The Mode of Inhibition by Calcium of Cell-Membrane Adenosine-Triphosphatase Activity

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1. The mechanism of the inhibition of Na^+ -plus- K^+ -activated adenosine triphosphatase by calcium was investigated with an enzyme preparation from rabbit kidney cortex and with membranes of human erythrocytes. 2. CaATP , rather than ionic Ca^{2+} , acts as a competitive inhibitor, competing with MgATP in the Na^+ -plus- K^+ -activated adenosine-triphosphatase reaction. 3. There appears to be no competition between calcium and Na^+ for the activation of adenosine triphosphatase. 4. The inhibition of Na^+ -plus- K^+ -activated adenosine triphosphatase of cell membranes by low concentrations of CaATP and the consequent need of intact cells to keep the cytoplasmic concentration of calcium low relative to that of magnesium suggests a *raison d'être* for the mitochondrial calcium pump.

Calcium inhibits the Na^+ -plus- K^+ -activated $\text{ATPase}^\dagger$ of cell membranes in low concentrations that do not inhibit ATPase activity in the absence of Na^+ or K^+ (Skou, 1957, 1960; Dunham & Glynn, 1961; Landon & Norris, 1963; Deul & McIlwain, 1961; Aldridge, 1962; Järnefelt, 1962; Whittam & Blond, 1964; Wheeler & Whittam, 1964). Indirect evidence suggests that its site of action is the inner surface of the membrane (Hoffman, 1962; Blond & Whittam, 1964; Wheeler & Whittam, 1964) but its mode of action is not clear. Possibilities are interference with activation of the enzyme either by Mg^{2+} (Skou, 1960; Wheeler & Whittam, 1964) or by Na^+ (Judah & Ahmed, 1962, 1964). The present experiments were designed to find out how calcium acts. They show that it is a complex of Ca^{2+} with ATP that inhibits, by competition with a similar complex of Mg^{2+} and ATP . Calcium does not appear to compete with Na^+ or to inhibit directly as free Ca^{2+} .

METHODS

Materials. ATP (disodium salt) and tris (Sigma 7-9) were from the Sigma Chemical Co., St Louis, Mo., U.S.A. All other compounds were AnalaR reagents from British Drug Houses Ltd., Poole, Dorset. Tris- ATP was prepared by adding 1 g. of $\text{Na}_2\text{ATP} \cdot 3\text{H}_2\text{O}$ in 15 ml. of water to 5.5 g. of Dowex 50W resin (X8; 200-400 mesh) that had been washed with 0.1N-HCl and then water. The mixture was shaken in ice for 15 min. and filtered under suction into

0.5 ml. of saturated tris. The resin was washed three times with 2 ml. of cold water, and the final pH of the filtrate was adjusted to 8.0 with tris.

Enzyme preparation from rabbit kidney cortex. To increase the relative amount of Na^+ -plus- K^+ -activated ATPase activity in membrane preparations of kidney cortex, these were treated with urea, as outlined by Kinsolving, Post & Beaver (1963). Rabbits were killed by a blow on the head and the kidneys removed. A portion (2 g.) of cortex was homogenized in 20 ml. of sucrose (0.25M) and EDTA (2mM) in 1mM-imidazole buffer, pH 7.4, in a glass homogenizer surrounded by ice and fitted with a Teflon pestle (clearance about 0.14 mm., 700 rev./min.). After 20 complete strokes of the pestle the homogenate was squeezed through a double layer of muslin. This material was used in experiments with fresh whole homogenate. The filtered homogenate was then spun at 1000g (2400 rev./min.) for 20 min. in an MSE Mistral 6L Refrigerator Centrifuge (69167 head) at 0°. The supernatant fluid was decanted, the fluffy white layer of the sediment resuspended in the homogenizing medium and the centrifugation repeated. The washed sediment was then suspended in a solution containing urea (1.3M), imidazole buffer (12mM), EDTA (2mM), MgCl_2 (0.1mM) and $(\text{NH}_4)_2\text{SO}_4$ (7.6mM), pH 7.4, and stored at 2° for 3 days. After storage in urea, the suspension was spun again at 1000g for 20 min. at 0° and the sediment washed twice in a solution containing imidazole buffer (25mM), histidine hydrochloride (125mM) and EDTA (13μM), pH 6.8. The final sediment was resuspended in 10 ml. of a solution containing imidazole buffer (100mM), histidine hydrochloride (50mM) and EDTA (0.3mM), pH 6.8, and stored in the refrigerator at 2°.

Preparation of fragmented erythrocyte membranes. These were prepared as outlined by Wheeler & Whittam (1964), but after being washed they were frozen and thawed twice before being stored in the frozen state.

ATPase activity. Reaction mixtures (5 ml. final volume in conical flasks) contained imidazole-HCl buffer, pH 7.6

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† Abbreviation: ATPase , adenosine triphosphatase.

(8mm), various amounts of the cations under investigation as their chloride salts and ATP as the tris or disodium salt. In experiments in which the concentrations of Mg^{2+} and ATP were varied, the final concentration of Na^+ was 80mm and that of K^+ 16mm; when K^+ was omitted the concentration of Na^+ was increased to 96mm. When ouabain was added the concentration was 0.2mm. Except where indicated in the text, Mg^{2+} and ATP were added in equimolar amounts. The reaction was started by adding 0.1 or 0.2ml. of the kidney preparation (containing 0.5–1.0mg. of protein) or 1.0ml. of erythrocyte-membrane suspension (containing 5–7mg. of protein) and incubation was carried out at 37° in a shaking water bath at 120 oscillations/min. for 30–60 min. The reaction was stopped by adding 1.0ml. of 35% (w/v) trichloroacetic acid, and the flasks were cooled in ice before transferring their contents to centrifuge tubes and centrifuging at 0°. P_i was determined in the supernatant by the Fiske–Subbarow reaction at room temperature in the modification recommended by Bartlett (1959). One unit of enzyme activity is defined as that liberating 1 μ mole of P_i /hr.; specific activity is expressed as units/mg. of protein (or ml. of suspension). Protein was determined by the biuret reaction (Layne, 1955). The Na^+ -plus- K^+ -activated ATPase activity was obtained as the difference between the P_i liberated in a medium containing Na^+ and K^+ , and that in a medium containing no K^+ or containing ouabain.

RESULTS

Properties of the preparations from kidney cortex

Table 1 shows that after treatment with urea some 70–80% of the total ATPase activity of the enzyme preparation was activated by Na^+ and K^+ together in a way that was sensitive to ouabain. Other preparations showed somewhat less Na^+ -plus- K^+ -activated ATPase activity, but the value did not fall below 60% of the total. Precise measurements of small degrees of inhibition are therefore possible after treatment with urea, which appears

Table 1. Activity of urea-treated membrane ATPase from rabbit kidney

Enzyme suspension (0.1 ml., containing 0.7 mg. of protein) was added to 4.9 ml. of solution containing (final concentrations) $MgCl_2$ (2mm), Na_2ATP (2mm), imidazole buffer, pH 7.6 (8mm), and Na^+ and K^+ as indicated. Incubation was carried out at 37° for 60 min. The values are means of two to four experiments.

Incubation medium	Total ATPase activity (units/ml. of suspension)	Inhibition (%)
80mm- Na^+ , 16mm- K^+	6.7	—
96mm- Na^+ , no K^+	1.6	76
96mm-Choline, 16mm- K^+	1.7	74
80mm- Na^+ , 16mm- K^+ , 0.2mm-ouabain	1.4	79

Fig. 1. ATPase activity of kidney preparation with various concentrations of MgATP. Conditions were as outlined in the Methods section and in Table 1. Samples of enzyme suspension (0.2ml. containing 5.3mg. of protein/ml.) were added to start the reaction. Incubation was carried out at 37° for 40 min. The amounts of $MgCl_2$ and of ATP added were such as to give a final concentration of 0.2, 0.5, 1.0, 2.0 and 5mm for total magnesium and total ATP. The concentrations of MgATP plotted on the abscissa were calculated from the formation constant, K_f , for MgATP given by Walaas (1958) as $10^{4.04}M^{-1}$. K^+ was omitted in one set of flasks to obtain, by difference, the Na^+ -plus- K^+ -activated ATPase activity. ▲, Total ATPase; ●, Na^+ -plus- K^+ -activated ATPase.

Fig. 2. ATPase activity of kidney preparation with various concentrations of Na^+ . Enzyme suspension (0.1ml., containing 0.7mg. of protein) was added to start the reaction. Incubation was at 37° for 15 min. The concentration of K^+ was 5mm. The Na^+ concentration was varied as shown on the abscissa. Tris-ATP and $MgCl_2$ were added to the medium to give a final concentration of 3mm each. K^+ was omitted in one set of flasks to obtain, by difference, the Na^+ -plus- K^+ -activated ATPase activity. ▲, Total ATPase; ●, Na^+ -plus- K^+ -activated ATPase.

to cause deterioration of the Na^+ -plus- K^+ -insensitive part of the total ATPase (Kinsolving *et al.* 1963).

Increasing concentrations of Mg^{2+} and ATP (equimolar) raised the Na^+ -plus- K^+ -activated ATPase activity and the concentration giving half-maximal activity was about 0.3 mM (range of four preparations was 0.28–0.33 mM) (Fig. 1). The total ATPase activity increased in a similar way and the two curves are in the form of a rectangular hyperbola, as would be expected for MgATP as the substrate.

Fig. 2 shows that with a constant K^+ concentration (5 mM) the ATPase activity increased with increase in Na^+ concentration, with a concentration of about 4 mM giving half-maximum activation (range of four preparations was 1.6–6.4 mM). It appears that the Na^+ -plus- K^+ -activated ATPase may be treated formally as if both MgATP and Na^+ are 'substrates' in the standard treatment of enzyme kinetics of Lineweaver & Burk (1934).

Inhibition by calcium. A calcium concentration corresponding to 10% (0.2 mM) of that of the MgATP concentration had no effect on the ATPase activity in the presence of ouabain or absence of K^+ . On the other hand, it caused about $(7.71 - 6.41) / (7.71 - 3.65) \times 100 = 32\%$ inhibition of the Na^+ -plus- K^+ -activated ATPase activity (Table 2). This result confirms that of Wheeler & Whittam (1964) in which 22% inhibition was obtained with a concentration of calcium equal to 5% of the MgATP concentration.

To test whether the inhibition due to calcium was competitive with respect to the MgATP concentration, the latter was raised when the calcium concentration was constant. Fig. 3 shows clearly that the inhibition by calcium at low MgATP

concentrations was not found at higher concentrations.

Fig. 3 does not distinguish between inhibition by free Ca^{2+} and a complex of Ca^{2+} with ATP (CaATP). Excess of ATP (an extra 5 mM) was therefore added to the reaction mixture as the sodium salt to depress the concentration of free Ca^{2+} from 0.07 to 0.01 mM without decreasing the concentration of CaATP (Table 3). The degree of inhibition by calcium was almost the same with 2.4 mM- and 7.4 mM-ATP (52 and 50%), indicating that a major decrease in the free Ca^{2+} concentration did not relieve the inhibition. It was therefore important to change the CaATP concentration to see if less CaATP caused less inhibition. Addition of equimolar CaATP to give a final concentration of 0.14 mM caused inhibition of only 23%, whereas when the final concentration was 0.33 mM the inhibition was 52%. Lowering the concentration of CaATP therefore substantially decreased the inhibition, even though free Ca^{2+} declined only slightly, from 0.07 to 0.056 mM. The MgATP concentration was the same under these two conditions. The results show that inhibition of the Na^+ -plus- K^+ -activated ATPase is correlated with the concentration of CaATP and not with that of free Ca^{2+} .

Table 2. Inhibition by CaATP of ATPase from rabbit kidney

Enzyme suspension (0.1 ml., containing 0.77 mg. of protein) was added to 4.9 ml. of medium containing (final concentrations) MgCl_2 (2 mM), ATP (2 mM), imidazole buffer, pH 7.6 (8 mM), and Na^+ , K^+ and ouabain in the concentrations indicated. Incubations were carried out at 37° for 30 min. Mean values $\pm$ s.d. are given for the numbers of experiments shown in parentheses.

Incubation medium	ATPase activity (units/mg. of protein)	
	No calcium	CaATP (0.2 mM)
80 mM- Na^+ , 16 mM- K^+	$7.71 \pm 0.31^*$ (5)	$6.41 \pm 0.45^*$ (4)
80 mM- Na^+ , 16 mM- K^+ , 0.2 mM-ouabain	3.65 (2)	3.49 (2)
96 mM- Na^+ , no K^+	3.54 (2)	3.55 (2)

* Significantly different ($P < 0.01$).

Fig. 3. Competitive inhibition by calcium of Na^+ -plus- K^+ -activated ATPase in a kidney membrane preparation. Enzyme suspension (0.2 ml., containing 0.54 mg. of protein) was added to start the reaction. Incubation was at 37° for 60 min. The amounts of MgCl_2 and ATP added to the 5 ml. reaction volume were such as to give final concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0 mM for total magnesium and total ATP. Reciprocals of the rate of hydrolysis and of MgATP concentration are plotted. The concentrations of MgATP referred to on the abscissa were calculated from a K_i for MgATP of $10^{4.04} \text{ M}^{-1}$ and K_i for CaATP of $10^{3.77} \text{ M}^{-1}$ (Walaas, 1958). ●, No calcium; Δ, CaCl_2 added to give a final concentration of 0.5 mM.

Table 3. Effect of adding ATP on the inhibition of rabbit-kidney Na⁺-plus-K⁺-activated ATPase by calcium

Enzyme suspension (0.1 ml., containing 0.79 mg. of protein) was added to 4.9 ml. of medium containing (final concentrations) Na⁺ (80 mM) and K⁺ (16 mM), imidazole buffer, pH 7.6 (8 mM), Na₂ATP and magnesium and calcium in the concentrations shown. Incubation was carried out at 37° for 30 min. The concentrations of 'free ATP', Mg²⁺, MgATP, Ca²⁺ and CaATP at the start of the reaction were calculated from formation constants, K_f, given by Walaas (1958) as 10^{4.04}M⁻¹ for MgATP and 10^{3.77}M⁻¹ for CaATP. Complexes of ATP with Na⁺ and K⁺ were neglected in these calculations. The values for Na⁺-plus-K⁺-activated ATPase are means ± s.d. of four observations.

Incubation medium	Total ATP (mM)	'Free ATP' (mM)	Total Mg (mM)	MgATP (mM)	Mg ²⁺ (mM)	Total Ca (mM)	CaATP (mM)	Ca ²⁺ (mM)	Na ⁺ -plus-K ⁺ -activated ATPase (units/mg. of protein)	Inhibition (%)
No Ca	2.0	0.40	2.0	1.60	0.40	—	—	—	4.18 ± 0.08	—
No Ca, 5 mM-ATP	7.0	5.04	2.0	1.96	0.04	—	—	—	4.08 ± 0.10	—
0.4 mM-CaATP	2.4	0.45	2.0	1.62	0.38	0.40	0.33	0.07	1.98 ± 0.28	52
0.4 mM-CaATP, 5 mM-ATP	7.4	5.05	2.0	1.96	0.04	0.40	0.39	0.01	1.99 ± 0.17	50
0.2 mM-CaATP	2.2	0.44	2.0	1.62	0.38	0.20	0.14	0.056	3.22 ± 0.21	23

Because it seemed possible that treatment with urea had altered the characteristics of the native enzyme, the inhibitory action of CaATP was tested on a fresh homogenate of rabbit kidney cortex. As illustrated in Fig. 4, when the concentration of MgATP was varied, the competitive nature of inhibition by CaATP was apparent in the fresh homogenate as well as with the urea-treated enzyme.

In contrast with the competitive effects in Figs. 3 and 4 no change in the inhibitory effect of calcium was found when the Na⁺ concentration was varied from 1 to 100 mM (Fig. 5). In these experiments the concentration of K⁺ was kept at 5 mM or less to avoid competition between K⁺ and Na⁺ as the Na⁺ concentration was lowered. The two parallel straight lines in Fig. 5 do not indicate a competitive relationship and are characteristic of 'uncompetitive inhibition'. They suggest that there is no connexion between the effects of Na⁺ and calcium. The uncompetitive plots may be a consequence of the competitive behaviour of CaATP towards MgATP, if Na⁺ is considered as a second substrate (Dalziel, 1963).

Properties of the erythrocyte-membrane preparation

The Na⁺-plus-K⁺-activated ATPase of human erythrocyte membranes is like that of kidney in

Fig. 4. Competitive inhibition by CaATP of Na⁺-plus-K⁺-activated ATPase in fresh kidney homogenate. A sample of fresh homogenate of rabbit kidney cortex (0.1 ml., containing 0.5 mg. of protein) was incubated in a final volume of 5 ml. for 15 min. Reciprocals of the rate of ATP hydrolysis and of MgATP concentration are plotted, showing competition between CaATP and MgATP. The concentration of MgATP was calculated from the formation constant, as in Fig. 3. ●, No calcium; Δ, CaCl₂ and Na₂ATP added to give a final concentration of 0.2 mM.

Fig. 5. Effect of Na^+ concentration on inhibition of kidney-cortex Na^+ -plus- K^+ -activated ATPase by calcium. There is no evidence for competition between Na^+ and calcium. The reciprocal of the velocity of the Na^+ -plus- K^+ -activated ATPase reaction is plotted on the ordinate against the reciprocal of the Na^+ concentration on the abscissa. A portion (0.1 ml.) of urea-treated enzyme suspension from rabbit kidney cortex was added to 4.9 ml. of medium to start the reaction. Incubation was at 37° for 35 min. Concentrations of MgCl_2 and tris-ATP were 3 mM, and the concentration of K^+ was 5 mM. ●, No calcium; Δ, CaCl_2 and ATP added to give a final concentration of 0.2 mM.

Fig. 6. Effect of MgATP concentration on the Na^+ -plus- K^+ -activated ATPase and ouabain-insensitive ATPase of erythrocyte membranes. A sample of membrane suspension (1.0 ml., containing 6.8 mg. of protein) was added to 4 ml. of medium and incubated at 37° for 60 min. At the start of the reaction all flasks contained Na^+ (80 mM), K^+ (16 mM) and imidazole buffer, pH 7.6 (10 mM). Ouabain (0.2 mM) was added to every other flask and Na^+ -plus- K^+ -activated ATPase activity was obtained by subtracting P_i liberated in the presence of ouabain from that liberated in its absence. The concentrations of MgATP plotted on the abscissa are those actually added and assume negligible dissociation of the MgATP complex. ○, Ouabain-resistant ATPase, no added calcium; Δ, ouabain-resistant ATPase, CaATP (0.25 mM) added; ●, ouabain-sensitive ATPase, no added calcium; ▲, ouabain-sensitive ATPase, CaATP (0.25 mM) added.

Fig. 7. Competition between CaATP and MgATP in the Na^+ -plus- K^+ -activated ATPase activity of human erythrocyte membranes. The Figure shows a Lineweaver-Burk plot of the Na^+ -plus- K^+ -activated ATPase activities given in Fig. 6, illustrating competition between CaATP and MgATP in the Na^+ -plus- K^+ -activated ATPase reaction. ●, No added calcium; ▲, CaATP (0.25 mM) added.

being inhibited by calcium. A difference, however, is that the Na^+ -plus- K^+ -insensitive ATPase is activated by Ca^{2+} instead of being inhibited (Dunham & Glynn, 1961; Hoffman, 1962). In seeing whether the inhibition by calcium is reversed by the addition of MgATP, allowance was made for the dual effects of calcium. Fig. 6 shows that, whatever the MgATP concentration, 0.25 mM-calcium stimulated ATPase activity in the presence of ouabain. In contrast, the Na^+ -plus- K^+ -activated ATPase was inhibited at low concentrations of MgATP but not in the presence of 5 mM-MgATP. A plot of reciprocals of Na^+ -plus- K^+ -activated ATPase activity and MgATP concentration in Fig. 7 shows that, as with kidney, the erythrocyte Na^+ -plus- K^+ -activated ATPase is inhibited by CaATP, which interacts competitively with MgATP. The concentration of MgATP required for half-maximum activation of the Na^+ -plus- K^+ -activated ATPase was about 0.2 mM. The addition of excess of ATP, as with kidney, did not alter the calcium inhibition of Na^+ -plus- K^+ -activated ATPase.

DISCUSSION

The results described above have a bearing on three aspects of membrane function: (1) the mechanism of action of the Na^+ -plus- K^+ -activated ATPase; (2) the way inhibition by calcium affects the active transport of Na^+ and K^+ ; and (3) the way calcium affects the function of the intact kidney.

The results show that calcium retards Na^+ -plus- K^+ -activated ATPase activity in membranes of

kidney cortex and erythrocytes by competing with its substrate, MgATP. Because CaATP rather than ionic Ca^{2+} is responsible for the inhibition, it seems likely that CaATP attaches reversibly to the enzyme at the same site as MgATP, but is less easily hydrolysed by the enzyme to ADP and P_i . Strong indirect support is thus provided for the hypothesis that MgATP, rather than ATP, is the 'true substrate' of the Na^+ -plus- K^+ -activated ATPase reaction. This is consistent with the finding that the optimum $\text{Mg}^{2+}/\text{ATP}$ ratio is 1:1 in membranes from rabbit kidney (Wheeler & Whittam, 1962) and human erythrocytes (Dunham & Glynn, 1961). Comparable studies do not appear to have been made with other ATPases, and although Ca^{2+} generally inhibits phosphatases (see Dixon & Webb, 1964) it is not known whether it acts competitively with respect to the substrate. The competition in the present experiments was found with relatively low calcium concentrations. Roughly tenfold higher concentrations are needed to inhibit the membrane ATPase that is insensitive to Na^+ and K^+ (Wheeler & Whittam, 1964). The mode of action of Ca^{2+} on the latter ATPase may be different from the action on the Na^+ -plus- K^+ -activated ATPase.

The physiological evidence of an interaction between Ca^{2+} and Na^+ (Hodgkin, Huxley & Katz, 1949; Niedergerke & Luttgau, 1957; Walser, 1961) has raised the question whether Ca^{2+} might inhibit the Na^+ -plus- K^+ -activated ATPase by competing with Na^+ for an enzymic site. Judah & Ahmed (1962, 1964) suggested that the two ions were antagonistic in a reaction involving the turnover of phosphoprotein in liver slices. More directly relevant, Portius & Repke (1962) reported that inhibition by Ca^{2+} of the Na^+ -plus- K^+ -activated ATPase of heart muscle was reversed by raising the Na^+ concentration. Our results with kidney cortex, however, show that the inhibition by calcium was unaffected by a large change in the concentration of Na^+ of the medium. There may be differences in different tissues, and it is an open question whether Ca^{2+} and Na^+ are generally antagonistic by virtue of direct competition.

As a physiological correlation of these experiments one might expect that a high cellular concentration of calcium would interfere with the utilization of energy for the active transport of Na^+ and K^+ across cell boundaries. The presence of calcium at the internal face of the cell membrane, rather than its concentration in extracellular fluid, would be crucial, since it is on the interior that MgATP is normally available to the membrane Na^+ -plus- K^+ -activated ATPase. These inferences are borne out in experiments on erythrocytes. High external concentrations of calcium do not alter transport of Na^+ or K^+ by human erythrocytes. When calcium

is incorporated into erythrocytes or erythrocyte 'ghosts', on the other hand, active transport of K^+ and Na^+ is depressed (Hoffman, 1962; Rummel, Seifen & Baldauf, 1963).

Calcium also appears to depress energy-linked transport in brain cells. When the calcium content of brain slices is increased above that of fresh tissue, their intracellular Na^+ content increases. This difference between calcium-loaded slices and those with normal calcium content is eliminated by electrical stimulation, which depolarizes the cell membrane and causes an influx of Na^+ . When the electric current is interrupted, the difference reappears, for Na^+ extrusion by the calcium-loaded slice is slower and less complete (Keeseey, Wallgren & McIlwain, 1965).

At the level of the whole organ, hypercalcaemia induced in intact animals retards the reabsorption of Na^+ by the kidneys while increasing the content of calcium in kidney tissue (Epstein, 1960). When this happens, tissue calcium is highest in the medulla of the kidney and it is here that function is most prominently impaired (Beck, Levitin & Epstein, 1959; Carone, Epstein, Beck & Levitin, 1960; Manitiis, Levitin, Beck & Epstein, 1960; Epstein, Beck, Carone, Levitin & Manitiis, 1959). Though interpretation of these experiments is complicated, they are at least consistent with the hypothesis that an increase in intracellular calcium inhibits active transport by interfering with the Na^+ -plus- K^+ -activated ATPase reaction.

The present results may be considered in relation to the normal content of calcium, magnesium and ATP in kidney cortex and erythrocytes. The concentration of calcium in human erythrocytes is negligible (Kramer & Tisdall, 1922), presumably because the permeability of erythrocyte membranes to calcium is so low (Rummel, Seifen & Baldauf, 1962). This serves an important physiological purpose because internal calcium would inhibit Na^+ -plus- K^+ -activated ATPase and the active transport of ions (Hoffman, 1962), and would therefore predispose to haemolysis.

In kidney and liver the concentration of calcium is about 1–3 m-moles/kg. of fresh tissue (Hofer & Kleinzeller, 1963; Widdowson & Dickerson, 1964). The content of magnesium is three to five times this (Hofer & Kleinzeller, 1963; Widdowson & Dickerson, 1964), and the content of ATP is 1–3 m-moles/kg. (Dianzani, 1957; Chance *et al.* 1965; Schenker & O'Donnell, 1965). Most of the calcium and magnesium in the cell is bound by the nucleus and mitochondria (Thiers & Vallee, 1952; Hofer & Kleinzeller, 1963) and only a small fraction of the remaining cytoplasmic calcium is ionized (Hodgkin & Keynes, 1957). If calcium and magnesium were distributed similarly throughout the cell, the CaATP/MgATP ratio at the cell membrane would

be close to that found in the present experiments to inhibit Na^+ -plus- K^+ -activated ATPase. Calcium and magnesium are not, however, evenly distributed, but are differentially bound by subcellular fractions. For example, Thiers & Vallee (1952) found that the proportion of cellular calcium bound to liver mitochondria was about twice that of magnesium, whereas the reverse relation held in the supernatant.

As well as inhibiting membrane Na^+ -plus- K^+ -activated ATPase, calcium inhibits phosphofructokinase (A. Underwood & E. Newsholme, personal communication) and pyruvate kinase (Kachmar & Boyer, 1953), key glycolytic enzymes located in the cytoplasm. The implication of these facts is that cells require mechanisms for decreasing the concentration of calcium in the cytoplasm and at the inner surface of the cell membrane. Under some circumstances the efficacy of these mechanisms might regulate cellular metabolism. Calcium could be excluded from entry into the cell at the limiting cell membrane (as in erythrocytes; Rummel *et al.* 1962) or actively transported out of the cell (Hodgkin & Keynes, 1957). Calcium might also be sequestered within the cell, away from the enzymes particularly susceptible to inhibition. Isolated mitochondria are known to accumulate calcium from the bathing medium in a reaction driven by respiration and requiring ATP (Vasington & Murphy, 1962; Drahota, Carafoli, Rossi, Gamble & Lehninger, 1965; Rasmussen, Chance & Agata, 1965). The preferential accumulation proceeds even at extremely low concentrations of ionized calcium and in the presence of an excess of magnesium. The function of the mitochondrial calcium pump in intact cells is not clear but it is tempting to suggest that it serves the need to decrease the calcium content of extramitochondrial portions of the cell.

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