The Cellular Transport of Calcium in Rat Liver

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ABSTRACT The bidirectional transport of calcium in rat liver was studied using slices labeled with Ca^{47} in a closed two compartment system. Steady-state conditions were observed with influx and efflux transfer coefficients of 0.070 and 0.018 per minute, respectively. The rapidly exchanging cell fraction of calcium existed at a concentration three times higher than the average cell concentration of calcium and occupied cell loci comprising less than 25% of the cell mass, suggesting that calcium associated with the cell membranes, nuclei, and mitochondria participated in the rapidly exchanging fraction. At pH 7.4 and 37°C, the influx transfer coefficient was 25% above the steady-state condition and accumulation of calcium by the slices occurred. Studies of the effects of varied physical and chemical conditions revealed that the influx transfer coefficient was increased by elevated pH, strontium, certain metabolic inhibitors, and 2 mM concentrations of cyclic adenosinemonophosphate and adenosinetriphosphate. The influx transfer coefficient was decreased by reduced temperature, decreased pH, magnesium, and 10 mM adenosinetriphosphate. The efflux transfer coefficient was increased by elevated pH, strontium, iodoacetate, and adenosinetriphosphate, and was decreased by reduced temperature and by *N*-ethylmaleimide. These data support the thesis that cell transport of calcium is accomplished by the attachment of calcium atoms to the cell surface and transport through the plasma membrane bound to either specific carriers or to membrane constituents. Conditions which change the affinities, capacities, and mobilities of plasma membrane ligands that bind calcium or cause extracellular chelation of calcium are capable of altering the rate of calcium transport.

60 to 90% of the calcium in various mammalian soft tissues is intracellular (1) and exists as discrete fractions bound to macromolecular constituents of plasma membranes, nuclei, mitochondria, and other cellular structures (2–4). These cellular calcium fractions are critically involved in membrane stabilization, excitation-contraction coupling, muscle contraction, endocrine and exocrine gland secretion, and mitochondrial function. In muscle and nerve, calcium effects a stabilization of the cell membrane to depolarizing

influences with little or no increase in resting membrane potential (5). In muscle, the imposition of a depolarizing stimulus sufficient to cause contractile activity is accompanied by increased calcium influx (6–8). Since calcium also stimulates contractile activity directly, movement of calcium atoms from the plasma membrane and its invaginations to the sarcomeres has been suggested as the mechanism responsible for the coupling of excitation and contraction (6, 9, 10). In lacrimal, salivary, adrenal medullary, and neurohypophyseal tissue, calcium is required for glandular secretion and an increase in calcium influx occurs during stimulation of secretion (11–15). In mitochondria, an excessive uptake of calcium leads to swelling and loss of oxidative functions (16).

Despite the variety of physiologic and biochemical processes (1, 5–22) which are influenced by calcium, the mechanisms by which cell concentrations of calcium are controlled are poorly understood. Most cell membranes are permeable to calcium and the extracellular concentration of calcium is therefore a determinant of cell calcium concentrations (1). In normocalcemia, however, factors which directly affect cell membrane transport of calcium would be expected to play a key role in the regulation of cell calcium concentrations. The present investigation was undertaken to devise and study a model system in which bidirectional cell transport of calcium could be measured simultaneously and the effects of varied physical, chemical, and metabolic conditions assessed. Liver was chosen for this work because it presumably lacks specialized mechanisms for calcium transport which pertain in tissues with irritability, contractility, or a specific role in calcium conservation, but nevertheless shows a high exchangeability with radiocalcium in vivo (1). An investigation of the characteristics of calcium transport in such a tissue would provide basic information regarding cell transport of calcium and comparative data for assessing the physiologic significance of calcium movements in the tissues of the neuromuscular, glandular, and gastrointestinal systems.

METHODS

Mature male Wistar rats, 5 to 10 months old and weighing 350 to 500 g, were decapitated. Their livers were removed and sliced free hand to a thickness of 0.3 mm with a Stadie-Riggs blade. 5 g of slices were placed in 50 ml of a buffered pH 7.4 medium of the following composition: 148 mM Na^+, 5.0 mM K^+, 1.5 mM Ca^{++}, 128 mM Cl^-, 25 mM HCO_3^-, 1.0 mM $PO_4^{\equiv}$, 5.5 mM glucose, and 10 to 15 μc Ca^{47} (Union Carbide Nuclear Co., Oak Ridge, Tenn.). The slices were incubated at 37°C in a Dubnoff metabolic shaker at 105 oscillations per min, gas phase 95% O_2, 5% CO_2. After 30 min, the labeled slices were removed and washed twice for 5 min periods in nonradioactive medium. 2½ g of washed, blotted slices were then incubated in 25 ml of nonradioactive medium for 180 min in a Dubnoff metabolic shaker at 37°C, 105 oscillations per min, gas phase, 95% O_2, 5% CO_2. For studies at pH 6.8, the gas phase was 80% O_2, 20% CO_2 and for studies at pH 8.2, the gas phase was 100% O_2. Periodically, 0.25 ml samples of medium were removed for Ca^{47} analysis. At the end of incubation, the

slices were removed and blotted. The water, calcium, and Ca^{47} concentrations of pre- and postincubation slices were determined. Calcium analyses and pH measurements were performed on pre- and postincubation samples of medium.

Radioactivity measurements were made at constant geometry in a scintillation spectrometer calibrated to detect gamma rays above 0.3 Mev, thus eliminating emissions from the Sc^{47} daughter of Ca^{47}. After counting, liver samples were dried at 105°C for 48 hr and their water content determined by differential weight. The dried slices and postincubation media were prepared for calcium analysis by homogenization in 10% trichloroacetic acid. Calcium analyses were performed by a modification of the method of Berger (23); calcium was precipitated as the oxalate salt and after washing, the oxalate content of the precipitate was determined by cerate oxidation. In experiments in which magnesium and acid-soluble phosphorus were measured, extraction in 10% trichloroacetic acid was performed as above. Magnesium was determined by complexometric titration with EDTA of extracts previously freed of calcium by oxalate precipitation (24). The acid-soluble phosphorus content of the extracts was determined by the method of Fiske and Subbarow (25).

To test for viability, the preparative and experimental procedures described above were repeated without addition of Ca^{47}. Periodically, 300 mg samples of slices were removed and their oxygen utilization measured in a Warburg apparatus.

CALCULATIONS

The slices were assumed to contain interstitial and cellular calcium fractions. After equilibration of the slice interstitium with the medium, calcium exchange between the cellular and total extracellular phases could be expressed by a differential equation describing a closed two compartment system,

$$\frac{d(\mathrm{Ca})_s}{dt} = -\mathrm{K}_{sb}(\mathrm{Ca})_s + \mathrm{K}_{bs}(\mathrm{Ca})_b \qquad (1)$$

where $(\mathrm{Ca})_s$ = radiocalcium concentration of slices (CPM/g)
$(\mathrm{Ca})_b$ = radiocalcium concentration of medium (CPM/ml)
K_{sb} = efflux transfer coefficient
K_{bs} = influx transfer coefficient

Since the system is closed, radiocalcium disappearing from one phase must appear in the other,

$$m\frac{d(\mathrm{Ca})_s}{dt} = -v\frac{d(\mathrm{Ca})_b}{dt} \qquad (2)$$

where m = mass of slices (2.5 g)
v = volume of medium (25 ml)

Substituting Equation 2 in Equation 1,

$$\frac{d(\mathrm{Ca})_b}{dt} = \frac{m}{v}\mathrm{K}_{sb}(\mathrm{Ca})_s - \frac{m}{v}\mathrm{K}_{bs}(\mathrm{Ca})_b \qquad (3)$$

Since all the radiocalcium was initially present in the slices,

$$m(\mathrm{Ca})_{so} = m(\mathrm{Ca})_s + v(\mathrm{Ca})_b \tag{4}$$

where $(\mathrm{Ca})_{so}$ = radiocalcium concentration of the slices at zero time. Substituting Equation 4 in Equation 3, the following differential equation is obtained,

$$\begin{aligned}\frac{d(\mathrm{Ca})_b}{dt} &= \frac{m}{v}\mathrm{K}_{sb}\left[(\mathrm{Ca})_{so} - \frac{v}{m}(\mathrm{Ca})_b\right] - \frac{m}{v}\mathrm{K}_{bs}(\mathrm{Ca})_b \\ &= \frac{m}{v}\mathrm{K}_{sb}(\mathrm{Ca})_{so} - \left[\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}\right](\mathrm{Ca})_b\end{aligned} \tag{5}$$

The solution of Equation 5 is,

$$(\mathrm{Ca})_b = \frac{\frac{m}{v}\mathrm{K}_{sb}(\mathrm{Ca})_{so}}{\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}}\left[1 - e^{-\left(\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}\right)t}\right] \tag{6}$$

At isotopic equilibrium,

$$(\mathrm{Ca})_{b\infty} = \frac{\frac{m}{v}\mathrm{K}_{sb}(\mathrm{Ca})_{so}}{\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}} \tag{7}$$

Substituting Equation 7 in Equation 6 and rearranging terms yields

$$1 - \frac{(\mathrm{Ca})_b}{(\mathrm{Ca})_{b\infty}} = e^{-\left(\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}\right)t} \tag{8}$$

$$\ln\left[1 - \frac{(\mathrm{Ca})_b}{(\mathrm{Ca})_{b\infty}}\right] = -\left(\mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs}\right)t \tag{9}$$

If experimental values of $1 - \frac{(\mathrm{Ca})_b}{(\mathrm{Ca})_{b\infty}}$ are plotted semilogarithmically against time, a straight line is obtained, the slope of which,

$$S = \mathrm{K}_{sb} + \frac{m}{v}\mathrm{K}_{bs} \tag{10}$$

Using Equations 7 and 10 and solving simultaneously for K_{sb} and K_{bs},

$$K_{sb} = \frac{S(\mathrm{Ca})_{b\infty}\frac{v}{m}}{(\mathrm{Ca})_{so}} \tag{11}$$

$$K_{bs} = [S - K_{sb}]\frac{v}{m} \tag{12}$$

In each experiment, a plot of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ against time yielded a linear relation between approximately 10 min and 90% of equilibrium, validating the assumption of a closed two compartment system. Between 0 and 10 min, a curve of greater slope was obtained which was assumed to represent equilibration of Ca^{47} initially in the slice interstitium with the medium. To eliminate interstitium-medium equilibration from consideration, the following equation is substituted for Equation 11,

$$K_{sb} = \frac{S[(Ca)_{b\infty} - (Ca)_{bo}]\frac{v}{m}}{(Ca)_{so} - (Ca)_{bo}\frac{v}{m}} \tag{13}$$

where $(Ca)_{bo}$ = the apparent extracellular radiocalcium concentration at time zero. This value could be determined by extrapolation of the linear portion of the curve to zero time and the use of the intercept value of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ to calculate $(Ca)_{bo}$. A digital computer program was devised to yield a least squares linear fit of values of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ against time beween 10 min and 90% of equilibrium. The slope and intercept of this line were then utilized to calculate K_{sb} and K_{bs}, employing Equations 12 and 13.[1] For the purpose of illustration, a semilogarithmic plot of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ against time for a representative study and the method of calculation is shown in Fig. 1.

RESULTS

General Features The calcium concentrations of the rat livers employed in these studies ranged from 1.25 to 1.92 mmole/kg. After slicing, incubation in radioactive media for 30 min, and washing, slice concentrations of calcium increased to the range of 2.00 to 3.50 mmole/kg. During incubation, uptake of Ca^{47} by the slices occurred rapidly and the liver slices assumed a specific activity approximately 70% that of the medium. The net transfer of Ca^{47} from medium to slices was 10 to 20%. In preliminary studies in which incubation in radioactive media was continued beyond 30 min, additional net transfer of Ca^{47} occurred at a very slow rate. Thus, the calcium present in the liver slices

[1] It was initially expected that $(Ca)_{so}$ could be obtained from the sample of radioactive slices not used for incubation. In approximately 20% of the experiments, it was found that $(Ca)_{so}$ so obtained did not agree with that obtained from the sum of slice and buffer radioactivities at the end of incubation. Since this indicated an occasional inhomogeneity in so far as Ca^{47} content was concerned, the sum of slice and buffer radioactivities was routinely used to obtain $(Ca)_{so}$.

at the end of incubation in radioactive media consisted of three fractions, an extracellular fraction of 0.3 mmole per kg, a rapidly exchanging cell calcium fraction comprising two-thirds, and a slowly exchanging cell fraction comprising one-third of the intracellular calcium.[2]

During subsequent incubation in nonradioactive media for 180 min, slice calcium concentrations increased further, as will be shown subsequently, and decreases of 0.05 to 0.15 mM occurred in the calcium concentrations of the media. The 2.5 g of washed slices used for incubation in nonradioactive media

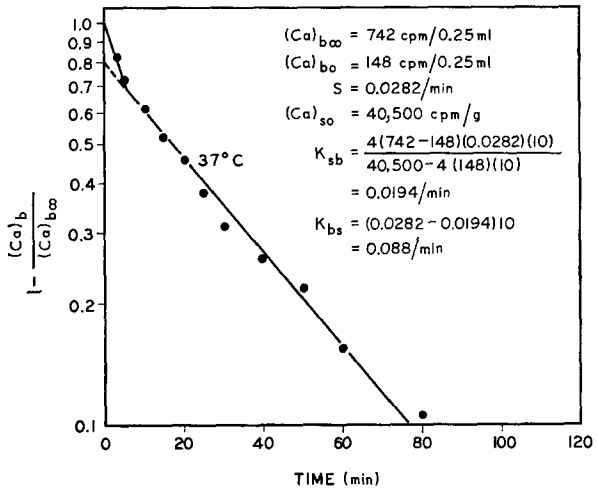

FIGURE 1. Semilogarithmic plot of $1-\frac{(Ca)_b}{(Ca)_{b\infty}}$ vs. time for a representative study at 37°C. The method of calculation of K_{sb} and K_{bs} is shown.

consisted of 0.54 to 0.63 g solids and 1.87 to 1.96 ml water. During incubation, losses of slice solids of 0.07 to 0.16 g occurred. Slice water decreased by 0.01 to 0.17 ml except when metabolic inhibitors were added. In such studies, gains of 0.06 to 0.24 ml water occurred. At the end of incubation, when no further increases in the Ca^{47} concentrations of the media were detectable, the specific activities of the slices exceeded those of the media by 24 to 68%.

[2] The extracellular space of the slices was assumed to comprise 22% of the wet weight of the slices (45). Using the control group for sample calculations:

Extracellular calcium = 1.5 mmole/liter × 0.22 liter/kg slices = 0.33 mmole/kg slices

Cell calcium = 2.65 mmole/kg − 0.33 mmole/kg = 2.32 mmole/kg slices

Since the rapid exchangeability of the slices was 70%, rapidly exchanging tissue calcium = 2.65 mmole/kg × 0.70 = 1.85 mmole/kg slices; rapidly exchanging cell calcium = 1.85 mmole/kg − 0.33 mmole/kg = 1.52 mmole/kg slices.

These values for cell calcium represent average cell values and do not take into account discontinuous distribution of calcium within the liver cell.

The viability of the liver slices during these studies was confirmed by collateral measurements of oxygen consumption. The results of a representative study are shown in Table I. Oxygen consumption was relatively constant

TABLE I

OXYGEN CONSUMPTION OF RAT LIVER SLICES (37°C, IN AIR)

Time*	Oxygen consumption
min	*μl/g dry weight/hr*
15–65	3800
45–95	3420
120–170	3900
180–230	3620
240–290	2440

* Time refers to minutes after sacrifice of animal. The oxygen consumption of each sample was measured for a 50 min period.

TABLE II

EFFECT OF TEMPERATURE AND pH ON BIDIRECTIONAL CALCIUM TRANSPORT IN RAT LIVER SLICES

	No. of experiments	K_{bs} per min	K_{sb} per min	Initial slice calcium	Change in slice calcium
				mmole/kg	*mmole/kg*
37°C, pH 7.4	16	0.087±0.0032	0.0182±0.00050	2.65±0.089	+0.88±0.10
22°C, pH 7.4	8	0.080±0.0027	0.0138±0.00092 $P < 0.001$	2.75±0.12	+0.82±0.19
7°C, pH 7.4	7	0.037±0.0036 $P<0.001$	0.0076±0.00089 $P<0.001$	2.58±0.099	
0°C, pH 7.4	6	0.015±0.0026 $P<0.001$	0.0029±0.00021 $P<0.001$	2.70±0.11	
37°C, pH 6.8	5	0.072±0.0046 $P<0.05$	0.0174±0.00031	2.75±0.18	+0.54±0.13
37°C, pH 8.2	7	0.146±0.012 $P<0.001$	0.0226±0.0015 $P<0.01$	2.89±0.14	+1.46±0.28 $P<0.05$

Values are means ± SEM.
P values indicate significance compared to studies conducted at 37°C and pH 7.4.

during the 4 hr period necessary for preparation and incubation of the slices but decreased when incubation was continued beyond this period.

Effect of Temperature and pH (Table II) At 37°C and pH 7.4, the mean influx and efflux transfer coefficients (K_{bs} and K_{sb}) were 0.087 and 0.0182 per min respectively. As the temperature of incubation was decreased, a progressively slower rate of loss of Ca^{47} from the slices occurred as shown in Fig. 2. At 22°C, K_{bs} was not significantly altered, K_{sb} was decreased by 24%, and

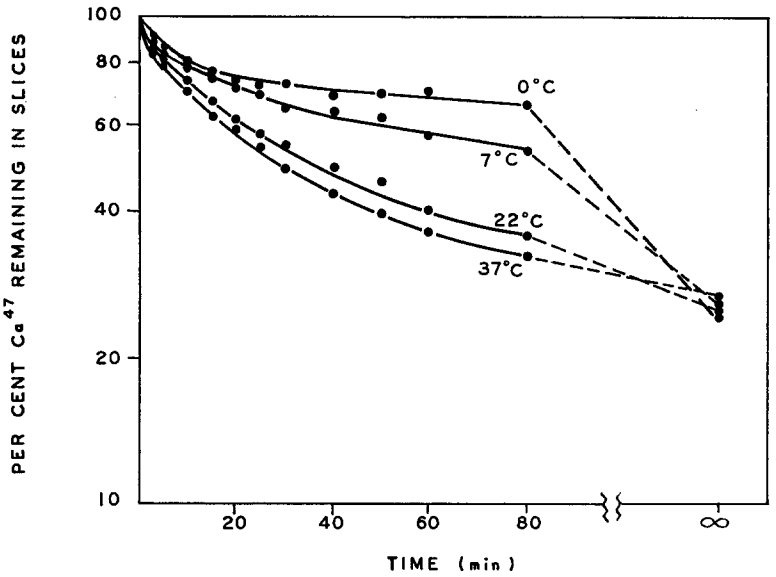

FIGURE 2. Effect of incubation temperature on the loss of Ca^{47} from liver slices. Incubation temperature was raised to 37°C after 80 min in studies conducted at 7°C and 0°C.

the accumulation of calcium by the slices was similar to that at 37°C. At 7 and 0°C, K_{bs} and K_{sb} were 42% and 17% of their values at 37°C respectively. The relation between K_{bs} and K_{sb} and temperature is shown in Fig. 3.

From Fig. 2 it is apparent that an inordinately long period of time would

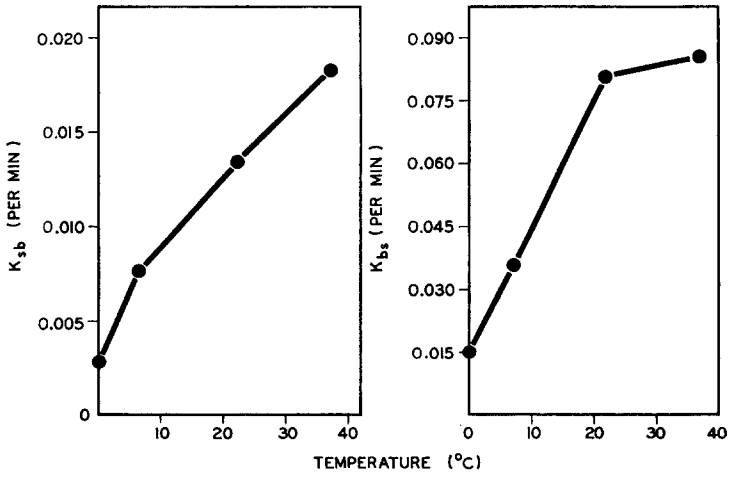

FIGURE 3. Effect of incubation temperature on efflux (K_{sb}) and influx (K_{bs}) transfer coefficients. A decrease in temperature from 37°C to 22°C had an insignificant effect on K_{bs}.

be required to achieve isotopic equilibrium at 7 and 0°C if it occurred at approximately the same point as at 37°C and 22°C. To obtain values for $(Ca)_{b\infty}$ within a shorter period, the temperature of these experiments was increased to 37°C after 80 min of incubation. The effect of rewarming on values of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ in a representative experiment conducted at 0°C is shown in Fig. 4. Because of the possibility that calcium transport might be qualitatively as well as quantitatively different or a different equilibrium point might pertain at reduced temperature than at 37°C, it was necessary to validate this

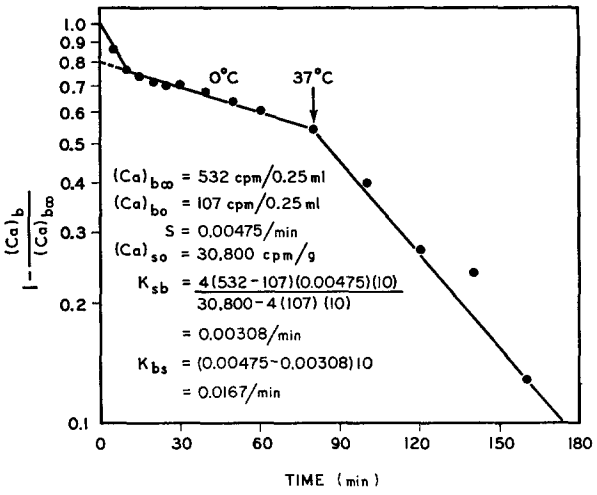

FIGURE 4. Semilogarithmic plot of $1 - \frac{(Ca)_b}{(Ca)_{b\infty}}$ vs. time for a representative study at 0°C. The increase in incubation temperature to 37°C after 80 min resulted in a marked acceleration of slice loss of Ca^{47}. The method of calculation of K_{sb} and K_{bs} is shown.

modification in procedure for reduced temperature studies. This was accomplished by comparing values of K_{sb} obtained by the usual method shown in Fig. 4 with those obtained by determining the rate of decrease of slice Ca^{47} concentration between 10 and 30 min. It was assumed that the latter would approximate the efflux transfer coefficient since the accumulation of Ca^{47} in the medium and the uptake of Ca^{47} by the slices would both be small. At 7°C, the mean fractional rate of decrease of slice Ca^{47} concentration was 0.0081 ± 0.00073 per min and at 0°C, 0.0029 ± 0.00033 per min. The concordance with values for K_{sb} at 7°C and 0°C indicated that despite the radically different rates of metabolic activity and intracellular water and electrolyte concentrations which exist at reduced temperature as compared to 37°C, calcium transport was not qualitatively altered.

A decrease in pH to 6.8 resulted in a 17% decrease in K_{bs} and an increase in pH to 8.2 in a 68% increase in K_{bs}. There was no significant alteration of

K_{sb} at pH 6.8 but an increase in pH to 8.2 resulted in a 24% increase in K_{sb}. The greater effect of pH on the influx than on the efflux transfer coefficient resulted in 39% less accumulation of calcium by the slices at pH 6.8 and 66% more accumulation of calcium at pH 8.2 than at pH 7.4.

Effect of Cation Addition (Table III) These studies were performed to ascertain whether calcium transport was influenced by other cationic species. The addition of 50 mM sodium or potassium chloride to the medium had no discernible effects despite the resultant increases in osmolality and ionic strength. The addition of 25 mM magnesium chloride caused a 36% decrease in K_{bs} but had no effect on K_{sb}. As a result, a 15% decrease in slice calcium concentrations occurred as compared to a 33% increase in the control studies.

TABLE III

EFFECT OF CATION ADDITION ON BIDIRECTIONAL CALCIUM TRANSPORT IN RAT LIVER SLICES (37°C, pH 7.4)

	No. of experiments	K_{bs} per min	K_{sb} per min	Initial slice calcium	Change in slice calcium
				mmole/kg	*mmole/kg*
Control	16	0.087±0.0032	0.0182±0.00050	2.65±0.089	+0.88±0.10
NaCl, 50 mM	5	0.085±0.0041	0.0172±0.00075	2.89±0.16	+0.61±0.099
KCl, 50 mM	4	0.089±0.0016	0.0174±0.0015	2.47±0.16	+0.97±0.42
$MgCl_2$, 25 mM	6	0.056±0.0017	0.0189±0.0011	2.82±0.16	−0.43±0.17
		$P<0.001$			$P<0.001$
$SrCl_2$, 12.5 mM	6	0.124±0.0068	0.0225±0.0012	2.82±0.17	+9.3±0.52*
		$P<0.001$	$P<0.01$		

Values are means ± SEM.
* Calcium + strontium.

The addition of 12.5 mM strontium chloride increased K_{bs} and K_{sb} by 42% and 24%, respectively, and the chemical analysis of the postincubation slices indicated a large accumulation of strontium by the slices.

Since the slice calcium loss which occurred with the addition of 25 mM magnesium chloride was entirely the result of a decrease in the influx transfer coefficient, the effect of lower concentrations of magnesium was studied by measuring changes in slice calcium concentrations in a nonradioactive system. The methods and results of these collateral studies are shown in Table IV and indicate that magnesium concentrations as low as 2.6 mM significantly inhibit slice calcium uptake. A proportionality of end-incubation slice concentrations of magnesium to the magnesium concentrations of the media was observed.

Effect of Metabolic Inhibitors (Table V) Metabolic inhibitors have been shown to alter calcium transport in other systems (26–28). The present studies were restricted to cyanide (NaCN) which inhibits cytochrome oxidase and

thereby reduces oxygen utilization and the production of adenosinetriphosphate (ATP); iodoacetate (IoAc) which primarily inhibits triosephosphate dehydrogenase and thereby interrupts glycolysis but in higher concentrations reacts with other cell proteins (29); and *N*-ethylmaleimide (NEM) and sodium parahydroxymercuribenzoate (PMB) which at high concentrations not only block sulfhydryl groups which might serve as binding sites for calcium (30), but are also capable of reaction with a variety of cell proteins (29). The addition of 10 mM NaCN to the medium increased K_{bs} by 29% but did not change K_{sb} significantly. Sodium iodoacetate (IoAc) in a similar concentration

TABLE IV

EFFECT OF MEDIUM MAGNESIUM CONCENTRATIONS ON CALCIUM AND MAGNESIUM CONCENTRATIONS OF RAT LIVER SLICES (37°C, pH 7.4)

Medium magnesium concentration	No. of experiments	Slice magnesium concentration		Slice calcium concentration	
		Initial	Final	Initial	Change
mM		*mmole/kg*			*mmole/kg*
0.75			4.82±0.12		+1.73±0.13
2.60	6	8.16±0.30	7.89±0.52	2.57±0.10	+1.24±0.13 ($P<0.05$)
6.25			11.2±0.13		+1.17±0.14 ($P<0.05$)
0.75	8	8.02±0.22	5.20±0.20	2.86±0.16	+1.18±0.12
13.3			16.1±0.55		+0.11±0.098 ($P<0.001$)

In each experiment, liver slices from the same animal were divided into three or four samples of 2.0 g each. One sample was analyzed for initial Ca and Mg concentrations. The remaining samples were incubated for 3 hr in 25 ml of a buffered medium of the following composition: 80 mM Na^+, 5.0 mM K^+, 1.5 mM Ca^{++}, 88 mM Cl^-, 50 mM Tris, 11.1 mM glucose, and the $MgCl_2$ concentrations shown above. Incubation was conducted in a Dubnoff metabolic shaker at 105 oscillations/min with 100% O_2. Values are means ±SEM.

resulted in 43% and 21% increases in K_{bs} and K_{sb} respectively. *N*-ethylmaleimide (NEM) in 5 mM concentration resulted in an insignificant increase in K_{bs} and decreased K_{sb} by 18%. The effect of PMB was studied in a pH 7.8 glycylglycine-buffered medium of the following composition: 250 mM glycylglycine, 79 mM Na^+, 5.0 mM K^+, 1.5 mM Ca^{++}, 6.5 mM Cl^-, 1.0 mM $PO_4^{\equiv}$, and 5.5 mM glucose. The addition of 2.5 mM PMB had no significant effects on K_{bs} or K_{sb}. The increases in influx transfer coefficients which occurred with NaCN and IoAc resulted in increased slice accumulations of calcium. The increases in slice accumulation of calcium observed with NEM and PMB may possibly have been due to the small increases in influx transfer coefficients which occurred. In the NEM studies, the concomitant decrease in the efflux transfer coefficient may also have been a factor.

Effect of Nucleotide and Orthophosphate Addition (Table VI) Various nucleotides have been shown to have a strong chelating affinity for calcium (31)

which might be of physiologic importance in calcium transport. Two of these, 3′, 5′ cyclic adenosinemonophosphate (C-AMP) and adenosinetriphosphate (ATP), play key roles in a variety of cellular processes and were therefore studied to determine their effects on calcium transport. The addition of C-AMP in 2 mM concentration increased K_{bs} by 18% but this effect was lost at 10 mM concentration. Neither concentration of C-AMP had a significant effect on K_{sb}. Adenosinetriphosphate (ATP) in 2 mM concentration increased

TABLE V

EFFECT OF METABOLIC INHIBITORS ON BIDIRECTIONAL CALCIUM TRANSPORT IN RAT LIVER SLICES (37°C)

	No. of experiments	K_{bs} per min	K_{sb} per min	Initial slice calcium	Change in slice calcium
				mmole/kg	*mmole/kg*
Control	16	0.087±0.0032	0.0182±0.00050	2.65±0.089	+0.88±0.10
NaCN, 10 mM, pH 7.4	8	0.112±0.0093 $P<0.01$	0.0208±0.0017	3.03±0.23	+1.55±0.22 $P<0.01$
Na iodoacetate, 10 mM, pH 7.4	7	0.124±0.0087 $P<0.001$	0.0220±0.0015 $P<0.01$	3.35±0.24	+1.59±0.12 $P<0.001$
N-Ethylmaleimide, 5 mM, pH 7.4	7	0.095±0.0064	0.0150±0.0014 $P<0.05$	2.66±0.19	+1.51±0.21 $P<0.01$
Glycylglycine medium, pH 7.8	6	0.091±0.0064	0.0161±0.0012	3.11±0.25	+0.58±0.23
P-Hydroxymercuribenzoate, 2.5 mM in glycylglycine medium, pH 7.8	7	0.107±0.0060	0.0186±0.0012	3.15±0.12	+1.52±0.22 $P<0.02$

Values are means ± SEM.

K_{bs} by 14% and in 10 mM concentration, decreased K_{bs} by 16%. At 2 mM concentration, ATP caused an insignificant increase in K_{sb} but at 10 mM concentration, K_{sb} was increased by 28%. The increases in the influx transfer coefficients produced by 2 mM concentrations of C-AMP and ATP did not increase slice accumulations of calcium significantly. The 66% decrease in slice accumulation of calcium observed with 10 mM C-AMP is unexplained since K_{bs} and K_{sb} were not significantly different from control values. In 10 mM ATP, the concomitant decrease in K_{bs} and increase in K_{sb} abolished accumulation of calcium by the slices.

The effect of high concentrations of inorganic orthophosphate (Pi) was

studied to ascertain whether the effects observed with C-AMP and ATP might be the result of the generation of Pi by hydrolysis. In addition, high concentrations of Pi frequently occur in the gastrointestinal contents and renal tubular urine. In certain pathologic states associated with disturbed calcium metabolism, such as uremia and hypoparathyroidism, high concentrations of Pi may be present in the extracellular fluid. The addition of 10 mM Pi to the medium resulted in spontaneous precipitation in approximately 1 hr if not

TABLE VI

EFFECT OF NUCLEOTIDES AND ORTHOPHOSPHATE ADDITION ON BIDIRECTIONAL CALCIUM TRANSPORT IN RAT LIVER SLICES (37°C, pH 7.4)

	No. of experiments	K_{bs} per min	K_{sb} per min	Initial slice calcium	Change in slice calcium
				mmole/kg	*mmole/kg*
Control	16	0.087±0.0032	0.0182±0.00050	2.65±0.089	+0.88±0.10
3′, 5′ cyclic adenosine monophosphate, 2 mM	7	0.103±0.0068 $P<0.05$	0.0193±0.00075	2.61±0.17	+0.94±0.15
3′, 5′, cyclic adenosine monophosphate, 10 mM	6	0.091±0.0056	0.0198±0.0012	2.89±0.16	+0.30±0.21 $P<0.02$
Adenosinetriphosphate, 2 mM	7	0.099±0.0034 $P<0.05$	0.0205±0.00056 $P<0.05$	2.87±0.13	+1.22±0.20
Adenosinetriphosphate, 10 mM	6	0.073±0.0043 $P<0.05$	0.0234±0.0011 $P<0.001$	2.72±0.14	−0.04±0.27 $P<0.001$
Orthophosphate, 10 mM	6			2.85±0.23	+2.58±0.56 $P<0.001$

Values are means ±SEM.

placed in contact with liver slices. During the first hour of incubation with liver slices, Ca^{47} was lost from the slices at a rate similar to that of the control incubations (Fig. 5). However, after 1 hr, reaccumulation of Ca^{47} by the slices occurred. These data indicate that in the presence of excess Pi, calcium transport is normal prior to a phase change. When colloidal calcium phosphate complexes are formed, these are rapidly taken up by the slices. Therefore, the effects observed with nucleotides are not the result of an excess of Pi generated by hydrolysis since a stimulation of transport is not apparent at high Pi concentrations in the absence of a phase change and totally dissimilar effects on liver slice accumulation of calcium are observed with added nucleotides and Pi.

The relation between cell concentrations of calcium and phosphate was further investigated in the collateral studies shown in Table VII. Incubation for 180 min in a medium containing 1.5 mM Ca^{++} resulted in a mean slice accumulation of calcium of 1.13 mmole/kg and a mean loss of slice total

TABLE VII

EFFECT OF MEDIUM CALCIUM AND ATP CONCENTRATIONS ON CALCIUM AND TOTAL ACID-SOLUBLE PHOSPHORUS (TAS-P) CONCENTRATIONS OF RAT LIVER SLICES (37°C, pH 7.4)

Medium calcium concentration	No. of experiments	Slice calcium concentration		Slice TAS-P concentration	
		Initial	Change	Initial	Final
mM		*mmole/kg*		*mmole/kg*	
1.5	8	2.79±0.17	+1.13±0.14	42.1±1.7	29.0±1.3
14.0			+17.9±0.99		33.6±1.5
1.5*	3	2.73±0.10	−0.37±0.14	41.8±1.8	37.6±3.4
14.0*			+9.6±1.7		40.2±2.7

See Table IV for experimental procedure. The media were Mg-free. Values are means ±SEM.
* 10 mM ATP added to medium.

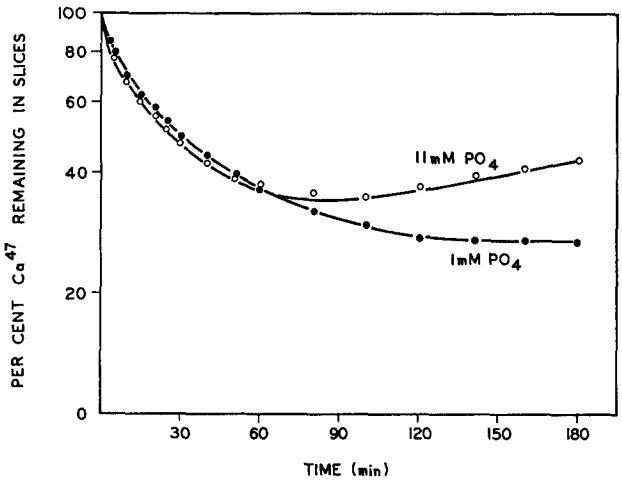

FIGURE 5. Effect of an increased $PO_4^{\equiv}$ concentration on the loss of Ca^{47} from liver slices. After 60 min the slices reaccumulated Ca^{47}.

acid-soluble phosphorus of 13.1 mmole/kg. An increase in the calcium concentration of the medium to 14 mM resulted in a mean slice accumulation of calcium of 17.9 mmole/kg and a mean slice loss of total acid-soluble phosphorus of 8.5 mmole/kg. The addition of 10 mM ATP prevented significant calcium accumulation in 1.5 mM Ca^{++} and markedly reduced the accumulation ordinarily seen in 14 mM Ca^{++}. At both the low and high calcium con-

centrations, ATP addition prevented significant phosphorus loss by the slices. These data indicate that at physiologic calcium concentrations, alterations in slice concentrations of calcium are unrelated to slice concentrations of phosphorus but at high calcium concentrations, a portion of the increased slice calcium accumulation occurs as a calcium phosphate complex.

DISCUSSION

The results of these experiments are referable to exchange between the extracellular calcium and the rapidly exchanging cell calcium fraction. The slowly exchanging fraction, which was not labeled during the initial 30 min of incubation, probably became partially labeled during the subsequent 3 hr of incubation in nonradioactive media. However, it appears that little recycling of Ca^{47} accumulated by the slowly exchanging fraction to the rapidly exchanging cell fraction or the extracellular space occurred since a significantly higher specific activity developed in the slices than in the medium upon incubation in nonradioactive media. It can therefore be assumed that exchange between the rapidly and slowly exchanging cell calcium fractions was slow compared to exchange between the rapidly exchanging cell fraction and the extracellular calcium, and the utilization of a closed two compartment model and the rate of accumulation of Ca^{47} in the extracellular phase is valid for determining transfer coefficients for the latter exchange process.

Since the calcium concentration of the media decreased very slightly during incubation, the derived influx transfer coefficients bear a direct relation to calcium influx rates. In Fig. 6, the linear relation between the mean influx transfer coefficients and mean changes in slice calcium concentrations of the control and experimental groups is depicted. In five experimental groups, the mean efflux transfer coefficient varied from the control value by more than $+14\%$ or -6% and these points were not utilized in determining the line of regression or correlation coefficient. Thus, if efflux is not appreciably altered, cell calcium concentrations can be maintained at in vivo levels in media containing 1.5 mM Ca^{++} if the influx transfer coefficient is below 0.070 per min. A fractional influx rate of calcium in this range has been observed in the livers of intact dogs. Injected Ca^{47} disappears from plasma at a rate of 5% per min in dogs with renal pedicle ligations (1). Since liver manifests a high in vivo exchangeability with Ca^{47} and since some recycling of accumulated cellular Ca^{47} back to the extracellular space is also occurring, an influx transfer coefficient slightly in excess of 0.05 per min must apply to liver. In liver slices, the mean influx transfer coefficient is 0.087 per min and accumulation of calcium by the slices occurs. Previous studies of calcium uptake during cell injury (8, 32, 33) indicate that this is probably coincident to the trauma of slice preparation. Since a standard preparation and labeling technique was employed and control values for influx and efflux transfer coeffi-

cients were established with an acceptable variance, an assessment of the effects of altered conditions on calcium transport could be made.

An effect of calcium efflux on cell concentrations of calcium is also ascertainable from Fig. 6. In four of the five experimental groups in which the efflux transfer coefficients were significantly different from control values and in four of the experimental groups in which they were not, considerable

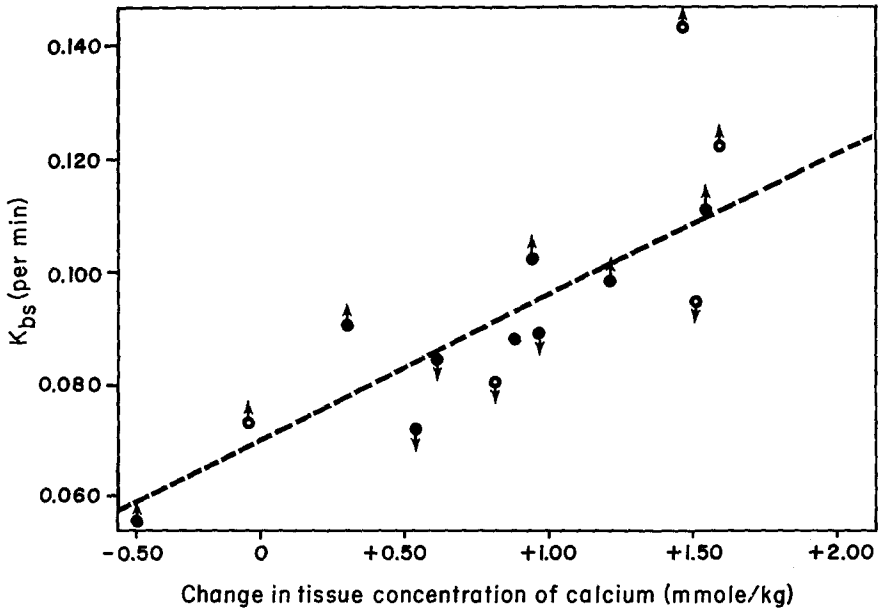

FIGURE 6. Relation of the influx transfer coefficient to tissue accumulation of calcium. The closed circles represent mean values for experimental groups in which the mean efflux transfer coefficients (K_{sb}) did not differ from the mean control value by more than +14% or −6%. The open circles represent groups in which K_{sb} was outside these limits. The arrows indicate the direction of change of K_{sb} with reference to the mean control value. The line of regression and its correlation coefficient, $r = 0.987$, are based on the closed circles only.

displacement from the line of regression occurred. In all eight instances, the direction of displacement agreed with the direction of change of the efflux transfer coefficients from control values. These data suggest that efflux transfer coefficients bear a direct, albeit variable, relation to rates of calcium efflux.

Since steady-state slice concentrations of calcium prevail at influx and efflux transfer coefficients of approximately 0.070 and 0.018 per min, respectively, the size of the rapidly exchanging cell fraction of calcium can be estimated as $0.070/0.018 \times 1.5 = 5.8$ mmole/kg slices. This value exceeds the average cell concentration of exchangeable calcium by almost 300% and suggests that the rapidly exchanging cell fraction of calcium is restricted to

cell loci comprising less than 25% of the total cell mass.[2] A discontinuous distribution of calcium within the cell has been indicated by microincineration and ultracentrifugal studies (2–4). In view of the large influx transfer coefficient observed in liver, cell calcium within and closely related to the plasma membrane is undoubtedly an important component of the rapidly exchanging cell fraction. Calcium within nuclei, mitochondria, and endoplasmic reticulum in continuity with the plasma and nuclear membranes probably participates as well.

In the small intestine, calcium transport has been characterized as active since movement occurs against a concentration gradient and an impairment of transport may be induced by cyanide, iodoacetate, and other metabolic inhibitors (28, 34–36). In liver, as in resting muscle and brain, the few studies which have been performed do not indicate a similar inhibition of calcium transport (6, 37), although additional studies of the effects of inhibitors of oxidative phosphorylation will be required before a final conclusion can be reached. Since much of the calcium in the rapidly exchanging cell fraction is bound to cell constituents, the ionic concentration of calcium within the cell is probably small and a sharp downhill electrochemical gradient therefore exists. Some of the present data suggest that calcium influx is an ionic diffusion with the superimposition of regulatory factors which can alter flow through pores in the cell membrane. An insignificant decrease in influx occurs with a reduction in temperature to 22°C and metabolic inhibition of influx is not observed. Magnesium and hydrogen ions may impede calcium flow by binding to pore surfaces. However, the sharp temperature dependence below 22°C and the stimulation of influx by strontium and 2 mM concentrations of C-AMP and ATP cannot be explained by ionic diffusion. In addition, except for a small effect of NEM, metabolic inhibition of efflux is not observed. These considerations, plus the finding that altered physical and chemical conditions have a greater influence on influx than on efflux transfer coefficients, and previous studies demonstrating stimulation or inhibition of calcium influx in muscle under appropriate conditions (6–8, 38), suggest that calcium influx does not occur by passive diffusion of ionic calcium but by a considerably more complex process.

The present data are compatible with the theory that calcium influx is largely accomplished by the attachment of calcium atoms to the cell surface and transport of the attached atoms through the plasma membrane bound to either specific carriers or to membrane substituents (31). The physical and chemical conditions which influence calcium transport are capable of altering the binding capacity, affinity, and mobility of plasma membrane ligands and of causing extracellular chelation of calcium. Reduced temperature reduces the mobility of binding ligands whereas hydrogen and magnesium ions may compete with calcium for ligand-binding sites. Sodium and

potassium apparently do not have this property in liver though they compete with calcium for binding sites in other tissues (39–41). Stimulation of calcium transport by strontium has not been previously observed in a cellular system but strontium does enhance calcium uptake by isolated mitochondria (42). Possibly in liver, strontium competitively blocks the binding of calcium to extraneous ligands. An increased influx of calcium during exposure to metabolic inhibitors has been observed in muscle (6) as well as liver and this is possibly due to the exposure of more binding sites by metabolic cell injury (33). The stimulatory effects of 2 mM concentrations of C-AMP and ATP may result from their ability to chelate calcium extracellularly (31) with subsequent interaction of the chelates with the cell surface and the transfer of calcium to membrane ligands involved in transport. Such a process would facilitate transport since the chelation reaction causes a reduction in charge and water of hydration of the calcium atoms (31). ATP has also been reported to stimulate calcium uptake in nerve and the shell gland of the hen (43, 44). The loss of stimulation at high nucleotide concentrations is probably due to an extracellular fixation of calcium by chelation to the excess concentrations of nucleotide which cannot interact with the cell membrane.

The present study supports the thesis that calcium influx, since it appears to be mediated by binding processes and not be an unregulated ionic diffusion, is a key factor controlling cell concentrations of calcium. However, cell efflux of calcium and calcium phosphate complex formation are probably physiologically important as well. In addition, metabolic and enzymatic processes, as yet undefined, may play a role in the binding reactions, ligand mobility, or in the maintenance of optimal ligand concentrations. This latter possibility cannot be adequately explored until membrane constituents capable of binding and transporting calcium are isolated and their characteristics investigated.

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