Calcium Distribution and Exchange in the Rat Uterus

CASEY VAN BREEMEN, E. E. DANIEL, and
DIANNE VAN BREEMEN

From the Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada. Dr. and Mrs. van Breemen's present address is the Department of General Physiology, University of Leicester, Leicester, England

ABSTRACT The calcium content and distribution of the rat uterus were determined employing flame photometry and Ca^{45} determinations. The total uterine calcium concentration was found to be 2.25 millimoles (mmoles) per kilogram wet weight, 0.45 of which was inexchangeable. The exchangeable Ca could be divided into 0.8 mmole/kg wet weight extracellular and 1.0 mmole/kg wet weight intracellular. The concentration of ionic Ca in rat serum was obtained by equilibrium dialysis as 1.5 mM or 53% of the total serum Ca. The observed Ca distribution required that its active transport be postulated, since the membrane was shown to be permeable to Ca and the internal Ca concentration was far below its electrochemical equilibrium value. Metabolic inhibition by iodoacetate or dinitrophenol caused a net Ca uptake, but cooling to 4°C and ouabain did not. Iodoacetate did not affect the Ca^{45} efflux, but did increase the influx, suggesting that active Ca transport is accomplished by an exclusion mechanism. In experiments with varied external sodium concentrations, no evidence was obtained that sodium competes with calcium for inward transport. Cellular Ca binding was measured under conditions of prolonged metabolic inhibition, which abolished both active transport and the membrane potential. The association constants obtained were compatible with intracellular Ca binding to proteins, but insufficient to account for the low level of intracellular ionic Ca believed essential for relaxation. Hence metabolically dependent intracellular Ca binding was postulated. The Ca^{45} efflux was slowed down by Ca-free efflux media. The presence of Sr or EDTA could completely prevent this decrease in efflux rate, and Ba could partly prevent it. Changes in Mg and Na concentration did not affect the rate of Ca^{45} efflux. A model to explain Ca exchange across smooth muscle membranes has been proposed.

INTRODUCTION

Knowledge about the roles of Ca in the function of uterine smooth muscle has thus far come mainly from studies of the effects of composition of the external bathing solution on electrical and contractile activity of isolated

tissue. Such studies have established that tissue Ca depletion by prolonged exposure to Ca-free solution and short exposures to EDTA-like Ca chelating agents leads to loss of contractility (1, 2), membrane depolarization, and inexcitability (3). Furthermore, extensive use of this general technique has led to the theory that a superficially bound Ca fraction functions in regulating membrane permeability particularly to Na (3) and in supporting depolarization-induced contractions, whereas another more tightly bound Ca fraction functions in supporting drug-induced contractions (4).

Although the above type of study has convincingly demonstrated that in the myometrium Ca functions in membrane excitability and contraction, studies on Ca content and on radiocalcium movements in isolated uteri and cellular fractions are necessary for the elucidation of the mechanisms of Ca action. The work reported here initiates this latter type of study in the rat uterus by investigating the total and exchangeable Ca concentrations, the Ca distribution between the extra- and intracellular spaces, several aspects of Ca movements across the cell membranes, and finally the presence of cellular Ca binding.

METHODS

Experimental Animals and Preparation of Tissues Female Wistar rats weighing between 50 and 100 grams were pretreated for 3 days with 100 micrograms diethylstilbestrol administered subcutaneously in oil in order to stimulate growth of the myometrium. Each was killed by a blow on the head, its peritoneal cavity was opened, and the uterine horns were dissected free, split open, and placed in Krebs-Ringer solution. This procedure lasted 2 minutes.

For some experiments the serosa and longitudinal smooth muscle were dissected from the circular smooth muscle and mucosa. The uterine horns as prepared above were placed on filter paper moistened with Krebs-Ringer bicarbonate solution with the mucosal side upward. The two layers to be dissected apart were separated at one end by scraping with a piece of razor blade held in a hemostat, whereupon the mucosa and circular smooth muscle layer were grasped with forceps and pulled away from the longitudinal smooth muscle layer. This dissection took from 5 to 8 minutes, and the ease with which the two layers came apart varied greatly. During the dissection the tissue was continually moistened with Krebs-Ringer.

Solutions Krebs-Ringer bicarbonate solution (K.R.): NaCl 115 mM, KCl 4.63 mM, $CaCl_2$ 1.5 mM, $MgSO_4$ 1 mM, $NaHCO_3$ 21.9 mM, NaH_2PO_4 1.16 mM, glucose 50 mM, pH 7.4, bubbled with 95% O_2 and 5% CO_2.

Tris buffered solution (T.S.): NaCl 115 mM, KCl 4.63 mM, $CaCl_2$ varied, $MgSO_4$ 1 mM, tris(hydroxymethyl)aminomethane 50 mM, glucose 50 mM, pH 7.4, bubbled with 100% O_2.

Na concentrations were varied by raising the NaCl in T.S. to 200 mM to give 200 Na T.S. The NaCl in 200 Na T.S. was replaced by choline chloride, LiCl, or sucrose to give solutions of different [Na] at the same osmolarity.

Drugs Iodoacetic acid (IAA), 2,4-dinitrophenol (DNP), ouabain, ethylenediaminetetraacetic acid (EDTA) Na salt at pH 7.

Total Ca Determinations The internal standard technique as worked out by Harrison (5) for the Unicam SP 900 flame spectrophotometer was applied as follows.

Serum: Fresh pooled rat serum was diluted 100 times with deionized water or was first deproteinized with 100% trichloroacetic acid and then diluted with deionized water. Galvanometer deflections were obtained in duplicate determinations for the unknown (diluted serum) at the Ca emission wavelength of 422.7 mμ (D_1) and for a mixture of 10 ml unknown and 1 ml standard at the same wavelength (D_2). The standard Ca solutions were prepared by dissolving $CaCO_3$ (certified reagent Fisher Scientific Co.) in 0.1 N HCl to a concentration (c) such that the concentration of the unknown with the standard added was approximately twice that of the unknown. The deflection due to nonspecific interference (D_m) was taken to be the galvanometer deflection of the unknown at the wavelength of 428 mμ. The concentration of the unknown (x) was then calculated from the equation

$$x = \frac{(D_1 - D_m)c}{(11D_2 - 10D_1 - D_m)}.$$

This method corrects for specific interference provided that the galvanometer deflection is directly proportional to the Ca concentration in the absence of changes in specific interference. This direct proportionality was verified in $CaCl_2$ solutions over the range of Ca concentrations used in the experimental Ca determinations (0 to 0.1 mM). Recovery of Ca from K.R. and T.S. solutions of different Ca concentrations by this method was 100 ± 1.9% (7). All data given in this manner represent: mean ± SE of the mean (number of determinations given in parentheses). Ca concentrations of experimental solutions were determined as for serum except that dilutions were made with 0.1 N HCl.

Tissues: After ashing overnight at 580–600°C, the ash was dissolved in 10 ml 0.1 N HCl. 5 ml of this was used to obtain D_1 and D_m, and a mixture of the remaining 5 ml with 0.5 ml of standard was used to obtain D_2.

Equilibrium Dialysis In a lucite chamber 7.3 ml of pooled rat serum was separated by a cellulose dialyzer membrane from 7.3 ml of T.S. The dialyzer membrane was immobilized between two lucite grids so that the volumes of the two compartments remained constant. Both compartments were stirred with a magnetic stirrer. The Ca concentrations of the T.S. before and after 12 hours of dialysis were determined. The serum concentration of dialyzable Ca was taken to equal the T.S. Ca concentration, which did not change during dialysis.

Calcium-45 Influx Uterine horns were incubated in Ca^{45}-labeled K.R. for specific periods. They then were blotted by lightly pressing between two sheets of filter paper for about 2 seconds, weighed, and ashed. The ash was dissolved in 2 ml deionized water, 1 ml of which was spread on a planchette and the remaining ml diluted to 10 ml with 0.1 N HCl for total Ca determination. The samples on the planchettes were dried with infrared light and counted in a Nuclear Chicago model D-47 gas flow counter using a mica film end window. The plating procedure was found to be reproducible with a standard deviation of 1.5%.

The radioactivity of the tissues was expressed as millimoles (mmoles) labeled Ca per kilogram wet tissue weight by dividing the counts per minute of the tissues by the

specific activity of the labeled K.R. in cpm/mmole Ca and the tissue weights in kilograms.

No corrections for self-absorption needed to be made, since the samples prepared from the dissolved ash and 100 times diluted labeled K.R. were found to be of infinite dilution for counting purposes.

Calcium-45 Efflux Uterine horns were suspended in test tubes containing 4 ml of K.R. as follows. The solution in the test tube was bubbled with carbogen (95% O_2, 5% CO_2) through a narrow glass tube (aerator) open at both ends and held by a rubber stopper, which rested on the top of the test tube. The uterine horn was suspended on a small U-shaped stainless steel wire. The ends of this steel wire could be brought together by means of a hemostat and then inserted into the free end of the aerator. This arrangement allowed transfer of the tissue from test tube to test tube as well as rapid transfer from one aerator to another. The uteri were loaded in a test tube containing Ca^{45}-labeled K.R. for a time long enough to exchange almost all the exchangeable Ca, which was 2 to 3 hours, unless stated differently. The tissues were then rinsed by dipping into two successive tubes containing nonlabeled K.R. for a total time of 1 to 2 seconds. They were then transferred to a clean aerator and passed through a series of test tubes containing 4 ml nonlabeled K.R. They remained in each for an accurately measured period of time. At the end of the efflux period the tissues were blotted, weighed, and ashed. One milliliter of each efflux tube was dried on a planchette and counted. To avoid the necessity of corrections for self-absorption, the ash was dissolved in K.R. instead of water, for plating and counting. Also the loading solution was diluted in K.R. to be counted.

Method for Estimating Total Ca from Ca^{45} Measurements Tissues were incubated in Ca^{45}-labeled K.R. or T.S. for 2 hours in order to label the exchangeable Ca before incubation in Ca^{45}-labeled experimental and control solutions. Since all the solutions had the same known specific activity, analysis at the end of the experiment yielded values for total exchangeable Ca. To obtain a value for total tissue Ca, 0.45 mmole/kg wet weight was added to correct for the inexchangeable fraction. This method (called Ca^{45} method) gives values comparable with those from the flame spectrophotometer (see Table II), but is more convenient and gives less variation. Where this method is used it will be specifically mentioned; all other total Ca determinations were done as described above.

Measurement of Resting Membrane Potential after 330 Minutes in IAA Intact uteri were mounted serosal side upward, by spreading and pinning on a wax surface in the bottom of a small jacketed constant temperature bath at 37°C. Two uterine horns were simultaneously mounted in this manner in two adjoining baths, one containing K.R. and the other containing K.R. with 1 mM IAA. The experimental tissues were exposed to 1 mM IAA for 5 hours prior to the membrane potential determinations. Single cell electrical activity of the experimental and control uteri was recorded by means of hand-pulled glass microelectrodes filled with 3 M KCl, using the floating electrode technique of Woodbury and Brady (6). The electrode tips were inspected under a microscope. Only electrodes whose tips did not leak and had a resistance greater than 20 megohms were used. Potential measurements were made through a Medistor negative capacitance electrometer, monitored on a Tektronix 502 oscilloscope and recorded on a Grass polygraph.

Hematoxylin-eosin–stained histological sections were prepared according to standard techniques (7).

Glassware All glassware used was Pyrex, which does not contaminate solutions contained in it with Ca, provided it is properly cleaned. All stock solutions were kept in polyethylene bottles under refrigeration.

The cleaning of glassware consisted in rinsing in tap water, soaking in detergent, rinsing in tap water and distilled water, soaking in 5 to 10% nitric acid made up with distilled water, and finally rinsing in distilled and demineralized water. The distilled water supply was checked and found to be free of Ca contamination. Later during the course of these experiments all glassware used for Ca determinations and for experiments with Ca-free solutions were, after the above procedure, soaked in Na_4EDTA solution made up with distilled water, then rinsed in distilled water and demineralized water.

Temperature All experiments were carried out at 37°C unless otherwise specified.

RESULTS

Total Ca Concentration of Uterine Horns when Incubated in K.R. Containing 2.5 millimolar Ca

Initial total tissue Ca determinations showed that uteri incubated in the usual Krebs-Ringer solution containing 2.5 mM Ca contained more Ca than uteri which were analyzed immediately after removal from the rats. The rise in total tissue Ca concentration occurred rapidly at first, from 2.25 (see below) to 3.3 ± 0.3 (3) mmoles/kg wet weight in 10 minutes, and then more slowly to 4.1 ± 0.2 (3) after 8 hours. The rise could not be prevented by omission of phosphate from the medium. Since the uteri were not damaged by dissection, and since 2.5 mM Ca is about twice as high as human ultrafiltrable plasma Ca (8), it was suspected that the in vitro rise in tissue Ca was due to too high a Ca concentration in the usual Krebs-Ringer solution. This possibility was further investigated.

Total and Free Serum Ca Determinations

The shifts in Ca concentration of the Tris buffered solution (T.S.) which took place during 12 hours' dialysis against pooled rat serum tended to an equilibrium value of 1.5 mM. This value was used in the K.R. as an approximation of the interstitial ionic Ca concentration.

The total serum Ca concentration determined by dilution in double-distilled water was 2.7 ± 0.18 mM (6), and that determined by prior deproteinization with 10% trichloroacetic acid, 2.9 ± 0.13 mM (5).

Ca Gained as a Result of Dissection

The longitudinal smooth muscle and some circular smooth muscle were dissected away from the remainder of the uterine horns. The smooth muscle was

then either immediately analyzed for total Ca or incubated in K.R. (1.5 mM Ca) before analysis. The damage due to dissection was sufficiently great to cause a net uptake of Ca to 3.5 ± 0.15 (4) mmoles/kg wet weight within 30 minutes. No significant increase occurred thereafter. Four other pieces of isolated uterine smooth muscle were incubated in K.R. for 30 minutes followed by an hour in Ca^{45}-labeled K.R., and were then analyzed for labeled Ca content. The Ca gained appears to be exchangeable, since 3.2 ± 0.3 (4) mmoles/kg wet weight had exchanged.

Examination of histological slides from immature estrogen-treated rats indicated that more than 75% of the uteri was smooth muscle.

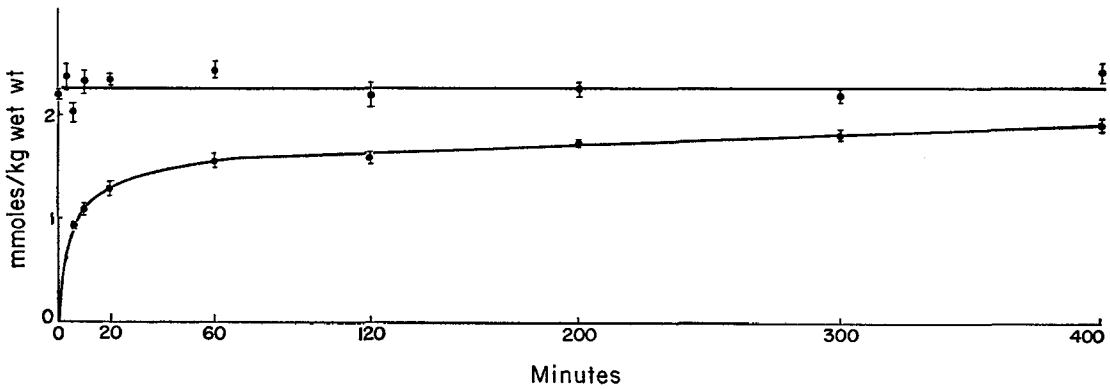

FIGURE 1. The upper line shows the total Ca concentration of rat uterine horns incubated, for times indicated, in K.R. (1.5 mM Ca). The lower curve shows the time course of Ca^{45} uptake expressed as mmoles labeled Ca per kg wet tissue. Each point is the average obtained from 5 or 6 tissues. The radioactive and total Ca values were obtained from the same uteri. Vertical bars indicate twice the SE.

Since results from dissected myometrium were more variable and would in part be attributable to an unphysiological Ca fraction (i.e. gains due to dissection), it was decided to use whole uterine horns in the Ca exchange studies.

Uptake of Total and Labeled Ca in Whole Uterine Horns

The change in tissue content of total and labeled Ca with time is given in Fig. 1. At an external Ca concentration of 1.5 mM the total calcium concentration remains constant at approximately 2.25 mmoles/kg wet tissue weight for many hours. The constancy of the total tissue Ca concentration allows the uptake or loss of radioactive Ca to be interpreted as true Ca exchange.

The uptake of labeled Ca as shown in the lower curve of Fig. 1 occurs at an initial rapid rate, which is followed by a continual slowing. After 2 hours the uptake of labeled Ca is so slow that it cannot be differentiated from a possible small net gain. At this time, between 0.4 and 0.5 mmole Ca per kg wet

weight has not exchanged. Thus about 0.45 mmole/kg wet weight is inexchangeable or very slowly exchangeable Ca. This fraction will, hereafter, be referred to as inexchangeable.

The uptake of labeled Ca in Fig. 1 plotted as the logarithm of the unexchanged fraction vs. time did not follow first order kinetics; i.e., it did not

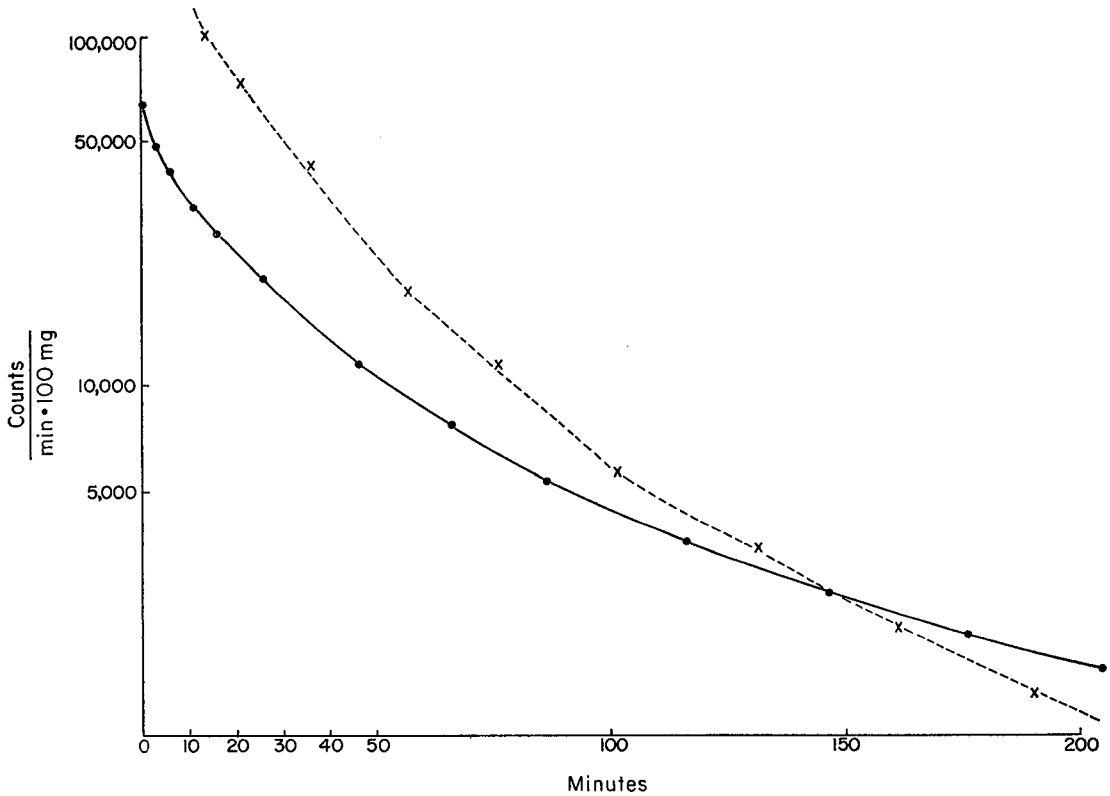

FIGURE 2. Ca^{45} efflux from rat uteri. Uterine horns were loaded in Ca^{45}-labeled K.R. for 3 hours and then passed through a series of inactive K.R. solutions. The solid curve represents the loss of tissue Ca^{45} with time, and the dashed curve the rate of Ca^{45} loss vs. time. Each point is the average of 5 determinations.

yield a straight line over the entire range of data. Also Ca exchange cannot easily be treated as a sum of a few exponential processes, since even the latter part of the curve was not a straight line.

Efflux of Labeled Ca

The loss of Ca^{45} from uteri incubated for 3 hours in Ca^{45}-labeled K.R. is illustrated in Fig. 2. The solid curve shows the log of the radioactivity in the tissue vs. time. The dashed line represents the rate of Ca^{45} loss vs. time. The plots are not linear and do not become parallel at any time, indicating that

the Ca was not emerging from a single well mixed compartment with first order kinetics.

The results obtained from Ca influx and efflux show that rat uterine exchange kinetics cannot be fitted to a unique sum of first order processes; therefore the division into tissue Ca fractions on this basis is unreliable.

Loss of Total Ca into Ca-Free Solution

The time course of the loss of total Ca into Ca-free solution is illustrated in Fig. 3. The main points of this experiment are that Ca is lost at an initially rapid rate, and that Ca-free medium is unable to extract a fraction of the tissue Ca which is approximately equal to the inexchangeable fraction, i.e. about 0.5 mmole/kg wet weight. The presence of 1 mM IAA does not affect the net Ca loss into Ca-free solution.

The possibility of binding of the more slowly exchangeable Ca in the extracellular space was investigated by comparing the loss of tissue Ca during a 10 minute exposure to Ca-free K.R. with the loss of tissue Ca during a 6 minute exposure to Ca-free K.R. containing 5 mM EDTA (i.e., at pH 7.4) followed by 4 minutes in Ca-free K.R. This experiment is based on the assumption that EDTA which has a volume of distribution resembling that of sucrose will remove only superficially bound extracellular Ca, for which some evidence is available (5, 6). However, in the above described experiment the loss of tissue Ca was the same, within experimental error, in both cases. The total Ca after Ca-free exposure was 1.09 ± 0.04 (4) mmoles/kg wet weight, and after Ca-free plus EDTA 1.08 ± 0.10 (4) mmoles/kg wet weight, in dicating that a large amount of extracellular bound Ca is unlikely.[1]

The Ca exchange and loss studies and other considerations elaborated in the "Discussion" lead to the following values for tissue Ca distribution: Extracellular Ca, 0.8 mmole/kg wet weight in an inulin space of 460 ml/kg (4). Cellular exchangeable Ca, 1.0 mmole/kg wet weight; inexchangeable Ca, 0.45 mmole/kg wet weight.

Effect of IAA on Ca^{45} Uptake and the Total Tissue Ca Concentration

Calculations given in the Discussion show that a large electrochemical gradient for Ca exists across the uterine cell membrane. Since the membrane

[1] A short experiment was performed to demonstrate that EDTA would remove extracellular bound Ca. Rat Achilles tendon and fascia weighing about 6 mg each were incubated in Ca^{45}-labeled K.R. for 1 hour. Tendon and fascia from one side of the animal were then exposed to Ca-free K.R. for 10 minutes, and the same tissues from the other side to Ca-free K.R. containing 5 mM EDTA also for 10 minutes. The Ca-free K.R. reduced the labeled Ca concentration in the tendon to 0.38 mmole/kg wet weight and in the fascia to 0.54 mmole/kg wet weight, whereas the respective values for EDTA-containing Ca-free K.R. were 0.075 and 0.161 mmole/kg wet weight. Since tendon and fascia are cellular but do contain bound Ca (7), this is considered to be a demonstration that EDTA is able to remove most of any extracellular bound Ca.

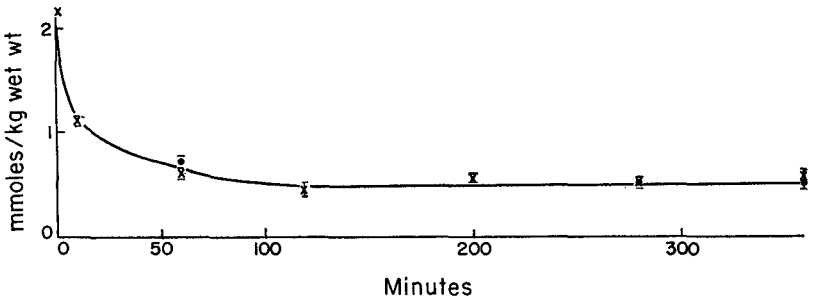

FIGURE 3. Loss of total Ca from rat uterine horns incubated in Ca-free solution (T.S.) (dots) and Ca-free T.S. containing 1 mM IAA (crosses). Points are averages of 4 determinations for each procedure. Where crosses and dots are superimposed, only crosses are drawn in. Vertical bars indicate twice the SE.

appears permeable to Ca, the maintenance of the Ca gradient requires metabolic energy. Fig. 4 shows the uptake of total and labeled Ca when uteri were incubated for various times in Ca^{45}-labeled K.R. containing 1 mM IAA. These curves demonstrate that the action of IAA has a latent period of about 70 minutes before it causes a net uptake of 2 mmoles Ca per kg wet weight. IAA also appears to abolish the inexchangeable fraction of calcium, but this was not proved. Similar results were obtained with 1 mM DNP. Lower con-

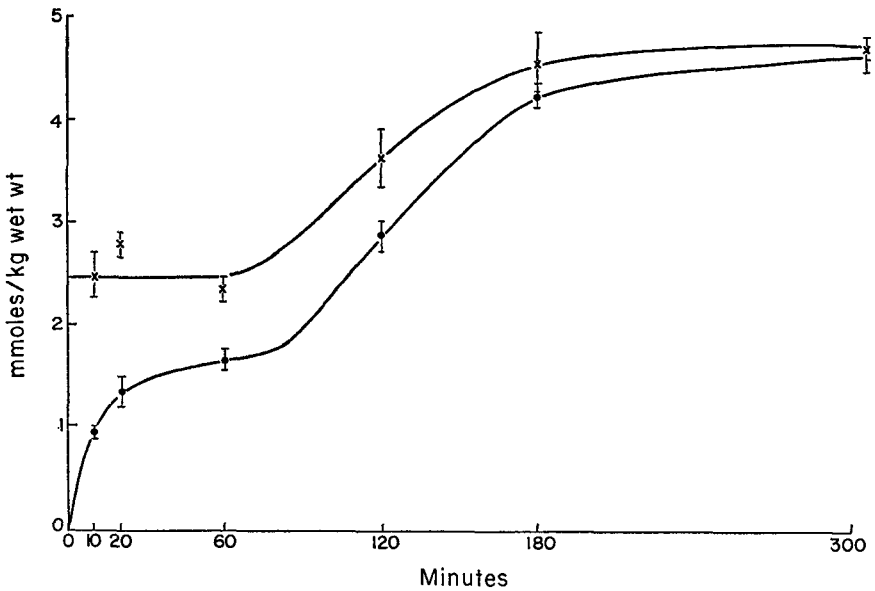

FIGURE 4. Uptake of labeled (dots) and total (crosses) Ca by rat uteri incubated in K.R. (1.5 mM Ca) labeled with Ca^{45} and containing 1 mM IAA. Radioactive and total Ca concentrations obtained from the same uteri. Each point is an average of 4 determinations. Vertical bars indicate twice the SE.

centrations (e.g., 0.1 mM) of IAA or DNP had no effect on net calcium concentrations in uteri except after several hours.

To test whether the IAA-induced Ca uptake could be reversed by reincubating in K.R., some uteri were exposed to IAA for 3 hours followed by K.R. for another 3 hours. Analysis showed these uteri to contain 4.9 ± 0.13 (4) mmoles Ca per kg wet weight. Uterine horns from the same rats incubated in K.R. containing 1 mM IAA for 3 hours and then analyzed contained 4.47 ± 0.18 (4) mmoles Ca per kg wet weight. Thus the IAA-induced Ca uptake was not reversed upon reincubation in K.R.

Effect of IAA on Ca^{45} Efflux

When the efflux medium is changed from K.R. to K.R. containing 1 mM IAA at various times during efflux, there is no immediate change in the rate of efflux, as demonstrated in 6 experiments. However, if the rise in total Ca were due to a decreased efflux, this would be expected, because of the delayed action of IAA. To study the efflux over the period during which the net Ca uptake occurred, the experimental uterine horns were loaded for 80 minutes in Ca^{45}-labeled K.R. and for an additional 40 minutes in Ca^{45}-labeled K.R containing 1 mM IAA. The experimental tissues were then effluxed in inactive K.R. containing 1 mM IAA for 95 minutes. These efflux curves were compared with control efflux curves obtained from uterine horns from the same rats and loaded and effluxed for the same times but not exposed to IAA. Fig. 5 represents an experiment typical of 6 such experiments. The tissue Ca concentration at the beginning of efflux was calculated by adding 0.45 mmole/kg wet weight for the unexchanged Ca to the labeled Ca concentration at that time; the tissue Ca concentration at the end of the 95 minute efflux period was determined by flame photometry. During the efflux period the Ca concentration of the experimental horn rose from 2.1 to 4.3 mmoles/kg wet weight, whereas the corresponding control values were 2.2 and 2.1 mmoles/kg wet weight. Fig. 5 shows clearly that, while IAA induced a net Ca gain, it had no effect on the rate of Ca^{45} efflux. Since the net Ca uptake during efflux would increase the dilution of Ca^{45} by Ca^{40} in the IAA-treated horns over that in the controls, the Ca efflux was probably raised during inhibition.

These experiments lead to the conclusion that the IAA-induced net Ca gain is caused by an increase in the Ca influx and not by a decreased efflux.

Effect of IAA on the Net Ca Uptake from Tris Buffered Solution Containing 10 Millimolar Ca

Uterine horns were incubated for various times in T.S. containing 10 mM Ca, and in T.S. containing 10 mM Ca plus 1 mM IAA. As can be seen in Fig. 6, the curves for the IAA-treated and control tissues are almost the same for the first hour. They show an initial fast uptake of about 4 mmoles/kg wet

weight, which is the amount expected for equilibration with the extracellular space. Then the tissue Ca concentration rises more slowly to an almost steady value of close to 7 mmoles/kg wet weight, which is maintained for about 30

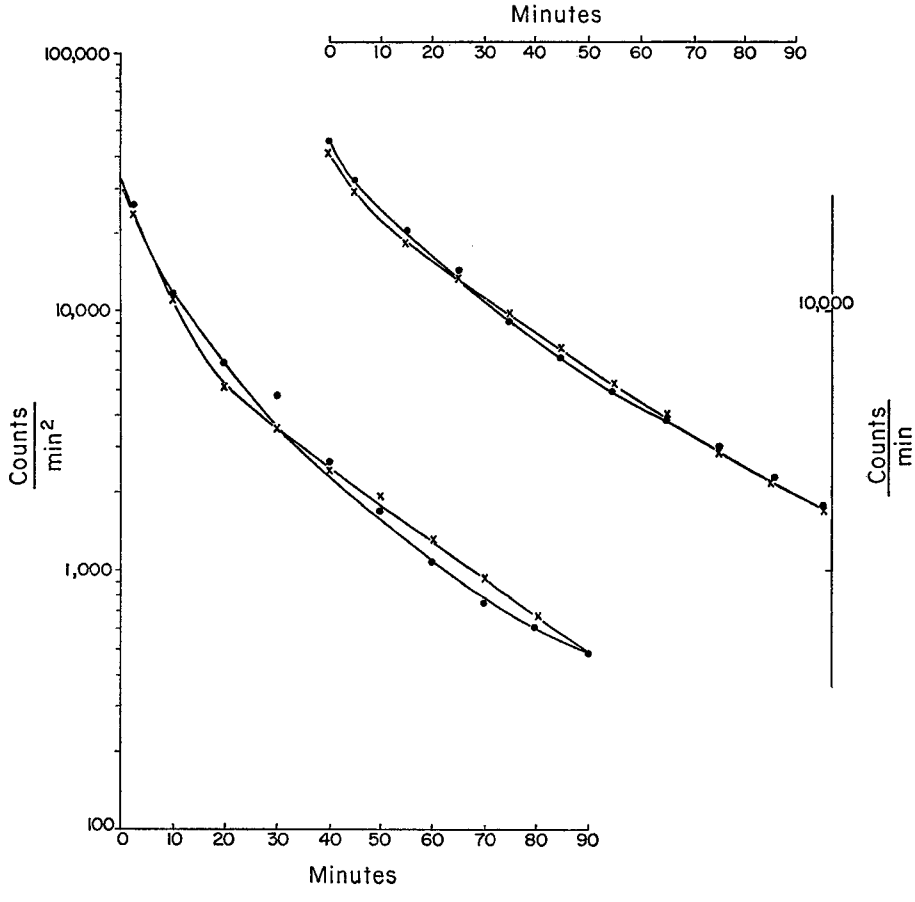

FIGURE 5. Typical experiment done on two uterine horns from the same rat, showing that IAA-induced net Ca uptake is not accompanied by a change in Ca^{45} efflux. The experimental horn (crosses) was loaded in Ca^{45}-labeled K.R. for 80 minutes and in labeled K.R. containing 1 mM IAA for an additional 40 minutes. It then was effluxed into K.R. containing 1 mM IAA for 95 minutes. The control horn (dots), loaded and effluxed for the same times, was not exposed to IAA. The curves on the left represent the rate of efflux vs. time (× 100), and those on the right Ca^{45} tissue content vs. time.

minutes in the IAA-treated, and somewhat longer in the control tissues. Calculations show that at a tissue Ca concentration of 7 mmoles/kg wet weight and an external Ca concentration of 10 mM, the cellular Ca concentration (including inexchangeable and bound Ca) is about 4 mmoles/liter cell water. The equilibrium value for intracellular Ca concentration is, however,

500 mM (assuming that the membrane potential (V) equals −50 mv). Thus 7 mmoles Ca per kg wet weight is a temporary steady-state level at which the exclusion pump is still effectively keeping Ca out of the cells. The effect of IAA is then seen to be a delayed net Ca uptake of about 14 mmoles/kg wet weight. Although more delayed and slower, the Ca concentration in the control tissues rises to the same final concentration of about 21 mmoles/kg wet weight.

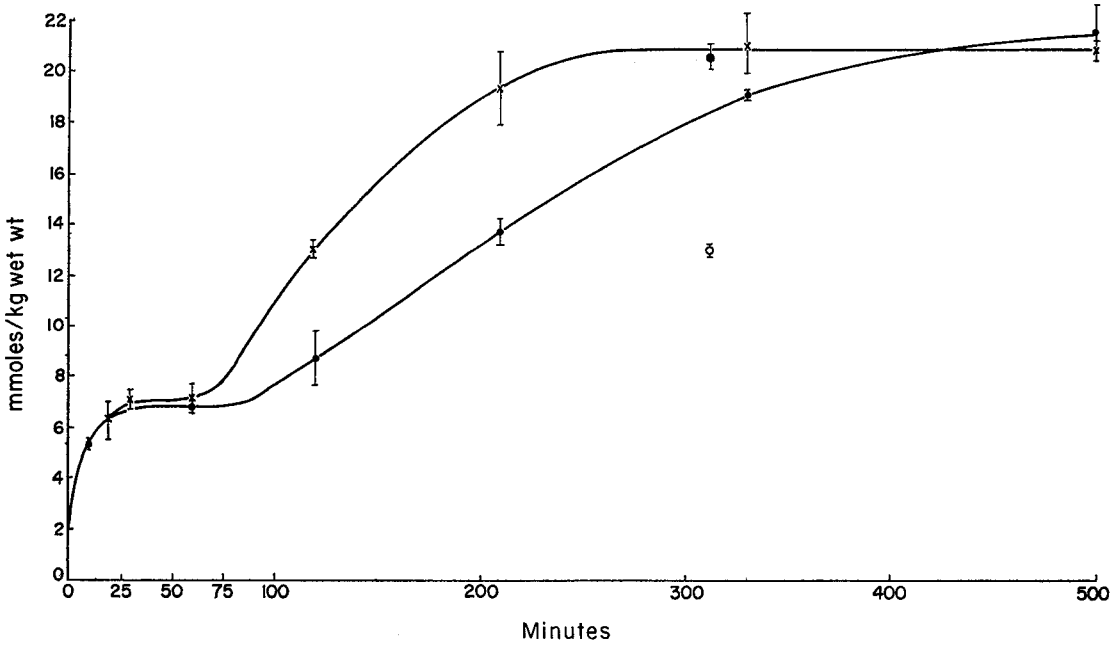

FIGURE 6. Uptake of total Ca by rat uteri from T.S. containing 10 mM Ca and 1 mM IAA (crosses) and from the same solution but without IAA (dots). For times 10 and 20 minutes one symbol is omitted since values overlapped. Vertical bars indicate twice SE of 4 determinations. In the absence of bars, twice the SE is less than the diameter of the symbol. The two circles represent values obtained from progesterone-dominated uteri (see text) at external Ca concentration of 10 mM with and without 1 mM IAA. Closed circles with and open circles without IAA.

At 21 mmoles Ca per kg wet weight the cellular Ca concentration would still be far below the equilibrium value if the potential were still −50 mv. At an external Ca concentration of 10 mM and in the presence of a normal membrane potential, electrochemical equilibrium would demand an intracellular Ca concentration of 490 mM. However, prolonged IAA treatment abolishes the membrane potential (see pages 1282–1285). Under these conditions the ionic tissue Ca concentration is approximately equal to the external Ca concentration, so that of the 21 mmoles Ca per kg wet weight about 11 mmoles/kg is in a bound form (see pages 1285–1287).

One explanation for the delay in the action of IAA is that during the delay period the store of ATP is being exhausted. Only after this has occurred does the Ca exclusion pump begin to fail, the result being a net Ca uptake. Using a similar argument the latent secondary rise of Ca concentration in the control tissue can be explained. The unusually high external Ca concentration places excessive demands on the energy (in the form of ATP) available for active transport. Since it is assumed that the metabolic energy supply is not inhibited in the absence of IAA, but only that the demands are excessive, it follows that a longer period of time is necessary for the ATP levels to be reduced sufficiently to interfere with active ion transport than in the presence of IAA. Also since metabolism is still taking place during the secondary Ca gain, this gain would be expected to be slower than in the IAA-treated uteri. These differences are apparent in Fig. 6.

This theory also predicts that prolonged exposure to 10 mM Ca will also cause a net downhill movement of Na and K. A number of uteri were exposed to T.S. containing 10 mM Ca for 5 hours. They were then blotted, wet ashed by heating to 200°C in concentrated nitric acid, and then analyzed for Na and K using the same internal standard technique as described for Ca (see "Methods"). The results showed a large net downhill movement for K to 13 ± 1.2 (5) mmoles/kg wet weight, and for Na to 104 ± 3.5 (5) mmoles/kg wet weight. The normal K concentration is about 75 mmoles/kg wet weight. The external Na concentration in these experiments using T.S. is 115 mM, so that the sodium space increased to 900 ml/kg wet weight. Large downhill ion movements in the uterus are accompanied by an increase in water content to about 900 ml/kg wet weight (9), so that the intracellular Na became equal to the external Na concentration. The membrane potential is dependent on the K gradient across the cell membrane and perhaps on electrogenic ion transport. Thus large downhill ion movements and inhibition of ion transport in the controls probably also led to complete membrane depolarization.

The lower (open) circle in Fig. 6 indicates that the secondary net Ca uptake occurs more slowly in progesterone-dominated uteri than in estrogen dominated ones.[2]

Effect of Ouabain and Cold on Ca Concentration

Rat uterine horns were incubated in K.R. containing 1 mM ouabain for 1, 2, and 6 hour periods and then analyzed for total Ca concentration. Uteri serving as controls were incubated for the same periods in K.R. Table I

[2] Values are averages from 2 rats treated 2 days with 100 μg diethylstilbestrol, followed by 1 day 100 μg diethylstilbestrol plus 50 μg progesterone, followed by 2 days 50 μg progesterone, and killed on the last day of treatment; and 4 older rats (120 g) treated 3 days with 100 μg diethylstilbestrol and left untreated for 2 more days.

demonstrates that ouabain does not cause a net uptake of total tissue Ca in this concentration which causes marked gain of Na and loss of K (48). It also had no effect on Ca^{45} efflux.

Table II demonstrates that 24 hours at 4°C does not raise the uterine Ca concentration.

Effect of Varying the External Na Concentration on Total Ca

To examine a possible competition between Na and Ca for membrane transport sites, the Na concentrations were varied by adding NaCl and by sub-

TABLE I

TOTAL CALCIUM CONCENTRATION OF RAT UTERI INCUBATED IN K.R. CONTAINING 1 MILLIMOLAR OUABAIN

Time	Total Ca	
	Controls	Ouabain treated
hr	*mmol/kg wet wt*	*mmol/kg wet wt*
1	2.0±0.13 (5)	2.0±0.07 (4)
2	2.1±0.20 (3)	2.1±0.08 (8)
6	2.3±0.07 (3)	2.2±0.15 (4)

TABLE II

TOTAL CALCIUM CONCENTRATION OF RAT UTERI INCUBATED IN K.R. AT 4°C

Time at 4°C	Total Ca	
	By flame photometry	By Ca^{45} method (see text)
hr	*mmol/ kg wet wt*	*mmol/kg wet wt*
0	2.0±0.19 (4)	2.1±0.11 (5)
15	2.4±0.09 (5)	2.2±0.04 (5)
24	2.1±0.04 (5)	2.3±0.08 (5)
36		2.6±0.04 (5)

stitution of NaCl by equimolar amounts of choline chloride in K.R. and T.S. Rat uterine horns were incubated in these media for 10, 60, and 120 minutes and analyzed for total tissue Ca by both flame photometry and the Ca^{45} method. Since the results of the two methods were the same, their averages are presented in Table III.

The results show that between 10 and 200 mM the Na concentration has no effect on the total Ca concentration of the uteri, indicating a lack of competition for transport sites in these cell membranes.

Further evidence which also excluded the possibility of Na and Ca competition for inward transport was the finding that decreasing the external Na concentration did not increase the rate of Ca uptake from solutions containing 10 mM Ca. Fig. 7 illustrates this point, using choline as the Na substitute. In

the 10 mM Na solution the secondary rise in Ca concentration is more delayed than in 50 or 200 mM Na. It was postulated earlier that this secondary rise was due to an inadequate or exhausted energy supply. Accordingly, the low external sodium concentration would have greatly reduced the energy consumption of the Na pump, which now could be used to operate the Ca exclusion mechanism effectively for a longer period of time. The results of experiments with Li and sucrose substituted for Na also failed to demonstrate Na and Ca competition for inward movement.

Effect of Temperature on Ca^{45} Efflux

To determine the effect of cold, the temperature was lowered from 38°C to 15°C at three different times during efflux. All uterine horns were loaded at 38°C for 2 hours and the experimental horns were effluxed either at 15°C for

TABLE III

EFFECT OF VARYING THE EXTERNAL SODIUM CONCENTRATIONS ON THE TOTAL CALCIUM CONCENTRATION OF RAT UTERINE HORNS

Time	Ca concentrations at various external Na concentrations			
	10 mM	50 mM	200 mM	138 mM (K.R.)
min	*mmol/kg wet wt*	*mmol/kg wet wt*	*mmol/kg wet wt*	*mmol/kg wet wt*
10	2.2±0.21 (4)	2.2±0.14 (6)	2.0±0.11 (4)	
60	2.3±0.23 (4)	2.3±0.20 (4)	2.3±0.11 (4)	2.1±0.11 (5)
120	2.2±0.15 (5)	2.3±0.10 (5)	2.3±0.09 (4)	

2 hours, at 38°C for 30 minutes followed by 15°C for 20 minutes, or at 38°C for 105 minutes followed by 15°C for 40 minutes. Fig. 8 shows one of two such experiments, both of which showed a relatively small temperature effect on Ca^{45} efflux.

Q_{10} values were calculated from the change in slope of the experimental curves in B and C and from the difference in slope between the experimental and control curves at times 30 minutes and 105 minutes in A. The Q_{10} at 30 minutes was 1.35 in both cases, and at 105 minutes it was 1.26 for A and 1.29 for C. These low Q_{10} values are consistent with a purely physical rate-limiting process for Ca^{45} efflux.

Sudden lowering of temperature will cause membrane depolarization and contracture (10) (uteri were observed to contract when the temperature was lowered in these experiments), but these factors will be shown in the following paper (30) to cause only very small changes in efflux and would not be expected to influence the above Q_{10} values.

Effects of External Ca, Sr, Ba, Mg, Na, and EDTA on Ca^{45} Efflux

Fig. 9 shows that Ca^{45} efflux into Ca-free medium is slow and that it can be accelerated by external Ca or EDTA. Ca and EDTA appear to accelerate

the efflux of cellular Ca^{45} rather than extracellular Ca^{45}, since the curves do not diverge until after about 8 minutes. When, after 30 minutes' efflux into K.R., Sr was substituted for Ca, the rate of efflux was not affected, but when Ba was substituted for Ca, the rate was decreased almost as much as when the efflux medium was changed to Ca-free K.R. without any substitute for Ca (Fig. 10). Na had no effect on Ca^{45} efflux into Ca-free K.R., as shown in Fig. 10. No significant increase in efflux into Ca-free K.R. was produced by Mg in 6 experiments.

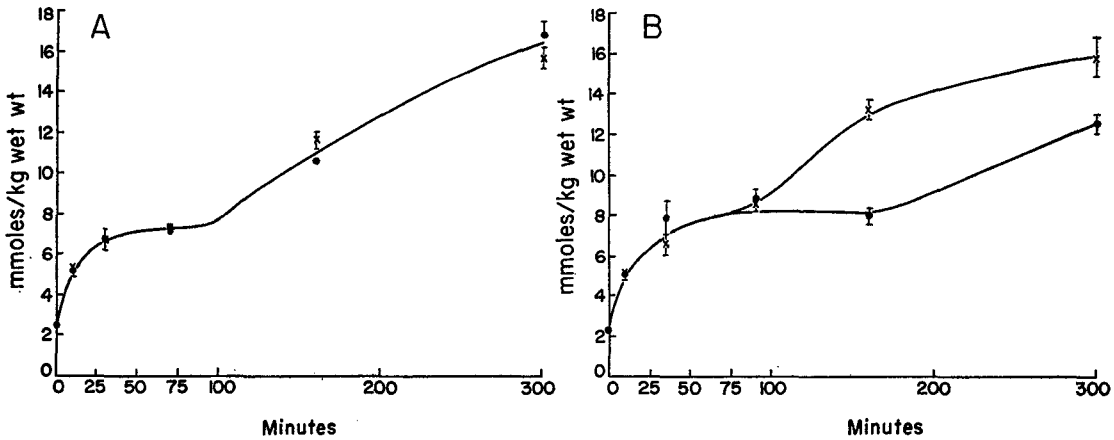

FIGURE 7. A. Total Ca uptake of rat uteri from T.S. containing 10 mM Ca and 200 mM Na (crosses) and uptake from T.S. containing 10 mM Ca and 50 mM Na (dots), choline substituted for Na.

B. Ca uptake from 10 mM Ca and 200 mM Na (crosses) and from 10 mM Ca and 10 mM Na (dots), choline substituted for Na. Vertical bars indicate twice the SE. Each point is the average of 4 determinations.

In an experiment illustrated in Fig. 11, uterine horns were effluxed into Ca-free K.R. for the first 65 minutes and then Ca was added to the efflux solution. The almost immediate increase in the slope of the efflux curve after inclusion of Ca in the efflux solution indicates that the sites upon which the added Ca ions act to increase the Ca^{45} efflux have a superficial location.

The increased Ca^{45} efflux induced by the presence of EDTA could not have been the result of its chelation of extracellular Ca, since reduction of the extracellular Ca concentration had an opposite effect on the Ca^{45} efflux. However, EDTA distributed extracellularly (11, 12) might act on the cell surface and might permanently damage the cell membrane. The reversibility of the EDTA effect was investigated by effluxing uterine horns for 35 minutes into Ca-free K.R. containing 2 mM EDTA followed by efflux into Ca-free K.R. without EDTA. The control horns were effluxed in the presence of EDTA for the entire efflux period. The results shown in Fig. 12 demonstrate

that the effect of EDTA is reversed upon its removal from the efflux solution, thus excluding permanent membrane damage. Experiments using EGTA (ethylene bis-glycol(β-aminoethylether)tetraacetic acid) instead of EDTA yielded similar results.

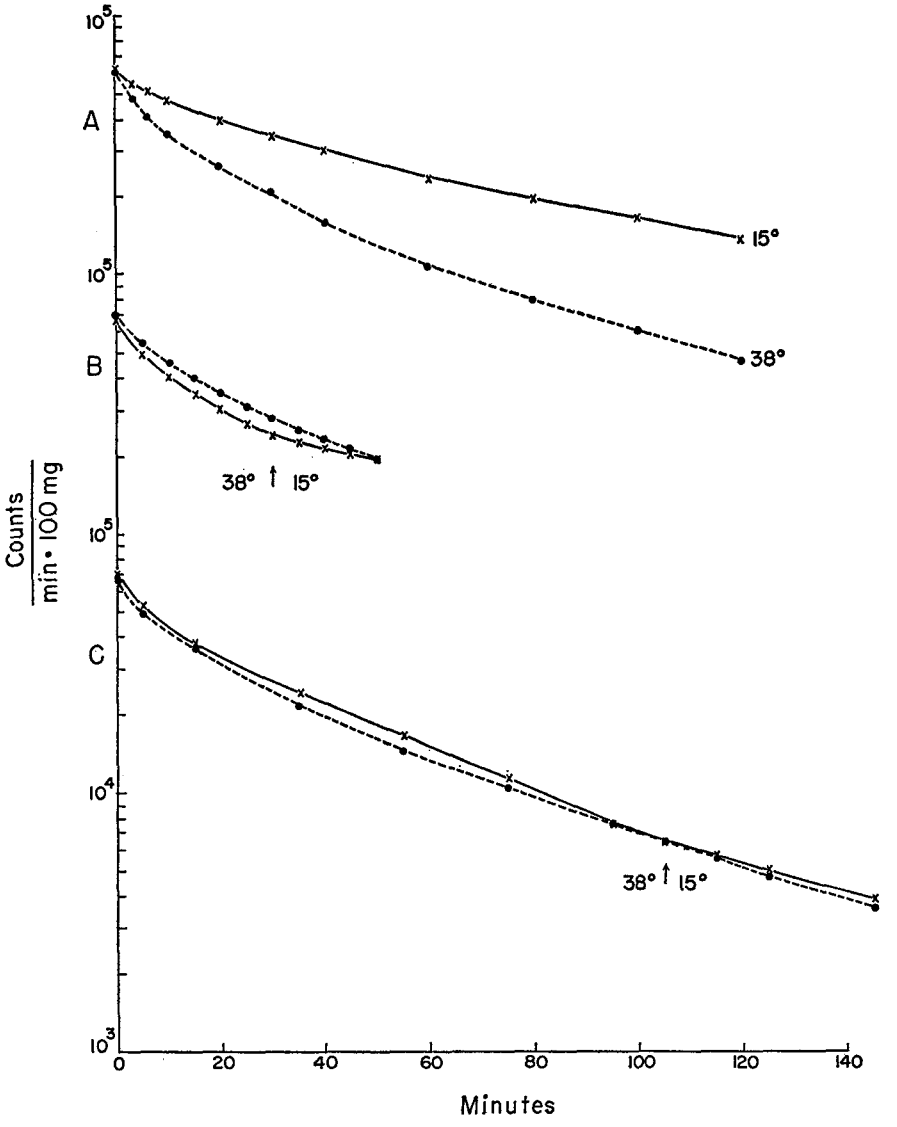

FIGURE 8. A, Ca^{45} efflux at 15°C (crosses). Temperature was lowered during efflux from 38°C to 15°C at the arrows in B and C for the experimental uteri (crosses). Control efflux curves at 38°C (dots) were obtained from uterine horns from the same rats as the experimental ones.

Estimation of Membrane Potential after 5 Hours of IAA Treatment

At a steady state for Ca, the tissue Ca concentration is determined by the external Ca concentration, the membrane potential, active Ca exclusion, and cellular Ca binding. Since active exclusion prevents equilibration of the in-

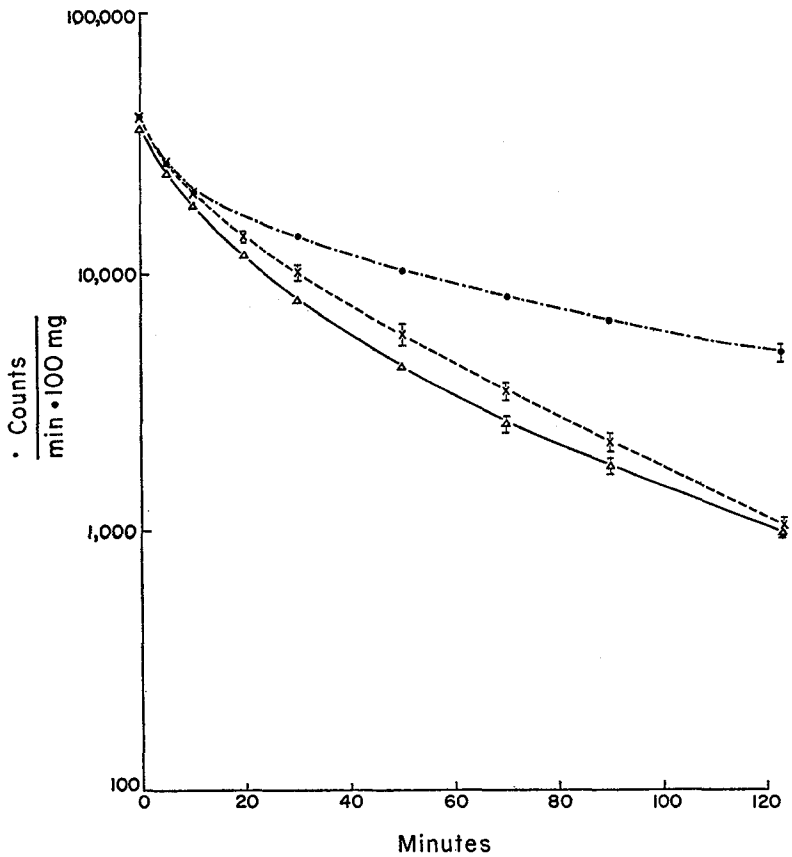

FIGURE 9. Ca^{45} efflux into K.R. (crosses), into Ca-free K.R. (dots), and into Ca-free K.R. containing 2 mM EDTA (triangles). Each curve is the average of 5 efflux curves. Vertical bars indicate twice the SE. In the absence of a bar the SE is less than the radius of the point.

tracellular with the external Ca, Ca binding cannot be studied by simply varying the external Ca concentration and measuring the tissue Ca concentration. If the active transport of ions is inhibited with 1 mM IAA for a sufficient length of time to reach Ca equilibrium across the cell membrane, and if the membrane potential can be estimated, then values necessary for quantitative treatment of cellular Ca binding can be obtained.

Daniel (9) reported data showing that 3 hours' exposure to 1 mM IAA reduced the uterine K concentration to 8.6 mEq/liter tissue water, as compared

with 5.8 mM in the bathing solution, and the Na concentration to 158 mEq/liter tissue water, as compared with 145 mM in the bathing solution. These values indicate that the K and Na gradients are completely abolished, the small excess of ions being due to a lower activity coefficient in tissue water,

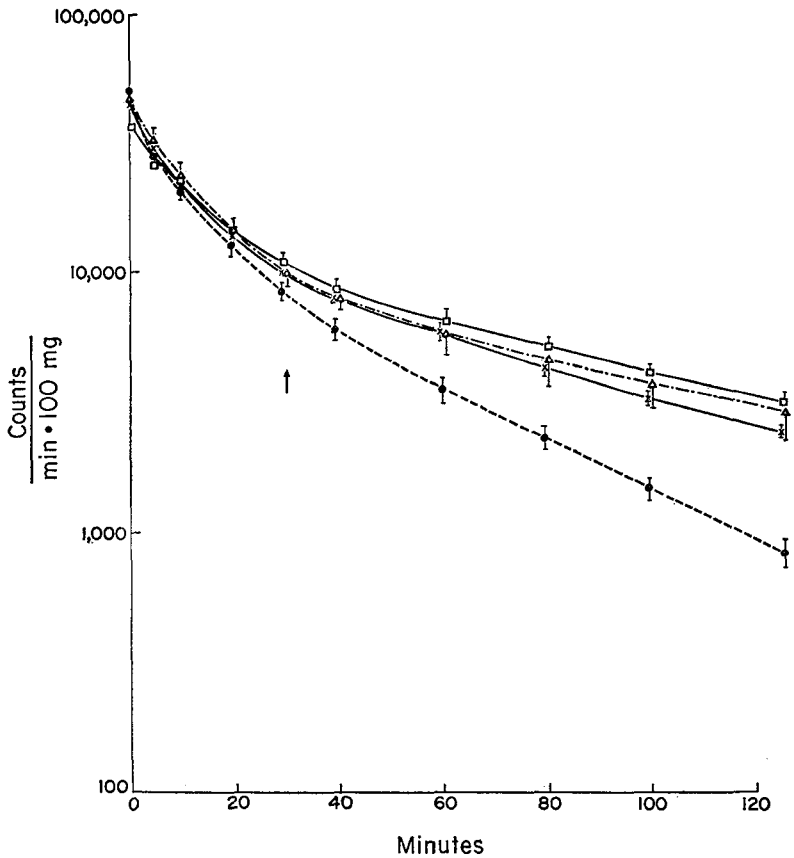

FIGURE 10. Ca^{45} efflux into K.R. for 30 minutes followed (after arrow) by Ca-free K.R. containing 1.5 mM Sr (dots), Ca-free K.R. containing 1.5 mM Ba (crosses), Ca-free K.R. (squares), and Ca-free K.R. with choline chloride substituted for NaCl (triangles). Each curve is the average of 5 efflux curves. A vertical bar projecting from one side of a point indicates the SE. In absence of a bar the SE is less than the radius of the point.

to binding, or to both. Since the two immediate causes of membrane potential are ionic gradients and electrogenic ion transport, and since IAA abolishes both, it would be expected that a 5 hour exposure to 1 mM IAA would also abolish the membrane potential.

During attempts at direct determinations of membrane potential of rat uterine smooth muscle, a resting membrane potential of about 40 mv could be frequently recorded in the control tissues, but no detectable membrane

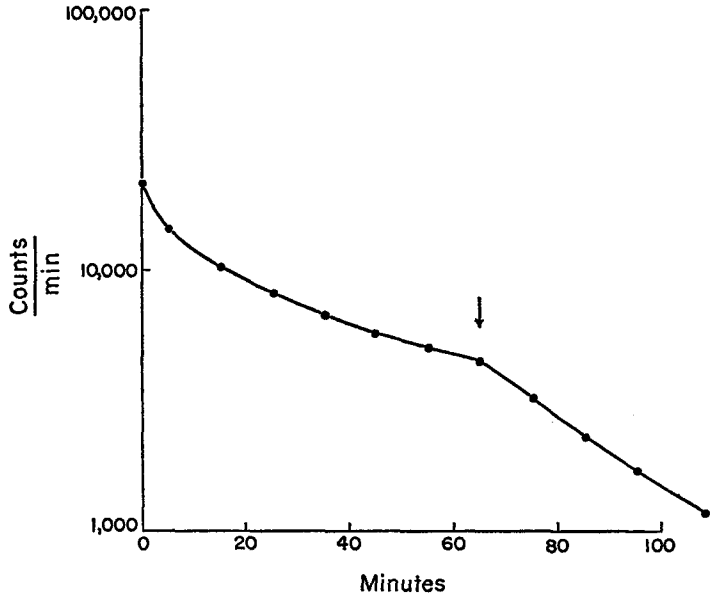

FIGURE 11. Ca^{45} efflux into Ca-free K.R. for 65 minutes followed by efflux into K.R. containing 1 mM Ca. Arrow indicates time at which Ca was introduced. The points represent means of experiments with 5 uterine horns.

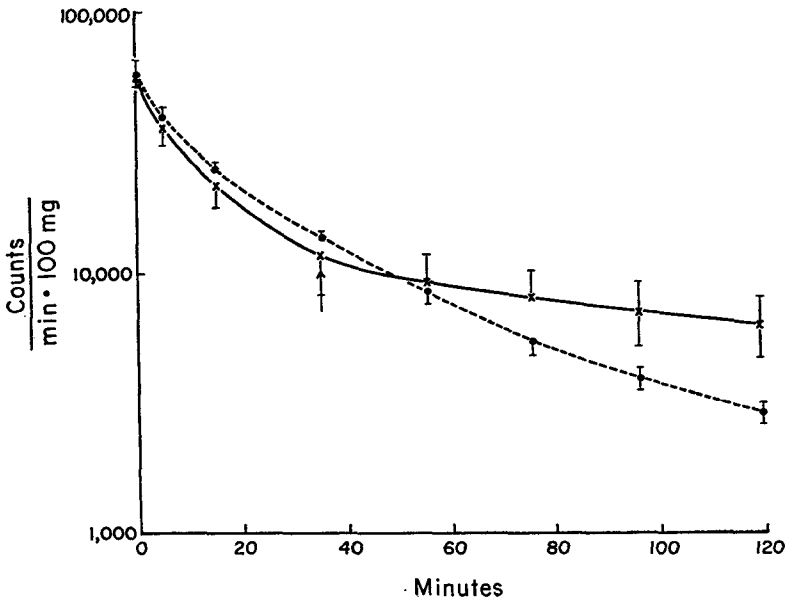

FIGURE 12. Ca^{45} efflux into Ca-free K.R. containing 2 mM EDTA (dots), and into same for 35 minutes followed (after arrow) by Ca-free K.R. without EDTA for the remainder of the efflux period (crosses). The two procedures were carried out on opposite uterine horns from the same 4 rats. Vertical bars indicate twice the SE. A bar projecting from one side of a point indicates the SE.

potential could be obtained from the IAA-treated ones. Some of these results are shown in Fig. 13. It is clear that genuine penetrations and potential recordings were made and that if the IAA-treated cells did have membrane potentials these should have been much easier to record, as IAA causes the cells to swell and inhibits spontaneous contractions.

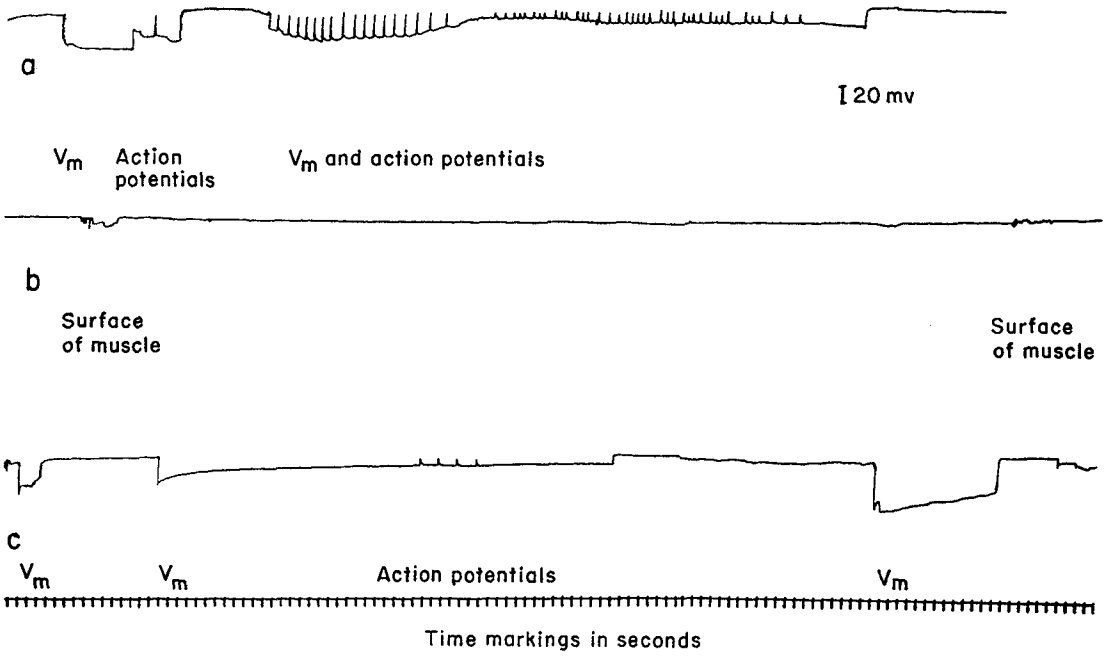

FIGURE 13. Intracellular potential recordings from uterine smooth muscle. *a* and *c* are controls recorded before and after recording *b* from uterine smooth muscle treated with 1 mM IAA for 5 hours. These recordings were made using the same microelectrode. V_m, membrane potential.

Uterine Ca Binding

The data given above establish with reasonable certainty that after 330 minutes' exposure to IAA the two important factors determining tissue Ca concentration are the external Ca concentration and Ca binding. Uteri were incubated for 330 minutes in T.S. containing 1 mM IAA and varying Ca concentrations. The relationship between the Ca concentration in the tissues after this treatment and the Ca concentration of the T.S. is illustrated in Fig. 14 (upper curve). The slope of this curve decreases continually until at Ca concentrations higher than 20 mM it remains constant at a value of 1. This final slope of 1 was confirmed as shown in the inset of Fig. 14, in an experiment using a different group of rats and higher Ca concentrations. The best explanation for this direct proportionality between the external and tissue Ca concentrations is that at an external Ca concentration of 20 mM all the

tissue binding sites are occupied, so that when the external Ca concentration is raised beyond this value the increase in tissue Ca is due solely to an increase in ionic Ca.

In agreement with Daniel's results (9), the tissue water in this case was 870

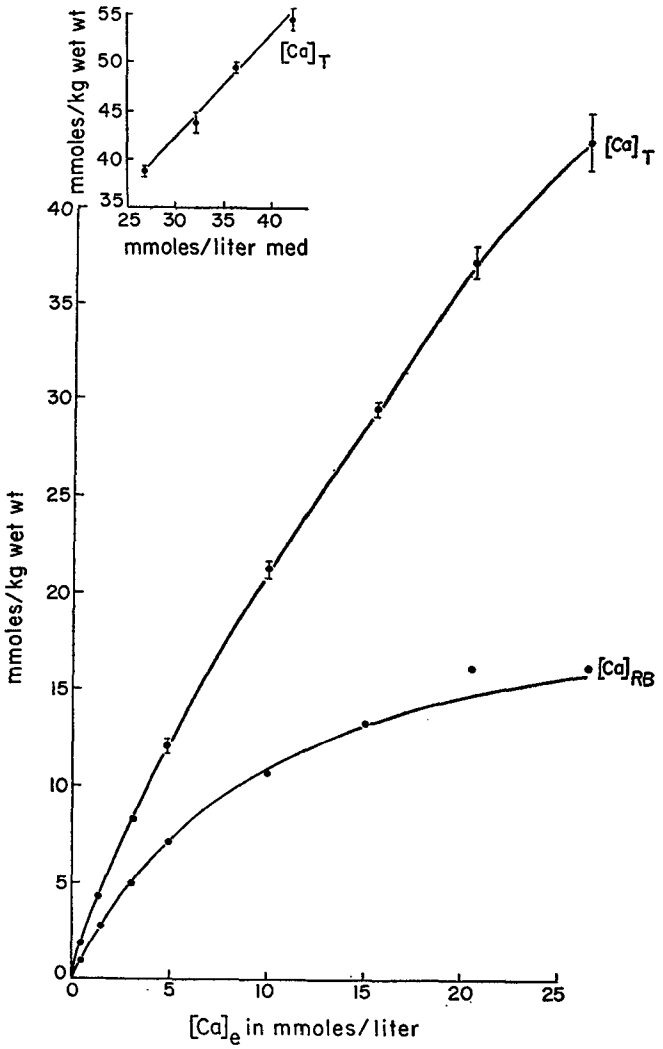

FIGURE 14. The upper curve and the inset give the tissue Ca concentration ($[Ca]_T$) against the external Ca concentration ($[Ca]_e$). These values were obtained from rat uterine horns incubated in T.S. containing 1 mM IAA for 330 minutes. The points of the lower curve show the relationship between external Ca concentration ($[Ca]_e$) and the Ca which is reversibly bound by the tissue ($[Ca]_{RB}$) under the same conditions. The curve fitted to these points demonstrates theoretical binding according to the law of mass action. Each point is the average of 4 determinations. Vertical bars indicate twice the SE. Where bars are absent, twice the SE is less than the diameter of the dots. No SE's are indicated in the lower curve.

ml/kg wet weight, so that at external concentrations above 20 mM the extra tissue calcium in mmoles per liter tissue water was 1.16 times the increase in the external solution. Such a relationship would be explained if the ionic tissue Ca has an activity coefficient of 0.87.

The observation that the ionic tissue Ca in mmoles per kg wet weight is numerically equal to the external Ca concentration in mmoles per liter allows calculation of the reversibly bound Ca by subtraction of the ionic and inexchangeable Ca from the total tissue Ca concentration. Inspection of the relationship between the reversibly bound Ca and the Ca concentration of the ambient solution suggested that the observed binding did follow the mass action law:

$$K \rightleftharpoons \frac{CaS}{Ca \cdot S}$$

where K is the apparent association constant for the reaction $Ca + S = CaS$ under experimental conditions; Ca is the Ca concentration of the ambient solution in mmoles per liter; S is the concentration of free sites in mmoles per kg wet weight; CaS is the concentration of Ca complexed with the binding sites in mmoles per kg wet weight. A theoretical curve was fitted to the observed relationship between the bound Ca and external Ca concentration according to this equation. The values of K and of CaS max $(= S + CaS)$ used to draw the theoretical curve (lower curve in Fig. 14) which gave the best fit to the experimental data were respectively 100 liters/M and 22 mmoles/kg wet weight.

To test whether the observed binding was specifically affected by the IAA, the experiment described above was repeated using DNP as a metabolic inhibitor instead of IAA. The curve obtained with IAA fitted the points obtained with DNP.

Although the association constant of 100 obtained above does not equal the thermodynamic equilibrium constant for Ca association with tissue binding sites, as concentrations instead of activities were measured, it does serve to demonstrate the extent of tissue Ca binding under conditions of metabolic inhibition.

DISCUSSION

Extracellular Uterine Calcium

Since it has been an established fact for many years that plasma Ca is only partly ionized, it is surprising that most in vitro bathing solutions contain Ca concentrations approximately equal to the total plasma Ca. Munday and Mahy (8) in a careful study in which temperature and pH were controlled found that ultrafiltrable Ca normally constituted 52% of the total plasma Ca. This is in close agreement with the value of 53% found by means of equi-

librium dialysis against pH buffered medium in this study on rat serum. Of the intravascular Ca, only ionic Ca and Ca bound to smaller molecules, mainly citrate, are freely diffusible through the capillary walls (14). The percentage of citrate-complexed Ca is small (13, 14) and was diluted 2 times during equilibrium dialysis. For these reasons the value of 1.5 mM $CaCl_2$, which was in equilibrium with Ca of rat serum on the other side of a dialyzer membrane, is taken as a measure of the ionic interstitial Ca concentration in vivo. Furthermore, the absence of a rapid change in tissue Ca concentration, and the prolonged maintenance of the in vivo Ca concentration when rat uteri are incubated at an external Ca concentration of 1.5 mM, strongly suggest that the interstitial ionic Ca concentration closely approximates the dialyzable serum Ca.

To determine the fraction of the total tissue Ca which is located extracellularly, the volume of the extracellular fluid and Ca binding to connective tissue must be known in addition to the ionic concentration of Ca. Bohr (15) discusses methods for measuring the extracellular space and indicates that the inulin method is the most acceptable and probably the most reliable. Using the inulin space of 460 ml/kg wet weight as reported by Daniel (10) for estrogen-pretreated rat uteri and assuming equal activity coefficients for Ca in Krebs-Ringer bicarbonate solution and extracellular fluid, the calculated extracellular ionic Ca represents 0.69 mmole/kg wet weight.

The initial rapid Ca exchange or loss of 0.9 mmole/kg wet weight and the fact that a short exposure to EDTA could not increase the initial rate of Ca loss from the uteri suggest a value for the exchangeable extracellular Ca fraction between that figure and 0.69 mmole/kg wet weight. Assuming a small amount of extracellular Ca binding, a value of 0.8 mmole/kg wet weight is a reasonable approximation. The excess over the ionic Ca in the extracellular space is then 0.11 mmole/kg wet weight or 0.24 mmole/liter extracellular volume. This corresponds to the value of 0.2 mmole loosely bound Ca per liter of extracellular fluid found by Bozler (16) in the frog stomach smooth muscle (the external Ca concentration in this case was 1 mM).

Bauer *et al.* (17) also found evidence for extracellular Ca binding in the guinea pig taenia coli, in that their extracellular Ca space exceeded the inulin space. Evidence for extracellular Ca binding has also been presented for skeletal muscle (18) and cardiac muscle (19, 20). As Bozler has pointed out (16), a portion of the inexchangeable calcium may also be bound in the extracellular space.

The Rate of Rat Uterine Ca Exchange in K.R.

Since the total Ca concentration remained constant during the uptake and efflux of Ca^{45}, the movements of tracer Ca were due to a true Ca exchange.

The observation that nearly all the exchangeable uterine cellular Ca becomes equilibrated with external Ca^{45} in 1 hour is in agreement with the

general finding that Ca exchange across smooth muscle membrane ($t/2$ = 2–60 min) (16, 17, 21–24) is more rapid than across cardiac muscle ($t/2$ = 30–186 min) (19, 20) and skeletal muscle sarcolemna ($t/2$ = 56–300 min) (25), and across squid axon neurolemna ($t/2$ = 20 hr) (26).

Rate constants for the Ca exchange of uterine cells could not be obtained, since the slopes of the plots of log Ca^{45} in the tissue vs. time for efflux and the log of the unexchanged fraction vs. time for influx decreased gradually with time. Furthermore, the slope of the curve relating the log of the counts emerging per unit time to time never became equal to the slope of the curve relating the log of the counts remaining in the tissue to time (27). The non-exponential nature of the Ca exchange has been observed in all types of muscle and could result from many factors (18, 20, 21, 28). Keynes (29) listed the following possible explanations for a similar exchange pattern for Na in frog skeletal muscle: (1) binding in the extracellular space; (2) trapping in some pockets of the extracellular space, which are less accessible than the remainder; these two factors would tend to flatten the initial part of the efflux curve; (3) size distribution of the muscle cells; (4) different membrane permeabilities for different types of cells; (5) gain in total ion concentration during the efflux period; (6) a drop in the absolute size of efflux with time; (7) intracellular binding; (8) binding at the cell surfaces.

For Ca exchange in the rat uterus factors 5 and 6 are not likely to contribute significantly to the flattening of the efflux curves, since there was no gain of Ca during the exchange experiments. However, a gradual decrease in both Ca influx and efflux was not ruled out.

In smooth muscle, besides the size distribution another morphological factor would be the irregular shape of the smooth muscle cells with their relatively wide centers and tapering ends. Factors 1, 7, and 8, relating to ion binding, are expected to be much more applicable to Ca than to Na. Harris (18) suggests the dissociation of Ca from Ca binding sites to be rate limiting, in a statement which seems applicable to the results reported here: "There is no justification for attempting to interpret Ca movements in terms of a small number of separate first order processes because behaviour is variable and probably results from a continuous gradation in strength of binding of the element." Thus it is postulated that the nonhomogeneity of the cellular Ca, which is exhibited in exchange with labeled Ca, is due mainly to the different rates of release of Ca from intracellular organelles, such as the mitochondria, nucleus, endoplasmic reticulum, and contractile actin and myosin fibrils, and also to different rates of dissociation from different molecular groups in the organelles and cytoplasm. No direct evidence for this postulate is available.

Inexchangeable Ca

In the rat uterus 0.45 mmole/kg wet weight of Ca, or about 20% of the total tissues Ca, was found to be inexchangeable or very slowly exchangeable.

Though definitive proof of its inexchangeable nature is lacking, we have so designated this fraction for convenience. Such a Ca fraction has been found in all three types of muscle. In the skeletal muscle the inexchangeable Ca is about 50% of the total tissue Ca (31), and in cardiac muscle the inexchangeable Ca is about 30% (16, 19, 32). In smooth muscle, values of 78% in guinea pig taenia coli (35) and 30 per cent in frog stomach muscle (16) have been obtained for the inexchangeable Ca fraction. However, in a recent publication Bauer *et al.* (17) reported the absence of a significant inexchangeable Ca fraction in smooth muscle. They attributed this to the use of a modified medium which contained no phosphate. Although this aspect has not been specifically investigated in this study, the omission of phosphate from the bathing solution did not lower the total Ca concentration of the rat uterus.

Several sources have been suggested for the inexchangeable tissue Ca. In the study mentioned above, Bozler (16) attributes some inexchangeable Ca to the connective tissue. Brink (33) suggests that some protein-bound Ca may be prevented from exchanging by virtue of phospholipid micelles surrounding the Ca protein complex. Hasselbach (31) states that Ca bound to actin fibrils and half of the Ca associated with myosin fibrils is inexchangeable, and that some but not all of this Ca becomes exchangeable when the two proteins are combined. In all probability the inexchangeable Ca is also heterogenous in that a number of different sources contribute to this fraction.

Active Membrane Transport of Ca in Rat Uterus

If the internal Ca ions were at electrochemical equilibrium with the extracellular Ca ions, the Nernst equation

$$Ca_i = Ca_o \exp -\frac{2VE}{RT}$$

would predict an intracellular Ca ion concentration of 67 mM.

Ca_i = intracellular Ca ion concentration
Ca_o = extracellular Ca ion concentration
V = electrical potential difference across the cell membranes, inside minus outside, and for this calculation taken to be −50 mv
F = the Faraday constant
R = the gas constant

The concentrations in this equation are used as approximations of the activities.

Even if all the cellular exchangeable Ca were ionized (1 mmole/kg wet weight), as is known to be incorrect, the intracellular Ca concentration would still only be 2.9 mM. Such a Ca gradient would not require an active trans-

port system if the cell membrane were impermeable to Ca ions. This possibility was excluded by the evidence presented for exchange of cellular Ca with external Ca^{45}.

However, if the Ca exchange occurred only by an exchange diffusion mechanism, whereby the passing of a Ca ion outward through the membrane is necessarily coupled to the inward movement of another Ca ion, the membrane would be essentially impermeable to Ca and no energy would be necessary to maintain the Ca gradient. This condition is believed not to apply to the rat uterus, since net outward movements were observed in high K (30) and Ca-free K.R., and net inward Ca movements in high Ca K.R. and during metabolic inhibition. Under these modified conditions the influx of one Ca ion was thus not coupled to the efflux of another Ca ion.

In order to explain an electrochemical Ca gradient across a membrane which is permeable to Ca, it was necessary to postulate an active membrane transport system for Ca which would derive its energy supply from cell metabolism. This postulate was further supported by the downhill Ca movement into the cells upon metabolic inhibition by iodoacetic acid and 2,4-dinitrophenol. However, the present data do not rule out the possibility that IAA and DNP may have altered membrane structure so that an obligatory coupling of inward and outward movement was eliminated. This consideration arises because of the fact that higher concentrations of these metabolic inhibitors were required to cause a net uptake of Ca than to cause net downhill movements of Na and K or to inhibit the ability to contract (9). Since no evidence of such an alteration in membrane structure is available, and since the chemical interactions of the two inhibitors differ, and since net Ca fluxes have been observed under other conditions, active transport of Ca is the preferred explanation.

The active Ca transport was shown to be an exclusion mechanism on the basis of the same evidence which led Shanes (34) first to postulate active exclusion in frog nerves; namely, that metabolic inhibition caused an increased influx but not a decreased efflux. The finding of a low Q_{10} of 1.34 for efflux indicates that Ca efflux is likely to be a physical process, which is also in agreement with active Ca exclusion but not with extrusion. If active Ca extrusion existed in the rat uterus, the efflux would necessarily have a component depending on chemical reactions, so that a higher Q_{10} value would be anticipated.

Evidence similar to that presented here for metabolism-dependent Ca exclusion has also been presented in the case of the guinea pig taenia coli (17, 21). Goodford (35), however, found it necessary to postulate an active Ca extrusion in this tissue on the ground that intracellular Ca is at a lower energy level than extracellular Ca. Active extrusion is not thought to be necessary to explain the present results, since the flux ratio M_i/M_o could just as readily

be decreased by active exclusion, which would reduce the influx (M_i), as by active extrusion, which would increase the efflux (M_o).[3]

The active Ca transport in the rat uterus seems remarkably resistant to procedures which in the same tissue inhibit active Na transport (48). Thus ouabain and cold had no effect on the total Ca concentration, and IAA had to be used at 10 times the concentration necessary to inhibit Na transport in order to cause a net Ca uptake. The observation that in the rat uterus active Na transport can be inhibited while Ca gradients are still being maintained suggests that these two cations do not have the same active transport mechanism. This suggestion is further strengthened by the failure to demonstrate any competition between Na and Ca. Even when excessive demands were made on the active Ca transport mechanism by high external Ca concentration, the external Na concentration had no effect on the net inward Ca flux, making it extremely unlikely that external Na and Ca ions were competing for transport sites. This finding necessitates modification of a model previously proposed by one of us (4).

The difference in IAA sensitivity of the Na and Ca gradients is quantitative rather than qualitative. Recent experiments by Daniel and Robinson (unpublished) demonstrate that the IAA-induced Na accumulation also has an initial lag period. Furthermore, the length of the lag period in the IAA-induced net Ca uptake appears related to the IAA concentration (van Breemen, unpublished results). If the length of the lag period is related to the degree of depletion of a metabolic energy source (perhaps ATP), then it could be concluded from the above evidence that the active Ca exclusion mechanism can function effectively at lower metabolic energy levels than can the active Na transport.

The absence of a net Ca gain in the cold was also unexpected and may be due to a combination of factors. Cold does not completely abolish metabolic energy sources for active transport (36). In the rat uterus, cold induces depolarization, and in this tissue it has recently been demonstrated that depolarization slows Ca influx (30). Thus it is possible that the combination of an ability to function at lower levels of metabolic energy and reduced passive influx are responsible for the maintenance of the Ca gradient at 4°C. However, these explanations require additional experimental verification. For example, it would be of great importance to know the levels of ATP under the

[3] Ussing (49) showed that where ions move across a membrane solely under the influence of their own kinetic energy, then, provided that the likelihood of penetration by each ion is unaffected by the presence of other ions of the same species, the ratio of fluxes in the two directions will be equal to the ratio of the activities in the two solutions if allowance is made for the membrane potential: $M_i/M_o = (\alpha_o C_o/\alpha_i C_i) \exp ZEF/RT$. M_i and M_o are inward and outward ion fluxes, respectively. α_o and α_i are the activity coefficients of the ions outside and inside the cells. C_o and C_i are the extra- and intracellular ion concentrations. The other symbols have their usual meaning. In the case of Ca transport across uterine smooth muscle cells, active transport (as well as exchange diffusion) would decrease this flux ratio.

experimental conditions described. The absence of ouabain sensitivity of the Ca active transport seems to indicate that the Na, K, Mg–activated ATPase, which is involved in active Na and K transport, does not function in Ca transport.

The Nature of Ca Exchange

The very rapid onset of the increased Ca^{45} efflux upon addition of Ca to the external solution indicated a superficial site of action. Phospholipids extracted from cell membranes (37, 38), isolated erythrocyte membranes (38, 39), and isolated skeletal muscle membranes (40) all show the presence of sites which associate with Ca ions. The nature of binding to these sites appears to be by ionic bond formation (38, 39), and Gent *et al.* (39) found the dissociation constant for the interaction of Ca and red cell membrane to be $2.8 \cdot 10^{-4}$ molar.

Assuming that these sites play a role in the transport of Ca through the membrane, the absence of external Ca may slow the Ca efflux at least in two ways. In one case it may prevent the exchange of extracellular ions for membrane-bound ions, and in the other case the absence of external Ca may lead to desaturation of the membrane binding sites, which in turn would lead to a slower passage of Ca ions through the membrane phase, probably because each Ca ion would be bound and released more times before it reached the other side of the membrane. Basically the two explanations are not very different, and both predict that only external ions with affinity for these membrane sites will be able to increase the Ca^{45} efflux. The present results show that these sites have high affinities for Ca and Sr, low affinity for Ba, and no affinity for Mg and Na. With respect to the latter explanation it is of interest that in the artificial phospholipid-cholesterol membrane of Mikulecky and Tobias (38) a low Ca concentration retarded the equilibration of membrane Ca with external Ca. In this case a greater number of vacant binding sites may have slowed the penetration of Ca into the phospholipid-cholesterol phase.

The relative affinities of Ca, Sr, and Ba for these superficial sites is in agreement with the findings of Daniel (2) and Marshall (3) that Sr is able to substitute for Ca in the maintenance of membrane excitability and polarization, but that when Ba is the Ca substitute the spontaneous action potentials disappear.

Calcium Binding

Although it is generally accepted that only a small portion of the cellular Ca is ionized, as has been demonstrated in intact skeletal muscle fibers (18) and squid axon (26), it is important to demonstrate that in the rat uterus intracellular Ca binding indeed occurs. Unfortunately it was not possible to measure such binding under control conditions, owing to the influence of the

membrane potential and active Ca exclusion on Ca distribution. However, upon prolonged metabolic inhibition cellular Ca binding was clearly demonstrated. The results indicated that Ca was probably bound to proteins.

The next important question is, How far could this type of binding, demonstrated after metabolic inhibition, lower the free cytoplasmic Ca concentration? An approximate calculation using an intracellular exchangeable Ca concentration of 1 mmole/kg wet weight or about 3 mmoles/liter cell water, an association constant of 100 liters/M, and a total concentration of cellular binding sites (assuming them to be located intracellularly) of 66 mmoles/liter cell water showed the expected free cytoplasmic Ca concentration to be 0.39 mmole/liter cell water. Hasselbach in a recent review (31) discusses the very compelling evidence for an intracellular ionic Ca concentration in relaxed skeletal muscle not exceeding 10^{-7} M. Filo *et al.* (41) showed that glycerinated smooth muscle has the same threshold concentration of Ca for contraction (between 10^{-6} and 10^{-7} M) as glycerinated skeletal muscle. It is thus very probable that the ionic Ca concentration in the cytoplasm of relaxed uterine smooth muscle is in the order of 10^{-7} M, 3900 times lower than the expected concentration if only passive Ca binding were responsible for lowering the cytoplasmic ionic Ca concentration. In this study no account was taken of Ca binding by ATP, since it was undoubtedly depleted after 5 hours' treatment with 1 mM IAA. Sanders and Daniel (unpublished results) have recently estimated the uterine ATP concentration to be about 1 mmole/kg, and calculations show that even in the presence of such an ATP concentration the free Ca concentration would still be more than three orders greater than that compatible with relaxed actomyosin.

What then is responsible for the reduction of ionic cytoplasmic Ca to a concentration below 10^{-7} M? In skeletal and cardiac muscle the answer to this question is almost an established fact (see review by Hasselbach, 31), namely, that the endoplasmic reticulum actively accumulates Ca from the cytoplasm. The above evidence and calculations show that such an explanation is also necessary for a low cytoplasmic free Ca concentration in rat uterine smooth muscle. The endoplasmic reticulum is not very well developed in smooth muscle (43–47), but since the rate of relaxation, which depends on the rate of removal of Ca (31, 42), is much slower than in skeletal muscle, this is not surprising. Smooth muscles exhibit a number of small vesicles close to the cell membranes which look like pinocytotic inpocketings of the membrane (43–47). That the endoplasmic reticulum may indeed play a role in active ion transport is suggested by the fact that it has ATPase activity, especially the vesicles close to the membrane and the inpocketings, as demonstrated by cytochemical staining techniques (45, 46).

In conclusion, the experiments on Ca binding and other evidence cited strongly suggest that intracellular active Ca accumulation by certain cellular

structures removes Ca from the cytoplasm of rat uterine smooth muscle. The structures which use metabolic energy to accumulate Ca are probably the endoplasmic reticulum and perhaps the inner part of the cell membrane. This indirect evidence for active intracellular Ca binding in the myometrium is of particular interest in this tissue, since no increased Ca influx could be demonstrated upon contraction (31).

The authors wish to thank Dr. G. B. Frank and Dr. C. P. Bianchi for helpful discussions related to this work.

The investigation was supported by the Life Insurance Medical Research Fund and the Medical Research Council of Canada.

This work was part of the thesis submitted by C. van Breemen in partial fulfillment of the requirements for the Ph.D. degree in the Department of Pharmacology of the University of Alberta.

Received for publication 1 November 1965.

REFERENCES

1. EDMAN, K. A. P., and SCHILD, H. O., The need for calcium in the contractile responses induced by acetylcholine and potassium in the rat uterus, *J. Physiol.*, 1962, **161**, 424.
2. DANIEL, E. E., On roles of calcium, strontium and barium in contraction and excitability of rat uterine muscle, *Arch. internat. Pharmacodyn.*, 1963, **146**, 298.
3. MARSHALL, J. M., Calcium and uterine smooth muscle membrane potentials, *in* Muscle, (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, editors), London, Pergamon Press, 1965, 229.
4. DANIEL, E. E., Attempted synthesis of data regarding divalent ions in muscle function, *in* Muscle, (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, editors), London, Pergamon Press, 1965, 295.
5. Unicam Instruments Ltd., Arbury Works, Cambridge, Method sheet 900/1, 1960.
6. WOODBURY, J. W., and BRADY, A. L., Intracellular recording from moving tissues with a flexibly mounted ultramicroelectrode, *Science*, 1961, **123**, 100.
7. Manual of Histologic and Special Staining Techniques, Washington, D. C., Armed Forces Institute of Pathology, 1957.
8. MUNDAY, K. A., and MAHY, B. W. J., Determination of ultrafiltrable calcium and magnesium on small quantities of plasma, *Clin. Chim. Acta*, 1964, **10**, 144.
9. DANIEL, E. E., Potassium movements in rat uterus studied in vitro. II. Effects of metabolic inhibitors, ouabain, and altered potassium concentrations, *Can. J. Biochem. and Physiol.*, 1963, **41**, 2085.
10. DANIEL, E. E., Potassium movements in rat uterus studied in vitro. I. Effects of temperature, *Can. J. Biochem. and Physiol.*, 1963, **41**, 2065.
11. PULLMAN, T. N., LAVENDER, A. R., and FORLAND, M., Synthetic chelating agents in clinical medicine, *Ann. Rev. Med.*, 1963, **14**, 175.
12. BIANCHI, C. P., The effect of EDTA and SCN on radiocalcium movement in frog rectus abdominis muscle during contractures induced by calcium removal, *J. Pharmacol. and Exp. Therap.*, 1965, **147**, 360.

13. CHABEREK, S., and MARTELL, A. E., Organic Sequestering Agents, New York, John Wiley and Sons, Inc., 1959.
14. BELL, G. H., DAVIDSON, J. N., and SCARBOROUGH, H., Textbook of Physiology and Biochemistry, Edinburgh, and S. Livingstone Ltd., 4th edition, 1959.
15. BOHR, D. F., Electrolytes and smooth muscle contraction, *Pharmacol. Rev.*, 1964, **16**, 85.
16. BOZLER, E., Distribution and exchange of calcium in connective tissue and smooth muscle, *Am. J. Physiol.*, 1963, **205**, 686.
17. BAUER, H., GOODFORD, P. J., and HÜTER, J., The calcium content and ^{45}calcium uptake of the smooth muscle of the guinea-pig taenia coli, *J. Physiol.*, 1965, **176**, 163.
18. HARRIS, E. J., The output of ^{45}Ca from frog muscle, *Biochim. et Biophysica Acta*, 1957, **23**, 80.
19. WINEGRAD, S., and SHANES, A. M., Calcium flux and contractility in guinea pig atria, *J. Gen. Physiol.*, 1962, **45**, 371.
20. LANGER, G. A., and BRADY, A. J., Calcium flux in the mammalian ventricular myocardium, *J. Gen. Physiol.*, 1963, **46**, 703.
21. GOODFORD, P. J., The loss of radioactive ^{45}calcium from the smooth muscle of the guinea-pig taenia coli, *J. Physiol.*, 1965, **176**, 180.
22. CHUJYO, N., and HOLLAND, W. C., Potassium-induced contracture and calcium exchange in the guinea pig's taenia coli, *Am. J. Physiol.*, 1963, **205**, 94.
23. SCHATZMANN, H. J., Calciumaufnahme und Abgabe am Darmmuskel des Meerschweinchens, Pflügers *Arch. ges. Physiol.*, 1961, **274**, 295.
24. SPERELAKIS, N., Ca^{45} and Sr^{89} movements with contraction of depolarized smooth muscle, *Am. J. Physiol.*, 1962, **203**, 860.
25. BIANCHI, C. P., Calcium movements in striated muscle during contraction and contracture, *in* Biophysics of Physiological and Pharmacological Actions, (A. M. Shanes, editor), Washington, D. C., American Association for the Advancement of Science, 1960, 281.
26. HODGKIN, A. L., and KEYNES, R. D., Movements of labelled calcium in squid giant axons, *J. Physiol.*, 1957, **138**, 253.
27. PERSOFF, D. A., A comparison of methods for measuring efflux of labelled potassium from contracting rabbit atria, *J. Physiol.*, 1960, **152**, 354.
28. SCHATZMANN, H. J., Excitation, contraction and calcium in smooth muscle, *in* Pharmacology of Smooth Muscle, (E. Bülbring, A. Kovarikovs and I. Seferns, editors), London, Pergamon Press, 1964, 57.
29. KEYNES, R. D., The ionic fluxes in frog muscle, *Proc. Roy. Soc., London, Series B*, 1954, **142**, 359.
30. VAN BREEMEN, C., and DANIEL, E. E., The influence of high potassium depolarization and acetylcholine on calcium exchange in the rat uterus, *J. Gen. Physiol.*, 1966, **49**, 1299.
31. HASSELBACH, W., Relaxing factor and the relaxation of muscle, *Progr. Biophysics*, 1964, **14**, 167.
32. GROSSMAN, A., and FURCHGOTT, R. F., The effects of external calcium concentration on the distribution and exchange of calcium in resting and beating guinea-pig auricles, *J. Pharmacol. and Exp. Therap.*, 1964, **143**, 107.

33. BRINK, F., The role of calcium ions in neural processes, *Pharmacol. Rev.*, 1954, **6**, 243.
34. SHANES, A. M., Electrochemical aspects of physiological and pharmacological action in excitable cells. I. The resting cell and its alteration by extrinsic factors, *Pharmacol. Rev.*, 1958, **10**, 59.
35. GOODFORD, P. J., The distribution of calcium in intestinal smooth muscle, *in* Muscle, (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, editors), London, Pergamon Press, 1965, 219.
36. WHITTEMBURY, G., Sodium extrusion and potassium uptake in guinea pig kidney cortex slices, *J. Gen. Physiol.*, 1965, **48**, 699.
37. DERVICHIAN, D. G., The physical chemistry of phospholipids, *Progr. Biophysics*, 1964, **14**, 263.
38. MIKULECKY, D. C., and TOBIAS, J. M., Phospholipid-cholesterol membrane model. I. Correlation of resistance with ion content. II. Cation exchange properties. III. Effect of Ca on salt permeability. IV. Ca-K uptake by sonically fragmented erythrocyte ghosts, *J. Cell. and Comp. Physiol.*, 1964, **64**, 151.
39. GENT, W. L. G., TROUNCE, J. R., and WALSER, M., The binding of calcium ion by the human erythrocyte membrane, *Arch. Biochem. and Biophysics*, 1964, **105**, 582.
40. KOKETSU, K., KITAMURA, R., and TANAKA, R., Binding of calcium ions to cell membrane isolated from bullfrog skeletal muscle, *Am. J. Physiol.*, 1964, **207**, 509.
41. FILO, R. S., BOHR, D. F., and RÜEGG, J. C., Glycerinated skeletal and smooth muscle: calcium and magnesium dependence, *Science*, 1965, **147**, 1581.
42. DAVIES, R. E., The role of ATP in muscle contraction, *in* Muscle, (W. M. Paul, E. E. Daniel, C. M. Kay, and G. Monckton, editors), London, Pergamon Press, 1965, 49.
43. LANE, B. P., and RHODIN, J. A. G., Cellular interrelationships and electrical activity in two types of smooth muscle, *J. Ultrastruct. Research*, 1964, **10**, 470.
44. BURNSTOCK, G., and MERRILLEES, N. C. R., Structural and experimental studies on autonomic nerve endings in smooth muscle, *in* Pharmacology of Smooth Muscle, (E. Bülbring, A. Kovarikovs, and I. Seferns, editors), London, Pergamon Press, 1964, 1.
45. ROSTGAARD, J., and BARRNETT, R. J., Fine structure localization of nucleoside phosphatases in relation to smooth muscle cells and unmyelinated nerves in the small intestine of the rat, *J. Ultrastruct. Research*, 1964, **11**, 193.
46. BARTOSZEWICZ, W., and BARRNETT, R. J., Fine structural localization of nucleoside phosphatase activity in the urinary bladder of the toad, *J. Ultrastruct. Research*, 1964, **10**, 599.
47. RHODIN, J. A. G., Fine structure of vascular walls in mammals, *Physiol. Rev.*, 1962, **42**, Suppl. 5, 48.
48. DANIEL, E. E., The interconnection between active transport and contracture in uterine tissues, *Can. J. Physiol. and Pharmacol.*, 1964, **42**, 453.
49. USSING, H. H., The distinction by means of tracers between active transport and diffusion, *Acta Physiol. Scand.*, 1949, **19**, 43.