Net Movements of Calcium and Magnesium in Slices of Rat Liver

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ABSTRACT Rat liver slices incubated at 1°C in phosphate (10 mM) or bicarbonate (25 mM) plus phosphate (2 mM)–buffered Ringer's solutions containing 1.2 mM Ca^{2+} underwent a 3-fold increase in Ca^{2+} content relative to their fat-free solids, and lost 10% of their Mg^{2+}. Upon subsequent incubation at 38°C, slices in the bicarbonate medium lost about half of the accumulated Ca^{2+}. This extrusion was less efficient in the phosphate medium. Succinate (40 mM), which strongly stimulated respiration, caused an accumulation of Ca^{2+} in slices incubated in the phosphate medium. The extrusion of Ca^{2+} was prevented by respiratory inhibitors, but not by inhibition of the Na^+ and K^+ transport (by ouabain or K^+-free medium). This suggests that the Ca^{2+} transport was itself directly dependent on high-energy compounds and was not due to a hetero-exchange diffusion of Ca^{2+} against Na^+ ions. Some evidence was obtained for the occurrence of an active accumulation of Mg^{2+} ions.

INTRODUCTION

The intracellular water of liver cells in vivo has a lower apparent concentration of Ca^{2+} (i.e. total cell Ca^{2+}/total cell H_2O), and a higher apparent concentration of Mg^{2+}, than the extracellular fluid (reviewed by Manery, 1954). Preparations of liver cells in vitro (i.e. perfused liver or slices) tend to accumulate Ca^{2+} (Dawkins et al., 1959; Judah and Ahmed, 1963; Wallach et al., 1966), particularly when incubated under metabolically unfavorable conditions. These findings suggest that the intracellular Ca^{2+} content may normally be controlled by an outwardly directed transport mechanism, and Judah and Ahmed (1964) have briefly reported that liver slices can indeed bring about an energy-dependent net extrusion of Ca^{2+}. Liver cells share this property with erythrocytes (Schatzmann, 1966; Olson and Cazort, 1969), cardiac muscle cells (Reuter and Seitz, 1968), and squid axons (Blaustein and Hodgkin, 1968), but it should be noted that these other cells show two apparently different types of transport. The Ca^{2+} extrusion from cardiac muscle and axons is linked by a hetero-exchange diffusion process to the entry of Na^+ into the cells, and

its dependence on energy results indirectly from the energy requirement of the Na^+ transport, which maintains the necessary concentration gradient of Na^+ between the cells and medium. On the other hand, Ca^{2+} extrusion by red cells requires ATP directly and is independent of the occurrence of Na^+ transport.

The possession by the liver cell of an outwardly directed Ca^{2+} transport mechanism is of interest in view of the ability of liver mitochondria to accumulate Ca^{2+} in the cell (Chance, 1965; Carafoli, 1967), and in view of the suggested importance of Ca^{2+} in the control of metabolism (Bygrave, 1966; Whittam, 1968; Kimmich and Rasmussen, 1969).

TABLE I

EXTRACTION OF Ca^{2+} AND Mg^{2+} FROM RAT LIVER SLICES

The slices were incubated for 100 min at 1 °C in the phosphate medium. After blotting and weighing, they were subjected to one of the following extraction procedures.

1. The fat-free solids from 80–150 mg wet weight of slices were allowed to stand for 16–20 hr at room temperature in 5 ml of 0.1 N HNO_3.
2. The fat-free solids were dry-ashed in a silica crucible, and finally dissolved in 5 ml of 0.1 N HNO_3.
3. The wet tissue was heated with 2% HNO_3 (5 ml) in a boiling water bath for 10–15 min. In this case, the fat-free solid content of the tissue was determined on duplicate tissue samples.

Extraction procedure	Ca^{2+}	Mg^{2+}
	mmoles/kg FFDW	
Experiment 1		
1. Dry tissue; 0.1 N HNO_3	16.9 ± 1.1 (10)	31.0 ± 0.4 (10)
2. Dry tissue; ashed	14.7 ± 0.7 (10)	30.4 ± 0.4 (10)
Experiment 2		
1. Dry tissue; 0.1 N HNO_3	15.6 ± 1.8 (8)	27.5 ± 1.6 (6)
3. Wet tissue; hot, 2% HNO_3	16.0 ± 0.9 (8)	29.0 ± 1.8 (6)

In the work described below, it is shown that liver slices can bring about a net extrusion of Ca^{2+} which requires energy from respiration, but which is independent of the occurrence of Na^+ and K^+ transport. There are indications that there may also be a system for Mg^{2+} accumulation.

METHODS

Incubation Procedure Liver slices (0.2–0.3 mm thick) were prepared from male albino rats weighing 200–250 g. Animals were fed ad libitum unless otherwise stated. In most experiments, the slices were first incubated for a total of 90 min at 1°C. For the first 20 min of this period they were incubated together in 20 ml of medium, after which they were distributed, in lots of 80–150 mg wet weight, among 8–10 Warburg manometric vessels. Each Warburg vessel contained 3 ml of medium. During the last 10 min of incubation at 1°C, the flasks were attached to manometers and gassed appropriately (see below). The manometers were then transferred to a bath maintained at 38°C and incubated for varying periods. If respiration was measured, the flasks

contained 0.2 ml of 20% KOH in the center well; readings were started after 5 min of equilibration. In experiments in which only the effects of incubation at 1°C were examined, the slices were incubated together in 20 ml of medium and were transferred to fresh portions of medium after 5, 20, and 50 min.

The phosphate-buffered medium contained Na^+, 161.5 mM; K^+, 5.0 mM; Ca^{2+}, 1.3 mM; Mg^{+2}, 1.0 mM; Cl^-, 151.5 mM; SO_4^{2-}, 1.0 mM; and phosphate (pH 7.4), 10.0 mM; this was gassed with O_2. The bicarbonate medium contained Cl^-, 147.5 mM; HCO_3^-, 25.0 mM; phosphate (pH 7.4), 2.0 mM; and other components as for the phosphate medium; this medium was gassed with 95% O_2–5% CO_2, to give a pH of 7.4. Other agents were added to these media as their Na^+ salts, in exchange for an osmotically equivalent amount of NaCl.

Analyses After the appropriate incubation period, slices were blotted and analyzed for their fat-free solids and water content (Elshove and van Rossum, 1963). Cations were extracted by treatment of the dried tissue with 0.1 N HNO_3 for 16–20 hr at room temperature (Whittam, 1955; Little, 1964). The extraction of Ca^{2+} and Mg^{2+} by this method gave recoveries which were not significantly different from those obtained by boiling the wet tissue with 2% HNO_3, or by dry-ashing the tissue (Table I). Cations were estimated by atomic absorption spectrophotometry. Lactate production was estimated by measuring the lactate content of the incubation medium after removal of the slices. The medium was deproteinized with $HClO_4$ and neutralized with K_2CO_3; lactate was estimated with lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27).

Results are expressed as means ± standard error (number of observations) and are related either to the fat-free dry solids, or to the slice water content.

RESULTS

Incubation at 1°C

Liver slices incubated at 1°C undergo an increase in their water, Na^+ and Cl^- contents, and lose about 75% of their K^+ (Leaf, 1956; Heckmann and Parsons, 1959a; Parsons and van Rossum, 1962a). It can be seen from Fig. 1 that these changes are accompanied by a small but statistically significant loss of Mg^{2+}, and a 3-fold increase in the Ca^{2+} per unit solids. These net changes approached completion after about 40 min. As a result of the simultaneous movements of ions and water, the apparent concentration of Ca^{2+} (i.e. total tissue Ca^{2+}/total tissue water) almost doubled, while the apparent Mg^{2+} concentration was halved (Table II). The net changes taking place in 90 min were very similar in both the phosphate and bicarbonate media (Table II).

Heckmann and Parsons (1959b) showed that the concentration ratios of Na^+ and Cl^- in the liver slice water and medium are compatible with these two ions being in a modified Donnan equilibrium after 60 min incubation at 1°C. The Donnan distribution ratios for other ions may therefore be compared with the Na^+ ratio in order to see if their distribution can also be attributed to a Donnan equilibrium. Table III shows that the distribution of K^+

and Ca^{2+} (like that of Na^{+}) reached equilibrium after 60–90 min, and that these two ions were retained in the tissue at greater concentrations than would be required for a Donnan distribution. This was probably also true for Mg^{2+}, since the distribution ratio for this ion after 120 min was not significantly different from the value at 60 min, and was much greater than the Na^{+} ratio;

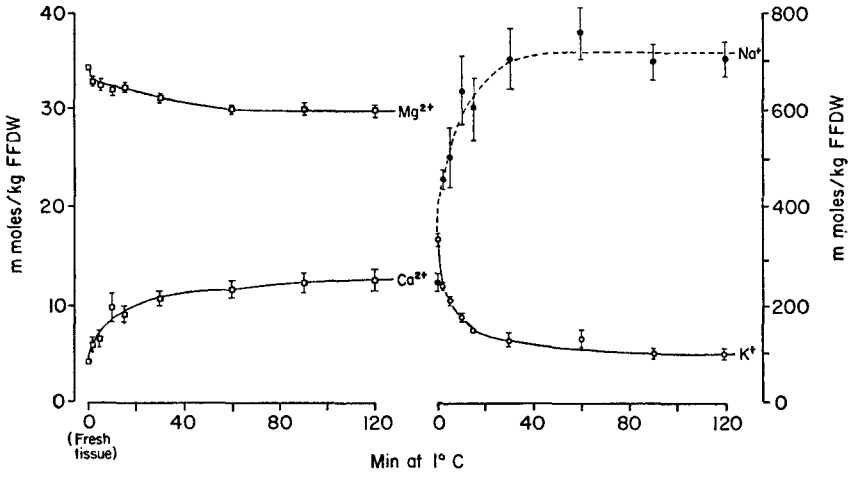

FIGURE 1. Changes in ion content of liver slices incubated at 1°C in phosphate-buffered medium for varying periods.

TABLE II

EFFECT OF DIFFERENT MEDIA ON THE COMPOSITION OF LIVER SLICES INCUBATED AT 1°C

Incubation was conducted for 90 min in either the "phosphate medium" (buffered with 10 mM phosphate) or the "bicarbonate medium" (25 mM bicarbonate plus 2 mM phosphate). For further details, see the text.

	n	Water	Ca^{2+}	Mg^{2+}	Ca^{2+}	Mg^{2+}
		kg/kg FFDW	*mmoles/kg FFDW*		*mmoles/kg water*	
Fresh tissue	6	2.27 ± 0.08	4.1 ± 0.1	34.4 ± 0.1	1.8 ± 0.1	15.2 ± 0.1
Phosphate medium	6	3.48 ± 0.07	12.3 ± 1.0	30.2 ± 0.6	3.3 ± 0.1	8.7 ± 0.4
Bicarbonate medium	8	3.77 ± 0.07	13.9 ± 0.9	32.5 ± 1.2	3.6 ± 0.2	8.6 ± 0.3

nevertheless, there was a small, downward drift of the mean values from 60 to 120 min, which may indicate that Mg^{2+} had not completely attained equilibrium. As suggested by Heckmann and Parsons (1959b) for the case of K^{+}, it may be supposed that Ca^{2+} and Mg^{2+} are each present in the tissue in a "free" form which is in Donnan equilibrium with the ion in the medium, and in a "bound" form which is retained in some way in the tissue. Possible factors involved in such retention may be binding to cellular structures, or presence

within a permeability barrier (e.g. the mitochondrial inner membrane). Calculations of these fractions for Ca^{2+} and Mg^{2+} are shown in Table IV; it should be noted that the calculated contents of "bound" Mg^{2+} would be maximal values if Mg^{2+} had, in fact, not completely reached its equilibrium distribution

TABLE III

DISTRIBUTION RATIOS OF CATIONS BETWEEN LIVER SLICE WATER AND MEDIUM AFTER INCUBATION AT 1°C FOR INCREASING PERIODS OF TIME IN PHOSPHATE MEDIUM

Results are taken from the experiments of Fig. 1. Each value is the mean of six observations.

$[X^{n+}]_i$ = (cation content of slices)/(water content of slices)

$[X^{n+}]_0$ = concentration in medium

Time at 1°C	$[Na^+]_i/[Na^+]_0$	$[K^+]_i/[K^+]_0$	$\sqrt{[Ca^{2+}]_i}/\sqrt{[Ca^{2+}]_0}$	$\sqrt{[Mg^{2+}]_i}/\sqrt{[Mg^{2+}]_0}$
min				
2	1.12 ± 0.04	17.3 ± 0.3	1.37 ± 0.07	3.43 ± 0.02
60	1.31 ± 0.06*	7.2 ± 1.1	1.72 ± 0.05	2.88 ± 0.02
90	1.24 ± 0.06	5.2 ± 0.4	1.66 ± 0.03	2.81 ± 0.05
120	1.23 ± 0.07	5.2 ± 0.4	1.71 ± 0.06	2.76 ± 0.06

* $n = 4$.

TABLE IV

"FREE" AND "BOUND" FRACTIONS OF Ca^{2+} AND Mg^{2+} IN LIVER SLICES INCUBATED FOR 90 MIN AT 1°C IN MEDIA OF VARYING Ca^{2+} CONCENTRATION

"Free" ionic content was calculated as the amount of ion, in the total tissue water, in Donnan equilibrium with the medium, assuming that the Na^+ distribution was in accordance with the Donnan equilibrium. The "bound" ionic content was taken to be the difference between total and "free" contents. All incubations were done in modified phosphate media.

Medium concentrations	n	Tissue contents			
		"Free" Ca^{2+}	"Bound" Ca^{2+}	"Free" Mg^{2+}	"Bound" Mg^{2+}
mM		*mmoles/kg FFDW*		*mmoles/kg FFDW*	
2.5 Ca^{2+}; 1.0 Mg^{2+}	8	11.8 ± 1.5	9.3 ± 1.9	6.3 ± 0.6	26.6 ± 0.5
1.2 Ca^{2+}; 1.0 Mg^{2+}	8	6.1 ± 0.3	8.1 ± 2.4	5.1 ± 0.2	28.0 ± 1.1
0.03 Ca^{2+}; 1.0 Mg^{2+}; 2.0 EDTA*	3	0.0	2.7 ± 0.5	5.5‡	19.1‡

* The medium was made up to be free of Ca^{2+}; the Ca^{2+} content shown here was derived from endogenous sources, and was determined by analysis of the medium after removal of the slices.

‡ $n = 1$.

(see above). Doubling the normal Ca^{2+} concentration of the medium to 2.5 mM did not increase the calculated "bound" fraction of this ion, so that the "binding" sites appeared to be already saturated at the lower concentration. About two-thirds of the "bound" Ca^{2+} was, however, lost upon incubation in a medium with a very low Ca^{2+} concentration and containing 2 mM EDTA. The

amount of Ca^{2+} retained under the latter conditions (2.7 ± 0.5 mmoles/kg FFDW[1]) may be compared to the slowly exchangeable fraction of Ca^{2+} of liver slices, which amounts to about 3.4 mmoles/kg FFDW (recalculation of results of Wallach et al., 1966). The "bound" Mg^{2+} content was not affected by raising the medium Ca^{2+}, indicating that there was no marked competition by Ca^{2+} for the Mg^{2+} sites. The "bound" Mg^{2+} was reduced about 30% in the presence of EDTA.

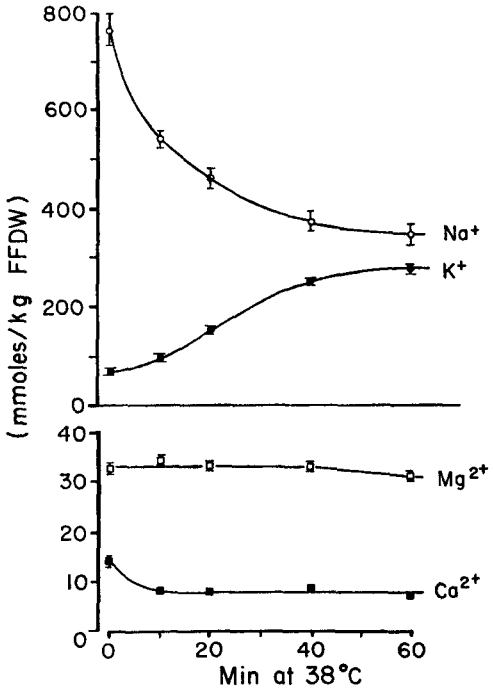

FIGURE 2. Changes in ion content of liver slices during incubation at 38°C in bicarbonate medium. Slices were preincubated for 90 min at 1°C; samples were then taken (time = 0 in figure), and the remaining slices were transferred to the bath at 38°C. For further details, see the text.

Recovery of Composition during Incubation at 38°C

The intracellular compartments of cold-incubated liver slices return nearly to their initial composition of K^+, Na^+, Cl^-, and water when subsequently incubated at 38°C in the well-oxygenated phosphate medium (Parsons and van Rossum, 1962b; Elshove and van Rossum, 1963). However, the results obtained with Ca^{2+} movements at 38°C in this medium were rather erratic. In some experiments a significant loss was seen (e.g. experiments with endogenous substrate in Table VIII), but in many there was no change in Ca^{2+} content of the slices.

A larger and more consistent extrusion of Ca^{2+} was obtained when incubation at 38°C was carried out in the bicarbonate medium.[2] A series of such

[1] Abbreviation used in paper: FFDW, fat-free dry weight of solids.

[2] Similar beneficial effects of bicarbonate-Ringer's solution have been observed for Ca^{2+} transport

experiments is summarized in Fig. 2. The Ca^{2+} content fell from 13.9 ± 0.9 (8) to 8.1 ± 0.3 (7) mmoles/kg FFDW by the first sampling time (10 min), and then remained constant. The apparent concentration of Ca^{2+} in the total tissue water also fell, from 3.6 ± 0.2 mmoles/kg water to 2.7 ± 0.1; it then remained constant at the latter level, which was still more than twice the concentration in the medium (1.3 mM). The changes in level of water (not shown), Na^+, and K^+, including the lag period before reaccumulation of the latter, were very similar to those observed previously in phosphate medium (Judah

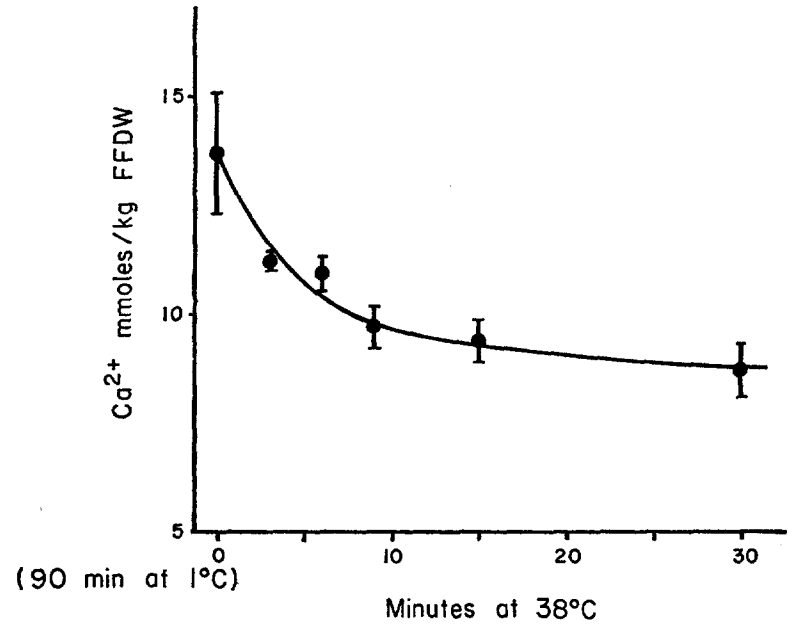

FIGURE 3. Changes in Ca^{2+} content of liver slices during initial stages of incubation at 38°C in bicarbonate medium. Details as for Fig. 2.

and McLean, 1962; Elshove and van Rossum, 1963). A study of the earlier phases of incubation at 38°C (Fig. 3) showed that the Ca^{2+} extrusion required about 10 min for completion.

Mechanism of Ca^{2+} Extrusion

The occurrence of net Ca^{2+} extrusion by a hetero-exchange diffusion process, of the type seen in cardiac muscle (Reuter and Seitz, 1968), requires that the ratio

(Na^+ concentration in cell water)/(Na^+ concentration in medium)

by kidney cortex slices (A. Kleinzeller, personal communication), and formed the basis for these experiments.

should be less than 1.0. However, the results of Elshove and van Rossum (1963) show that because of the simultaneous loss of Na^+ and water, the concentration of Na^+ in the intracellular water of slices incubated at 38°C does not fall below that of the medium until 20 min have elapsed, i.e. after the net loss of Ca^{2+} is complete. Similar results were obtained from the experiments of Fig. 2 when intracellular concentrations were calculated from the inulin space measurements of Elshove and van Rossum (1963). Furthermore, the establishment of a Na^+ gradient may be completely prevented by incubating the slices in a K^+-free medium, since the Na^+ transport is then inhibited

TABLE V

EFFECT OF K^+-FREE MEDIUM ON CATION MOVEMENTS IN LIVER SLICES AT 38°C

The slices were all preincubated for 80 min at 1°C in K^+-free bicarbonate medium, which was changed six times in order to remove as much endogenous K^+ from the system as possible. They were then transferred to a seventh portion in Warburg vessels kept at 1°C. The manometers were gassed and transferred to the bath at 38°C. K^+ (to give a final concentration of 5 mM) was tipped in from the side arms of the vessels in line 2 during the transfer to the bath at 38°C. K^+ was tipped into the flasks of line 4 after 20 min at 38°C.

Incubation	Ca^{2+}	Na^+	Water	*n*
	mmoles/kg FFDW		*kg/kg FFDW*	
90 min at 1°C, K^+-free	12.7 ± 0.9	840 ± 44	4.00 ± 0.04	8
90 min at 1°C, then at 38°C for:				
20 min, 5 mM K^+	8.1 ± 0.6	551 ± 9	2.96 ± 0.07	6
20 min, K^+-free	9.0 ± 0.8	779 ± 54	3.65 ± 0.10	8
40 min (20 min K^+-free plus 20 min with 5 mM K^+)	9.3 ± 0.9	553 ± 39	2.99 ± 0.09	8

(Judah and McLean, 1962; Elshove and van Rossum, 1963; van Rossum, 1966). Table V shows that slices incubated for 20 min at 38°C in a K^+-free bicarbonate medium extruded as much Ca^{2+} as did slices in the medium containing 5 mM K^+, although their Na^+ and water contents were the same as those of cold-incubated slices. Subsequent addition of K^+ to the K^+-free slices initiated Na^+ transport without a further effect on the Ca^{2+} content.

In order to obtain more evidence on this point, experiments were done in which reestablishment of the Na^+ gradient was prevented by using the inhibitors cyanide and ouabain. Both these agents inhibit Na^+ and K^+ transport in the bicarbonate medium (Table VI), but whereas ouabain does so by direct inhibition of the transport mechanism, cyanide acts by inhibiting the respiration required to support Na^+ transport (Elshove and van Rossum, 1963). It is apparent that cyanide inhibited Ca^{2+} extrusion (Table VI), but that, in contrast to the brief report of Judah and Ahmed (1964), ouabain did not.

Similar results with the inhibitors were obtained when liver slices from rats fasted for 40 hr were incubated in a medium containing glucose. The amount

of Ca^{2+} entering the tissue during incubation at 1 °C was considerably greater than in slices from fed rats, but the net extrusion of Ca^{2+} was similar (Table VII). The small amount of Ca^{2+} extrusion by liver slices from fed rats, in the phosphate medium, was also inhibited by the respiratory inhibitors cyanide

TABLE VI

EFFECTS OF INHIBITORS ON IONIC MOVEMENTS DURING INCUBATION AT 38°C IN BICARBONATE MEDIUM

Slices were incubated for 90 min at 1 °C, followed by 20 min at 38 °C. Inhibitors were added after the first 20 min of incubation at 1 °C. Each value is the mean (± standard error) of eight observations.

Inhibitor	Incubation	K^+	Na^+	Mg^{2+}	Ca^{2+}
	°C	*mmoles/kg FFDW*			
None	1	62 ± 2	624 ± 21	30.6 ± 0.6	15.4 ± 0.7
None	38	120 ± 6	486 ± 34	30.2 ± 0.3	9.1 ± 0.4
Ouabain, 0.75 mM	38	51 ± 3	650 ± 52	30.4 ± 0.8	10.1 ± 0.8
Cyanide, 2 mM	38	67 ± 4	676 ± 41	27.3 ± 0.7	15.8 ± 1.1

TABLE VII

EFFECTS OF INHIBITORS ON Ca^{2+} MOVEMENTS AND GLYCOLYSIS IN SLICES OF LIVER PREPARED FROM FASTED RATS

Animals were fasted for 40 hr prior to the experiment, but were allowed free access to water. The slices were incubated in bicarbonate medium for 90 min at 1 °C, followed by 70 min at 38 °C. Additions of inhibitors and substrate were made 15 min before the end of incubation at 1 °C. Lactate production was measured in the medium after removal of slices, the lactate content of the cold-incubated samples (corrected for differing tissue weights) being subtracted.

Incubation	Additions	n	Ca^{2+}	Lactate
°C			*mmoles/kg FFDW*	*mmoles/kg FFDW/ 70min*
None (fresh tissue)		14	3.4 ± 0.4	
1	None	14	20.4 ± 1.6	
38	None	10	13.1 ± 0.8	16.3 ± 5.3
38	Glucose (20 mM)	14	11.6 ± 0.5	22.2 ± 4.0
38	Glucose + CN^- (2 mM)	14	21.3 ± 0.9	35.3 ± 3.1
38	Glucose + ouabain (0.75 mM)	10	14.1 ± 0.9	36.3 ± 8.3

(2 mM) and Amytal (5 mM), but not by ouabain. The complete inhibition of Ca^{2+} extrusion by respiratory inhibitors in the presence of adequate glycolytic substrate (endogenous glycogen in slices from fed rats, or added glucose in slices from fasted rats) shows that glycolysis is not able to provide the requisite energy for the transport process.

It is clear from these results that the net, outward transport of Ca^{2+} has a

TABLE VIII

EFFECT OF SUBSTRATES ON RESPIRATION AND IONIC COMPOSITION OF SLICES INCUBATED AT 38°C IN PHOSPHATE MEDIUM

Slices were incubated for 90 min at 1°C, followed by 70 min at 38°C. Substrates were added after 20 min at 1°C. Rates of respiration are shown for the initial and final 10 min periods of manometric readings. Numbers of observations quoted for Ca^{2+} apply to other columns also.

Substrate	Ca^{2+}		Mg^{2+}		Q_{O_2} (38°C)	
	1°C	38°C	1°C	38°C	Initial	Final
	mmoles/kg FFDW		*mmoles/kg FFDW*		*µl/mg FFDW/hr*	
Experiment 1						
Endogenous	13.5 ± 0.3 (7)	10.8 ± 0.5 (12)	29.5 ± 0.5	29.4 ± 0.5	7.1 ± 0.5	7.5 ± 0.7
Succinate, 40 mM	16.9 ± 0.5 (7)	19.6 ± 1.4 (8)	26.6 ± 0.8	22.1 ± 0.4	42.0 ± 1.0	35.6 ± 2.0
Citrate, 20 mM		6.2 ± 1.0 (6)		20.5 ± 1.1	15.0 ± 0.7	7.5 ± 0.7
Octanoate, 20 mM	12.7 ± 1.0 (7)	8.3 ± 0.7 (6)	29.8 ± 0.7	33.2 ± 0.4	14.7 ± 0.6	9.7 ± 0.9
Experiment 2						
Endogenous	18.1 ± 0.1 (3)	13.0 ± 0.6 (6)	26.4 ± 0.7	31.9 ± 1.0	8.8 ± 0.1*	
Ethanol, 170 mM		12.3 ± 1.7 (3)		32.2 ± 1.4	9.6 ± 0.1*	

* Mean value for 60 min observation period. The rate was constant throughout.

requirement for respiratory energy, but that it is not dependent on the occurrence of net Na^+ transport or on the existence of a concentration gradient of Na^+ between the cells and medium.

Effects of Respiratory Substrates

Since the extrusion of Ca^{2+} from the cells, like its accumulation by the mitochondria, is respiration-dependent, it was of interest to see what effect stimulation of respiration by added substrates would have on the rather variable Ca^{2+} extrusion in the phosphate medium. The results of these experiments are shown in Table VIII. The large fall in both Ca^{2+} and Mg^{2+} contents caused by citrate was presumably due largely to its chelating properties. Octanoate caused a small stimulation of the net Ca^{2+} extrusion, but succinate, which caused the greatest stimulation of respiration (see van Rossum, 1969), gave a large increase in the tissue Ca^{2+} content.

Neither the addition of ethanol to the slices from fed rats (Table VIII), experiment 2) nor of glucose to slices from fasted rats (Table VII) had any effect on Ca^{2+} extrusion.

Movements of Mg^{2+} at 38°C

The loss of a small amount of Mg^{2+} during incubation at 1°C (Fig. 1) suggested that the maintenance of at least part of the liver cell content of this cation may be dependent upon active metabolism. A relation to respiration is suggested by the further, small loss of Mg^{2+} during incubation at 38°C in the presence of CN^- (Table VI). In some experiments a recovery of lost Mg^{2+} was seen during incubation at 38°C, as in the presence of octanoate and ethanol, or in the presence of endogenous substrate in experiment 2 of Table VIII. However, this last result was an exception, since in the majority of experiments with endogenous substrate alone, no uptake of Mg^{2+} was seen (Fig. 2; Table VI; experiment 1 of Table VIII).

DISCUSSION

Extrusion of Ca^{2+} by Cells

Most previous reports on the movements of Ca^{2+} in rat liver preparations in vitro have shown a net accumulation in the tissue under metabolically favorable conditions, with larger accumulation under a variety of less favorable conditions (Dawkins et al., 1959; Judah and Ahmed, 1963; Wallach et al., 1966). The present results confirm the occurrence of a rapid entry when metabolic activity is reduced, but also show that much of the accumulated Ca^{2+} can subsequently be extruded under metabolically favorable conditions (see also Judah and Ahmed, 1964). That the uptake took place at 1°C, and that extrusion at 38°C was inhibited by respiratory inhibitors, suggests that the

former process is passive and the latter an active, energy-requiring transport. Further indications of this from the literature are (*a*) that the unidirectional efflux of Ca^{2+} is lowered by reduced temperature (Wallach et al., 1966; but contrast their unexpected finding that cyanide increases the unidirectional influx rather than inhibiting the efflux) and (*b*) that net Ca^{2+} extrusion is inhibited by 2,4-dinitrophenol (Judah and Ahmed, 1964).

For reasons discussed in the "Results" section, particularly in connection with the differing effects of cyanide and ouabain, the present findings strongly suggest that the Ca^{2+} extrusion mechanism, like that of red cells (Schatzmann, 1966), is dependent directly upon high-energy compounds, and is not related either to the active transport or to the exchange diffusion of Na^+. However, the finding of Judah and Ahmed (1964) that Ca^{2+} extrusion is inhibited when the medium Na^+ concentration is lowered remains to be explained.

The question arises as to whether this energy-dependent loss of Ca^{2+} can be considered formal active transport, as defined by Ussing (1960). The apparent concentration of total Ca^{2+} in the slice water remained well above the external concentration after completion of the maximal Ca^{2+} extrusion. However, the Ca^{2+} content of fractionated liver tissue is largely associated with the particulate fractions, so that the concentration of free Ca^{2+} in the cytosol is normally probably very low (Thiers et al., 1960; Carafoli, 1967). A further indication of this may, perhaps, by given by the present finding that the final Ca^{2+} content of the slices, after completion of extrusion at 38°C (9–10 mmoles/kg FFDW; e.g. Table VI), was barely in excess of the calculated "bound" fraction of Ca^{2+} at the end of incubation at 1°C (8–9 mmoles/kg FFDW). Since, moreover, liver cells exhibit a membrane potential with the cell interior negative (Li and McIlwain, 1957), there seems to be a possibility that free Ca^{2+} had been transported across the plasma membrane against its electrochemical gradient during incubation at 38°C. However, a complete answer to this problem must involve clarification of the concentrations of free Ca^{2+}, and of the electrical potentials, in the various subcellular water compartments of the slices, as well as of the effect of Ca^{2+}-binding materials leaking from the slices (e.g. proteins) on the external concentration of free Ca^{2+}.

The ability to bring about an energy-dependent net extrusion of Ca^{2+} is a property of the liver cell which is retained in the rapidly growing Morris Hepatoma 3924A.[3] However, it is not found in all types of cells, since an energy-dependent accumulation of Ca^{2+} has been observed in slices of kidney (Höfer and Kleinzeller, 1963) and intestinal mucosa (Schachter et al., 1960).

Role of Mitochondrial Ca^{2+} Transport

Rat liver mitochondria are able to bring about an energy-dependent accumulation of Ca^{2+} both in vitro (Chance, 1965) and in vivo (Carafoli, 1967), and it

[3] Van Rossum, G. D. V., T. Galeotti, M. Gosalvez, and H. P. Morris. Manuscript in preparation.

is relevant to consider whether this could have tended to counteract the net extrusion of Ca^{2+} from the cells in the present work. There were two conditions under which the actively respiring cells tended to show less efficient Ca^{2+} extrusion: (*a*) when the phosphate content of the medium was raised to 10 mM and (*b*) on the further addition of 40 mM succinate to the phosphate medium, when the highest rate of respiration observed was accompanied by an accumulation of Ca^{2+}. Both these conditions may have exerted their effects by increasing the efficiency of mitochondrial Ca^{2+} accumulation. Thus, phosphate is required for massive accumulation of Ca^{2+} by mitochondria (Lehninger et al., 1963), and succinate, because of its rapid, one-step oxidation, greatly increases the availability not only of high-energy intermediates but also of dicarboxylate anions, which can accompany Ca^{2+} entering the mitochondria (Quagliariello and Palmieri, 1968; van Rossum, 1969). However, mitochondria undergoing "massive loading" can accumulate up to 2 mmoles Ca^{2+}/g mitochondrial protein (Lehninger et al., 1963; Greenawalt et al., 1964), and if this had occurred in the slices, their Ca^{2+} content would have been about 390 mmoles/kg slice FFDW (assuming 200 g mitochondrial protein/kg FFDW). In fact, the total Ca^{2+} content of the slices never exceeded 5% of this value under any of the conditions studied. This failure of the intracellular mitochondria to accumulate much Ca^{2+} appears to be an indication that the Ca^{2+}-extruding activity of the liver cells (presumably situated at the plasma membrane) competes favorably with them for Ca^{2+} diffusing into the cell, although the balance of the competition can be altered slightly by the two conditions mentioned. It is noteworthy that Reynolds (1963, 1964) has provided evidence that the Ca^{2+} content of intracellular mitochondria in vivo is low unless the plasma membrane is damaged.

Mg^{2+} Transport

The Mg^{2+} content of the slices remained much more constant than that of any of the other three cations studied, and much of it must be either bound or retained within an impermeable membrane. However, the loss upon respiratory inhibition, and the finding that the loss could be made good in at least some experiments, show that some of the Mg^{2+} is in a diffusible form and tentatively suggest that the cells may possess a mechanism for Mg^{2+} accumulation.

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