The Effect of Calcium Ions on the Leakage of Protein and Enzymes from Rat-Liver Slices

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1. The effects of Ca^{2+} ions and the nutritional state of the animals on the distribution of soluble protein between the medium, cytoplasmic and particulate fractions after incubation of rat-liver slices have been studied. 2. The Ca^{2+} ions decreased the leakage of cytoplasmic protein into the medium and increased the loss of protein from the particles; the effects were more marked with livers from starved animals and animals fed on a low-carbohydrate diet. 3. The results obtained for protein were confirmed by measurements of the changes in distribution of compartment-specific enzymes, and these indicated that both the cytoplasmic and particulate fractions contributed to the protein found in the medium. 4. EDTA decreased the loss of protein and enzymes from both the cytoplasmic and particulate fractions; this effect was independent of the Ca^{2+} ion concentration. 5. The inclusion of pyruvate, succinate or citrate in the medium had no marked effects on the leakage of protein.

The presence of Ca^{2+} ions in the incubation medium is known to decrease the loss of total nitrogen (Bernheim & Bernheim, 1946) and protein (Kaufmann & Wertheimer, 1957; Mellanby & Williamson, 1963) from rat-liver slices. To obtain more information on the nature of this effect, both the leakage of protein into the medium and the intracellular distribution of soluble protein has been studied in this present work. In addition, the activities of several enzymes have been determined in the incubation medium, and in the cytoplasmic and particulate fractions of the liver slices, to discover the source of the protein released into the medium. Lactate dehydrogenase and aldolase were chosen as cytoplasmic enzymes, glutamate dehydrogenase as a mitochondrial enzyme (Delbrück, Schimassek, Bartsch & Bücher, 1959) and glutamate-oxaloacetate transaminase as an example of an enzyme that occurs in both compartments of liver cells (Eichel & Bukovsky, 1961). Further, in view of the greater loss of protein from liver slices of starved rats observed by Kaufmann & Wertheimer (1957), the influence of the nutritional state of the animals on the action of Ca^{2+} ions has also been investigated.

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METHODS

Enzymes and chemicals. All the enzymes, coenzymes and substrates used in this work were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Animals. The male and female albino rats were of the Wistar strain and weighed 150–230 g. Details of the normal diet and the low-carbohydrate diet are given by Mellanby & Williamson (1963). The starved animals had their food withheld for 24 hr.

Incubation technique. The rats were killed by a blow at the back of the neck, and the livers were excised immediately and placed on ice. Tissue slices (0.3–0.4 mm. thick) were cut free-hand and transferred to a Petri dish containing ice-cold saline medium (see below) and left in the saline medium for about 5 min. The slices were then blotted on filter paper to remove excess of saline medium and weighed on a torsion balance. The weighed slices (60–80 mg.) were placed in manometer flasks containing 4.0 ml. of saline medium. Each experiment was set up in duplicate and the mean values of the results were used for calculating the standard deviations. The manometers were gassed with O_2 and incubated at 37° with shaking for 2 hr. unless otherwise stated. Substrates were added to the main compartment of the manometer flasks. The centre wells contained 0.2 ml. of 2N-KOH and filter papers. At the end of the incubation period the manometer flasks were placed on ice, and the slices were removed and immediately fractionated as described below. The incubation medium was centrifuged for 5 min. at 3000g to

remove insoluble material and the supernatant fluid used for the determination of protein and enzyme activities.

Incubation medium. The liver slices were normally incubated in either a 'tris saline A' containing: 100 ml. of 0.154 M-NaCl; 4 ml. of 0.154 M-KCl; 3 ml. of 0.11 M-CaCl₂; 1 ml. of 0.154 M-MgSO₄; 12 ml. of 0.10 M-tris-HCl buffer, pH 7.4; or a Ca²⁺ ion-free 'tris saline B': as tris saline A, but with the omission of CaCl₂. Tris buffer was used in the composition of these saline media instead of phosphate buffer because preliminary experiments indicated that protein could be lost by adsorption on to the calcium phosphate that is precipitated out of saline media containing Ca²⁺ and phosphate ions. No significant difference was found between the O₂ uptake of slices incubated in tris saline A and those incubated in Krebs-Ringer phosphate saline (Krebs, 1933).

Fractionation of slices. All the operations described in this section were carried out at 0-4°. The slices were transferred to a Potter-Elvehjem all-glass homogenizer (capacity 3 ml.) and homogenized for exactly 30 sec. with either 2.0 ml. of ice-cold 0.25 M-sucrose or 0.154 M-KCl (both in 10 mm-tris-HCl buffer, pH 7.4). The homogenate was centrifuged for 15 min. at 20000g and the supernatant fluid (the cytoplasmic fraction) and pellet were collected. The pellet (the particulate fraction) was suspended in 2.0 ml. of 10 mm-tris-HCl buffer, pH 7.4, and exposed to ultrasonic vibration for two 20 sec. periods. The soluble protein of the particulate fraction was obtained by centrifuging for 15 min. at 20000g and collecting the supernatant fluid. Homogenization in 0.154 M-KCl was only carried out when the activities of the cytoplasmic enzymes (aldolase and lactate dehydrogenase) were to be determined; the use of this medium gave better recoveries of these enzymes, especially aldolase. For example, homogenization of unincubated liver slices from a fed animal in the sucrose medium gave a value for aldolase activity of 2.2 units/g. fresh wt. of tissue, whereas the result obtained with the KCl medium was 6.8 units/g. This method of fractionation gave reproducible results and the total activities of the enzymes measured were similar to those reported by other workers (Delbrück *et al.* 1959) for rat-liver tissue.

Determination of enzyme activities. The units of enzyme activity are expressed as μ moles of substrate transformed/min. (corrected where necessary to 25°) and the specific activity as units/g. fresh wt. of tissue. The assay procedures were based on those described by Delbrück *et al.* (1959), with the modifications in substrate concentration and pH outlined below. To simplify the calculations a total volume of 3.1 ml. in a cuvette of 1 cm. light-path was used (division of extinction changes by 2 gives the units of activity). Each cuvette contained (final concentrations): tris-HCl buffer of the stated pH (33 mm); NADH (0.25 mm). In addition, for the lactate-dehydrogenase assay (pH 7.4), it contained: pyruvate (2.4 mm); for the aldolase assay (pH 7.4): fructose 1,6-diphosphate (0.65 mm); α -glycerophosphate dehydrogenase (0.75 unit/ml.); triose phosphate isomerase (5 units/ml.); for the glutamate-oxaloacetate-transaminase assay (pH 8.0): L-aspartate (6.5 mm); α -oxoglutarate (3.2 mm); malate dehydrogenase (6 units/ml.); for the glutamate-dehydrogenase assay (pH 7.7): ammonium acetate (32 mm); EDTA (1.6 mm); α -oxoglutarate (10 mm). The assays were usually started by the addition of the enzyme sample and readings were taken at 340 m μ in a Unicam SP.500 spectrophotometer at suitable time-intervals. The activities were obtained from the linear portion of the reaction curves after

correction for any blank reactions. All determinations of enzyme activities were carried out immediately, since storage of samples overnight resulted in loss of activity.

Determination of protein. Protein was determined by the colorimetric method of Lowry, Rosebrough, Farr & Randall (1951). The results are expressed as mg. of protein/g. fresh wt. of tissue. Crystalline bovine serum albumin dried to constant weight was used as a protein standard.

RESULTS

Leakage of protein and enzymes without added substrate. To determine the effect of time of incubation on protein leakage, slices from livers of well-fed and starved rats were incubated at 37° for various times in the presence and absence of Ca²⁺ ions and the distribution of protein between the medium, cytoplasm and particles was determined. In the absence of Ca²⁺ ions the amount of protein appearing in the medium during the first 40 min. of incubation was slightly (about 20%) higher with liver from starved animals; the addition of Ca²⁺ ions caused no appreciable change in the values (Fig. 1). This is perhaps not surprising since the leakage of protein occurring during the initial period of incubation (usually 30 min.) probably represents protein lost from damaged cells (Kline, 1949; Gassner & Wertheimer, 1961). During the next 80 min. of incubation, when the rate of protein leakage was virtually linear, the higher rate of loss of protein from slices from starved animals (18 mg. of protein/hr./g. fresh wt.) as compared with that from slices from fed animals (12 mg. of protein/hr./g. fresh wt.) was maintained, but in this case Ca²⁺ ions decreased the values to 10 and 8 mg. of protein/hr./g. fresh wt. respectively (Fig. 1). At least 75% of the protein found in the medium

Fig. 1. Effect of Ca²⁺ ions on the leakage of protein from rat-liver slices. The results are the mean values of two experiments. Liver from well-fed animals: ●, in the absence of Ca²⁺ ions; ○, in the presence of Ca²⁺ ions. Liver from starved animals: ▲, in the absence of Ca²⁺ ions; △, in the presence of Ca²⁺ ions.

after the first 40 min. of incubation could be accounted for by loss of protein from the cytoplasm; the addition of Ca^{2+} ions did not significantly alter this situation. During the next 80 min. of incubation the apparent contribution of the cytoplasm was less (40% for liver from fed animals; 65% for liver from starved animals) and the effect of Ca^{2+} ions was to decrease this dramatically (2 and 20% respectively). This suggested that Ca^{2+} ions were increasing loss of protein from the particulate fraction during the second period of incubation, and the results bear this out (Fig. 2). In the absence of Ca^{2+} ions the loss of protein from the particulate fraction (calculated with reference to the protein content of particles from unincubated slices) was similar for liver from fed and starved animals (5.0 and 5.2 mg. of protein/hr./g. fresh wt. respectively); Ca^{2+} ions increased the rate considerably (8.0 and 9.2 mg. of protein/hr./g. fresh wt. respectively).

Further evidence for this loss of protein from the particulate fraction was obtained by determining the distribution of a mitochondrial enzyme, glutamate dehydrogenase, in the same experiment. In agreement with other workers (Delbrück *et al.* 1959) at least 95% of the total activity of this enzyme was found in the particulate fraction from unincubated liver slices, and therefore any increase in glutamate dehydrogenase activity in the cytoplasm plus medium after incubation must be due to loss of enzyme from the particulate fraction. The rate of appearance of glutamate dehydrogenase in the medium plus cytoplasm was the same for slices from fed and starved animals (1.5 units/hr./g. fresh wt.)

Fig. 2. Effect of Ca^{2+} ions on the loss of protein from the particulate fraction of rat-liver slices. The results are the mean values of two experiments and were calculated with reference to the protein content of particles from unincubated slices. Liver from well-fed animals: ●, in the absence of Ca^{2+} ions; ○, in the presence of Ca^{2+} ions. Liver from starved animals: ▲, in the absence of Ca^{2+} ions; △, in the presence of Ca^{2+} ions.

incubated without Ca^{2+} ions (Fig. 3). As with the protein loss from particles, Ca^{2+} ions increased the rate significantly (liver from fed animals: 2.3 units/hr./g. fresh wt.; liver from starved animals: 2.1 units/hr./g. fresh wt.).

As mentioned above, one of the effects of Ca^{2+} ions was to maintain the concentration of protein found in the cytoplasmic fraction during the second period of incubation at values nearer to those of unincubated slices, and this effect was even more pronounced with glutamate-oxaloacetate transaminase, which in unincubated slices is distributed between the cytoplasm and the particles in the ratio 1:4. The changes in distribution of this enzyme on incubation are given in Table 1. The results show that after the initial equilibration period (40 min.) the concentration of glutamate-oxaloacetate transaminase in the cytoplasm actually increased in all cases except for slices from starved rats incubated without Ca^{2+} ions (decrease of about 20%), although the amount of enzyme found in the medium at the end of the incubation was greater than that originally present in the cytoplasm. Plots of the loss of glutamate-oxaloacetate transaminase from the particulate fraction against time showed that the rate was similar for liver from fed and starved rats (11 and 10 units/hr./g. fresh wt. respectively), and the presence of Ca^{2+} ions increased the values slightly (14.5 and 12.3 units/hr./g. fresh wt. respectively).

For the remainder of the experiments reported below it was decided to incubate for a fixed period of 120 min., although this had the disadvantage that changes in rate of protein or enzyme distribution

Fig. 3. Effect of Ca^{2+} ions on the loss of glutamate dehydrogenase from the particulate fraction of rat-liver slices. The results are the mean values of two experiments and represent the total activity found in the cytoplasm plus medium after correction for the glutamate-dehydrogenase content of the cytoplasm of unincubated slices. Liver from well-fed animals: ●, in the absence of Ca^{2+} ions; ○, in the presence of Ca^{2+} ions. Liver from starved animals: ▲, in the absence of Ca^{2+} ions; △, in the presence of Ca^{2+} ions.

Table 1. *Effect of Ca^{2+} ions and time of incubation of rat-liver slices on the distribution of glutamate-oxaloacetate-transaminase activity*

Liver slices from fed and starved rats were incubated in tris saline A (Ca^{2+} ions present) or tris saline B (Ca^{2+} ions absent) for various times. Other experimental details are given in the text. The activities (means of two experiments) are expressed in $\mu\text{moles/min./g. fresh wt. of tissue}$ and the changes in distribution are calculated with reference to the values for unincubated slices.

Fraction	Liver slices from fed rat						Liver slices from starved rat					
	- Ca^{2+} ions			+ Ca^{2+} ions			- Ca^{2+} ions			+ Ca^{2+} ions		
	Unincubated	0-40 min.	40-120 min.	0-40 min.	40-120 min.	Total	Unincubated	0-40 min.	40-120 min.	0-40 min.	40-120 min.	Total
Medium	0	+9.0	+11.3	+11.4	+13.0	+24.4	0	+8.7	+17.6	+13.5	+11.0	+24.5
Cytoplasm	15.6	-2.3	+5.1	-0.9	+5.5	+4.6	22.0	-2.2	-3.6	+1.0	+5.4	+4.4
Particles	77.6	-8.7	-12.4	-11.4	-14.9	-26.3	91.8	-7.4	-16.2	-8.0	-17.6	-25.6

could not be measured. However, this procedure allowed the scope of the investigation to be widened to include the determination of two cytoplasmic enzymes, lactate dehydrogenase and aldolase.

To confirm the effects of Ca^{2+} ions described above further experiments with livers from fed and starved animals were carried out. In addition the leakage of protein from livers from rats fed on a low-carbohydrate diet was studied because of the recent reports of increased gluconeogenesis (Krebs, Bennett de Gasquet, Gascoyne & Yoshida, 1963) and ketogenesis (Mellanby & Williamson, 1963) when tissue slices from such animals were incubated in a Ca^{2+} ion-containing medium. The results of the effect of Ca^{2+} ions and the previous nutritional state of the animals on the distribution of soluble protein and enzyme activities after incubation of the liver slices for 120 min. are given in Table 2. In the main the results for liver from fed and starved animals agreed with those found in the time-course experiments; the values for liver from animals fed on a low-carbohydrate diet were similar to those for liver from starved animals. The presence of Ca^{2+} ions caused no statistically significant change in the leakage of protein into the medium during incubation of slices from well-fed animals, but decreased (by about 20%) the loss from livers from starved animals and animals fed on a low-carbohydrate diet. In all cases Ca^{2+} ions maintained a higher concentration of protein in the cytoplasm and caused a loss of protein from the particles, though the effects were more marked with liver from starved animals and animals fed on a low-carbohydrate diet. As might be expected from the decrease in protein leakage into the medium, Ca^{2+} ions caused an appreciable decrease in the leakage of the cytoplasmic enzymes, lactate dehydrogenase and aldolase, with liver slices from starved animals, but there was no statistically significant difference between the aldolase values for liver slices from fed animals. The activities of glutamate dehydrogenase and glutamate-oxaloacetate transaminase found in the medium plus cytoplasm confirmed that Ca^{2+} ions increased the leakage of mitochondrial protein. It is also clear from Table 2 that the total recovery of protein and enzyme activity after incubation for 120 min. was about 90% of the values found for unincubated slices, except for aldolase (recovery about 80%).

Leakage of protein and enzymes with added substrate. Pyruvate, succinate and citrate tended to decrease protein leakage, but the results were only of statistical significance with liver slices from fed and starved animals incubated with pyruvate in the absence of Ca^{2+} ions (Table 3). This is in agreement with the work of Bernheim & Bernheim (1946), who found a decrease in the loss of nitrogen from liver slices incubated in the presence of pyruvate. Pyruvate also caused an appreciable decrease in the

Table 2. *Effect of Ca^{2+} ions and previous nutritional state on distribution of soluble protein and enzyme activities after the incubation of rat-liver slices*

Liver slices from rats fed on a normal diet, fed on a low-carbohydrate diet or starved for 24 hr. were incubated in tris saline A (Ca^{++} ions present) or tris saline B (Ca^{++} ions absent). Other experimental details are given in the text. The protein values are expressed in mg. of protein/g. fresh wt. of tissue ($\pm$ s.d.), the enzyme activities in $\mu\text{moles/min./g.}$ fresh wt. of tissue ($\pm$ s.d.) and the O_2 uptake in $\mu\text{l./hr./g.}$ fresh wt. of tissue ($\pm$ s.d.). The numbers of experiments in each series are shown in parentheses. The results were examined by Student's *t* test and values of *P* are given when the difference between two mean values is statistically significant ($P < 0.05$).

	Fraction	Liver slices from rat fed on normal diet (17)				Liver slices from rat starved for 24 hr. (17)				(4)			
		Unincubated		$-\text{Ca}^{++}$ ions		Unincubated		$-\text{Ca}^{++}$ ions		Unincubated		$-\text{Ca}^{++}$ ions	
		<i>P</i>		$+\text{Ca}^{++}$ ions		<i>P</i>		$+\text{Ca}^{++}$ ions		<i>P</i>		$+\text{Ca}^{++}$ ions	
		0-01		2050 $\pm$ 310		0-01		1890 $\pm$ 360		0-001		1920 $\pm$ 90	
O_2 uptake	Medium	—	—	1740 $\pm$ 300	35.9 $\pm$ 3.6	—	—	1280 $\pm$ 210	1890 $\pm$ 360	0-001	—	1300 $\pm$ 170	1920 $\pm$ 90
	Cytoplasm	—	—	35.9 $\pm$ 3.6	33.7 $\pm$ 3.6	—	—	48.5 $\pm$ 2.6	38.4 $\pm$ 2.6	0-001	—	42.7 $\pm$ 3.0	33.1 $\pm$ 2.8
	Particles	61.0 $\pm$ 5.6	61.0 $\pm$ 5.6	35.3 $\pm$ 4.0	40.3 $\pm$ 3.6	0-005	64.9 $\pm$ 5.9	26.2 $\pm$ 2.8	39.5 $\pm$ 3.3	0-001	62.0 $\pm$ 5.6	28.2 $\pm$ 1.1	39.9 $\pm$ 1.6
	Total	69.0 $\pm$ 7.2	69.0 $\pm$ 7.2	53.1 $\pm$ 3.1	49.9 $\pm$ 3.0	0-02	74.7 $\pm$ 12.1	55.3 $\pm$ 3.8	51.5 $\pm$ 4.2	0-025	69.1 $\pm$ 8.4	53.5 $\pm$ 3.0	49.0 $\pm$ 3.5
Lactate dehydrogenase	Medium	130.0 $\pm$ 8.6	130.0 $\pm$ 8.6	124.3 $\pm$ 6.0	123.9 $\pm$ 5.9	—	139.6 $\pm$ 15.0	130.0 $\pm$ 6.9	129.4 $\pm$ 7.3	—	131.1 $\pm$ 10.8	124.4 $\pm$ 5.0	122.8 $\pm$ 5.6
	Cytoplasm	—	—	163 $\pm$ 16	125 $\pm$ 12	0-001	—	234 $\pm$ 32	164 $\pm$ 29	0-001	—	257 $\pm$ 21	179 $\pm$ 14
	Total	287 $\pm$ 25	287 $\pm$ 25	112 $\pm$ 26	146 $\pm$ 17	0-01	329 $\pm$ 32	81 $\pm$ 27	142 $\pm$ 34	0-001	352 $\pm$ 28	86 $\pm$ 10	179 $\pm$ 25
				275 $\pm$ 24	271 $\pm$ 16	—	329 $\pm$ 32	315 $\pm$ 38	306 $\pm$ 31	—	352 $\pm$ 28	343 $\pm$ 22	358 $\pm$ 24
Aldolase	Medium	—	—	2.4 $\pm$ 0.4	2.2 $\pm$ 0.4	—	—	3.5 $\pm$ 0.4	2.3 $\pm$ 0.2	0-001	—	2.7 $\pm$ 0.4	1.8 $\pm$ 0.3
	Cytoplasm	6.8 $\pm$ 0.8	6.8 $\pm$ 0.8	3.0 $\pm$ 0.5	3.3 $\pm$ 0.5	—	7.4 $\pm$ 0.7	2.3 $\pm$ 0.7	3.3 $\pm$ 0.4	0-005	7.4 $\pm$ 0.7	2.9 $\pm$ 0.8	3.6 $\pm$ 0.7
	Total	6.8 $\pm$ 0.8	6.8 $\pm$ 0.8	5.4 $\pm$ 0.6	5.5 $\pm$ 0.7	—	7.4 $\pm$ 0.7	5.8 $\pm$ 0.7	5.6 $\pm$ 0.5	—	7.4 $\pm$ 0.7	5.6 $\pm$ 0.6	5.4 $\pm$ 0.5
						0-001	—	23.4 $\pm$ 2.9	22.8 $\pm$ 2.2	—	—	24.9 $\pm$ 4.5	24.7 $\pm$ 4.9
Glutamate-oxaloacetate transaminase	Medium	17.6 $\pm$ 3.5	17.6 $\pm$ 3.5	16.5 $\pm$ 2.6	18.0 $\pm$ 3.4	—	23.8 $\pm$ 2.6	15.4 $\pm$ 2.0	28.1 $\pm$ 3.9	0-001	18.9 $\pm$ 2.4	13.9 $\pm$ 2.2	24.7 $\pm$ 4.2
	Cytoplasm	80.4 $\pm$ 7.6	80.4 $\pm$ 7.6	54.5 $\pm$ 4.5	49.7 $\pm$ 6.6	0-05	90.8 $\pm$ 15.1	60.3 $\pm$ 4.9	50.7 $\pm$ 6.1	0-001	85.1 $\pm$ 9.6	55.9 $\pm$ 3.8	45.5 $\pm$ 5.0
	Particles	98.0 $\pm$ 9.1	98.0 $\pm$ 9.1	86.9 $\pm$ 6.1	88.2 $\pm$ 7.9	—	114.6 $\pm$ 17.1	99.1 $\pm$ 5.9	101.6 $\pm$ 7.3	—	104 $\pm$ 10.4	94.7 $\pm$ 5.7	94.9 $\pm$ 6.3
	Total	—	—	15.9 $\pm$ 3.3	20.5 $\pm$ 3.0	0-001	—	23.4 $\pm$ 2.9	22.8 $\pm$ 2.2	—	—	24.9 $\pm$ 4.5	24.7 $\pm$ 4.9
Glutamate dehydrogenase	Medium	—	—	1.8 $\pm$ 0.7	2.5 $\pm$ 0.6	0-01	—	2.5 $\pm$ 0.4	2.6 $\pm$ 0.3	—	—	1.7 $\pm$ 0.6	1.7 $\pm$ 0.3
	Cytoplasm	1.6 $\pm$ 0.4	1.6 $\pm$ 0.4	2.2 $\pm$ 0.3	2.5 $\pm$ 0.5	—	1.8 $\pm$ 0.5	2.6 $\pm$ 0.9	4.7 $\pm$ 1.6	0-005	1.7 $\pm$ 0.3	2.8 $\pm$ 0.7	5.1 $\pm$ 0.5
	Particles	32.7 $\pm$ 2.9	32.7 $\pm$ 2.9	27.1 $\pm$ 1.6	25.2 $\pm$ 2.2	0-02	35.5 $\pm$ 3.8	27.6 $\pm$ 3.4	24.6 $\pm$ 3.4	0-02	31.4 $\pm$ 3.8	26.6 $\pm$ 2.3	24.0 $\pm$ 2.2
	Total	34.3 $\pm$ 2.8	34.3 $\pm$ 2.8	31.3 $\pm$ 2.1	30.2 $\pm$ 2.8	—	37.3 $\pm$ 3.9	32.7 $\pm$ 2.7	31.9 $\pm$ 3.0	—	33.1 $\pm$ 3.7	31.1 $\pm$ 3.1	30.8 $\pm$ 3.6

Table 4. *Effect of EDTA on distribution of soluble protein and enzymes after the incubation of rat-liver slices*

Liver slices from fed and starved rats were incubated in tris saline A (Ca^{2+} ions present) or tris saline B (Ca^{2+} ions absent) with and without EDTA (1 mM). Other experimental details are given in the text. The protein values are expressed in mg. of protein/g. fresh wt. of tissue ($\pm$ S.D.), the enzyme activities in $\mu\text{moles/min./g.}$ of tissue ($\pm$ S.D.) and the O_2 uptake in $\mu\text{l./hr./g.}$ fresh wt. of tissue ($\pm$ S.D.). The results (three experiments in each series) were examined by Student's *t* test and the values of *P* are given when the difference between two mean values is statistically significant ($P < 0.05$).

	Fraction	Liver slices from fed rat				Liver slices from starved rat			
		— Ca^{2+} ions		+ Ca^{2+} ions		— Ca^{2+} ions		+ Ca^{2+} ions	
		— EDTA	<i>P</i>	+ EDTA	<i>P</i>	— EDTA	<i>P</i>	+ EDTA	<i>P</i>
O_2 uptake	—	1850 $\pm$ 130	1410 $\pm$ 200	2320 $\pm$ 110	1600 $\pm$ 110	1330 $\pm$ 70	0.001	2300 $\pm$ 40	1310 $\pm$ 120
	Medium	36.8 $\pm$ 1.5	30.8 $\pm$ 2.8	31.9 $\pm$ 2.3	22.7 $\pm$ 2.2	47.7 $\pm$ 3.6	0.001	36.1 $\pm$ 2.0	22.5 $\pm$ 1.8
	Cytoplasm	38.2 $\pm$ 2.4	36.7 $\pm$ 3.1	44.0 $\pm$ 4.0	42.3 $\pm$ 4.5	28.2 $\pm$ 3.8	0.002	43.5 $\pm$ 5.2	47.8 $\pm$ 3.2
Protein	Particles	52.8 $\pm$ 5.6	59.8 $\pm$ 2.1	50.5 $\pm$ 3.1	58.9 $\pm$ 3.3	54.2 $\pm$ 1.7	0.05	51.5 $\pm$ 2.0	58.6 $\pm$ 2.6
	Medium	140 $\pm$ 26	104 $\pm$ 10	119 $\pm$ 14	99 $\pm$ 10	201 $\pm$ 12	0.05	137 $\pm$ 14	114 $\pm$ 10
	Cytoplasm	140 $\pm$ 22	165 $\pm$ 13	157 $\pm$ 17	174 $\pm$ 12	105 $\pm$ 19	0.05	162 $\pm$ 16	176 $\pm$ 19
Lactate dehydrogenase	Medium	2.0 $\pm$ 0.3	1.5 $\pm$ 0.3	2.1 $\pm$ 0.5	1.1 $\pm$ 0.6	3.4 $\pm$ 0.5	0.001	2.3 $\pm$ 0.3	0.8 $\pm$ 0.2
	Cytoplasm	3.4 $\pm$ 0.3	3.6 $\pm$ 0.2	3.3 $\pm$ 0.4	4.1 $\pm$ 0.5	2.0 $\pm$ 0.3	0.005	3.0 $\pm$ 0.2	4.2 $\pm$ 0.4
Aldolase	Medium	14.5 $\pm$ 2.5	10.1 $\pm$ 0.5	18.8 $\pm$ 3.9	7.0 $\pm$ 0.7	21.5 $\pm$ 0.8	0.01	22.7 $\pm$ 2.7	9.9 $\pm$ 2.0
	Cytoplasm	16.4 $\pm$ 0.8	16.4 $\pm$ 1.8	17.6 $\pm$ 1.9	17.9 $\pm$ 1.9	13.4 $\pm$ 1.3	0.001	25.4 $\pm$ 2.1	32.5 $\pm$ 3.0
	Particles	57.1 $\pm$ 3.4	61.8 $\pm$ 2.5	53.4 $\pm$ 7.0	61.6 $\pm$ 4.5	57.9 $\pm$ 5.1	0.001	51.4 $\pm$ 6.5	58.0 $\pm$ 5.4
Glutamate-oxaloacetate transaminase	Medium	1.6 $\pm$ 0.6	0.5 $\pm$ 0.2	2.5 $\pm$ 1.1	0.7 $\pm$ 0.5	2.4 $\pm$ 0.5	0.05	2.8 $\pm$ 0.5	0.5 $\pm$ 0.9
	Cytoplasm	2.2 $\pm$ 0.2	2.5 $\pm$ 0.4	2.5 $\pm$ 0.2	2.3 $\pm$ 0.4	2.2 $\pm$ 0.3	0.05	3.9 $\pm$ 0.4	3.9 $\pm$ 0.6
	Particles	24.2 $\pm$ 3.6	25.9 $\pm$ 3.8	22.7 $\pm$ 4.3	25.8 $\pm$ 4.0	28.0 $\pm$ 1.9	—	26.6 $\pm$ 2.1	28.9 $\pm$ 1.9

leakage of lactate dehydrogenase under the same conditions, whereas citrate had a marked effect only with liver from starved animals (in the absence of Ca^{2+} ions), and succinate had no effect. However, with glutamate dehydrogenase and glutamate-oxaloacetate transaminase, the situation was reversed; succinate and citrate caused a marked decrease in the leakage of these enzymes and the effect was independent of Ca^{2+} ions and the nutritional state of the animals, whereas the addition of pyruvate caused either a much less pronounced or no decrease in leakage.

Effect of EDTA on the distribution of soluble protein and enzymes. EDTA might be expected to increase leakage of protein from slices, since it is known to alter the permeability of cells, and this is generally assumed to be the result of its reaction with Ca^{2+} ions (Wu, 1959; Zimmerman, Devlin & Pruss, 1960; Kalant & Miyata, 1963). In the absence of added Ca^{2+} ions, EDTA (1 mM) lowered the oxygen uptake, but, surprisingly, this was accompanied by a decrease in protein leakage into the medium (Table 4). Perhaps even more surprising was the fact that in the presence of Ca^{2+} ions the addition of EDTA caused a more pronounced decrease in protein leakage than in their absence, indicating that the decrease was the result of the action of both Ca^{2+} ions and EDTA. The other marked effect of EDTA was to maintain a higher protein concentration in the particles and this was independent of Ca^{2+} ions (Table 4). These effects of EDTA on protein distribution were confirmed by the results obtained for the marker enzymes, since leakage of lactate dehydrogenase and glutamate-oxaloacetate transaminase into the medium and the amount of glutamate dehydrogenase found in the medium plus cytoplasm were decreased by EDTA (Table 4).

DISCUSSION

Although previous workers have studied protein or enzyme leakage from rat-liver slices (Kaufmann & Wertheimer, 1957; Gassner & Wertheimer, 1961; McLean, 1963) this is the first study in which changes in the intracellular distribution of protein have been measured. The incubation of liver slices resulted in loss of protein and enzymes from both the cytoplasmic and particulate fractions; the magnitude of the losses was dependent on the concentration of Ca^{2+} ions in the incubation medium and on the nutritional state of the animals. In confirmation of the work of Kaufmann & Wertheimer (1957) the loss of protein was greatest with liver slices from starved animals and animals fed on a low-carbohydrate diet incubated in a Ca^{2+} ion-free medium.

Attempts to increase the protein loss from liver slices from well-fed animals by incubation in media containing Ca^{2+} ion-chelating agents (citrate or

EDTA) were unsuccessful. This is surprising, since Kalant & Miyata (1963) found that slices from rat livers previously perfused with Krebs-Ringer phosphate containing citrate or EDTA lost more protein into the medium than did slices from untreated livers or livers perfused with Krebs-Ringer phosphate alone.

The net effect of the addition of Ca^{2+} ions to the incubation medium was to maintain the ratio of cytoplasmic to particulate protein at a level similar to that of unincubated slices, although there was a decrease (about 27%) in the total soluble protein found in the two fractions. The ability of Ca^{2+} ions to maintain the concentration of protein in the cytoplasm at higher levels appears to be due to two opposite effects: Ca^{2+} ions decreased leakage of protein into the incubation medium but increased protein loss from the particles. An analogous situation occurs with the leucocidin-treated leucocyte, where incubation in a Ca^{2+} ion-containing medium resulted in decreased leakage of aldolase but increased extrusion of β -glucuronidase from the leucocyte granules (Woodin & Wieneke, 1963). This selective action of Ca^{2+} ions in decreasing the permeability of one membrane while increasing that of another is illustrated in the present work, where leakage of a cytoplasmic enzyme, lactate dehydrogenase, into the medium was considerably lower in the presence of Ca^{2+} ions, whereas loss of glutamate dehydrogenase from the particles was higher. It is possible that this greater loss of protein from the particulate fraction in the presence of Ca^{2+} ions may be connected with the well-established effects of these ions on the permeability of isolated mitochondria (Tapley, 1956).

The results of the present study suggest that the increased ketogenesis (Mellanby & Williamson, 1963) and gluconeogenesis (Krebs *et al.* 1963) observed with tissue slices (liver and kidney respectively) from starved rats and rats fed on a low-carbohydrate diet when incubated in a Ca^{2+} ion-containing medium are probably due to the action of Ca^{2+} ions on membrane permeability and not to a direct effect of the ions on the activity of a particulate enzyme. These diverse metabolic effects of Ca^{2+} ions are most likely to be the result of the maintenance of the intracellular distribution of enzymes and nucleotide cofactors.

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