ATP Splitting and Calcium Binding by Brain Microsomes Measured with a Rapid Perfusion Method

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ABSTRACT Rat brain microsomes, immobilized on a filter, were perfused with ATP-containing solutions in a device which made possible rapid change of perfusion media and frequent sampling of effluent. Inorganic phosphate production could be measured 10 times per sec. When ATP, sodium, or potassium was absent from the first perfusion medium and present in a second, and introduced without interrupting flow, phosphate output rose within a few tenths of a second. Inhibition by ouabain began within 0.3 sec but did not become maximal for at least 10 sec. Rapid binding of ouabain was minimal or absent, as was rapid release of ouabain on introducing potassium abruptly. Although the preparation bound some calcium reversibly, no measurable uptake of calcium occurred coincident with activation by ATP or by potassium, and no measurable release of calcium occurred coincident with the onset of ouabain inhibition. However, activation by sodium was consistently associated with simultaneous release (within <1 sec) of calcium, averaging 46 pmole per mg of protein. Calcium release in response to sodium also occurred in the absence of ATP or in the presence of ouabain. At 0°C sodium produced neither activation nor calcium release. The results are consistent with the possibility that sodium and calcium are competitively bound, even in the absence of ATP, to an active site on the enzyme distinct from the sites of potassium activation or glycoside inhibition.

INTRODUCTION

Enzyme systems which play a role in transport processes utilize metabolic energy in the translocation of solutes. It seems axiomatic that the transported solutes must become bound and subsequently released during this process. More precisely, the enzyme system must be capable of altering the chemical potential of the solute(s) in an anisotropic fashion.

A central question is how the utilization of chemical energy, for example

ATP, leads to translocation of solutes, for example sodium and potassium. To answer this question, it is first necessary to determine the reaction sequence in which ATP uptake, sodium and potassium binding, inorganic phosphate release, and sodium and potassium release at opposite sides of the membrane occur. However, the minute amounts of these cations bound by such enzyme systems are difficult to measure. The affinity of proteins and phospholipids for calcium is generally much higher than for alkali cations, and there is now considerable evidence pointing to the existence of common transport mechanisms for calcium and sodium, requiring ATP and inhibited by cardiac glycosides (see Discussion). Thus uptake or release of calcium might reflect the activity of such enzyme systems.

Although many workers have demonstrated calcium binding or uptake by membrane preparations from a variety of tissues (see Discussion), processes which are often facilitated by ATP and occasionally inhibited by cardiac glycosides, it seems possible that such binding or uptake is generally a secondary result of the activity of these systems rather than an integral part of the mechanism by which they bring about transport. This is suggested by the rate of calcium accumulation, which is usually measured in minutes, while the response to sodium and potassium is much more rapid (1). Furthermore, calcium accumulation usually is demonstrable only in the presence of anions which form sparingly soluble calcium salts, such as phosphate or oxalate.

In the present work a technique for measuring activity and changes in cation binding simultaneously several times per second is presented. Results of study of brain microsomal ATPase by this method suggest that many of the previously reported changes in calcium binding or uptake in response to ATP or ouabain occur later than, and are therefore secondary to the concomitant changes in inorganic phosphate release. Activation by sodium, however, appears to involve rapid displacement of calcium from the active site.

METHODS

The principle of the technique was to perfuse the particulate enzyme, immobilized on a filter, with ATP-containing media and to analyze the effluent for inorganic phosphate (Pi) and cations. During a steady state, concentrations of Pi and cations in the outflow should remain constant; a change in the rate of ATP hydrolysis induced by altering the perfusion medium could then be correlated with cation uptake or release. A fundamental difference between this technique and others is that changes in cation binding, rather than the total quantity bound, are determined.

In order to achieve optimal resolution in this method, the maximal rate of perfusion per unit weight of enzyme should be combined with minimal mixing of solutions before or after the enzyme. In the technique described here, the rate of perfusion is relatively slow, but the problems of mixing upstream and downstream have been solved satisfactorily.

A Millipore filter holder was modified as in Fig. 1 by sealing Teflon tubing (I.D. 1.1 mm) into both halves with epoxy resin. When tightly closed, the interior volume was about 120 μl. Particulate enzyme, prepared from rat brain as described by Skou ("II $Sed_{20,000}$," reference 2), and stored at −20°C until use, was placed on Millipore filters, type GS (0.22 μ pore size), by passing enzyme suspension through the apparatus. Approximately 70% of the protein content of the suspension remained on the paper. Usually we used three papers, one on top of the other, each supporting about 0.3 mg of enzyme protein, in order to obtain higher flow rates. This was done by successive insertions of papers followed by filtrations of enzyme suspension. Several milliliters of buffered salt solution (see below) were then perfused to remove traces of enzyme suspension medium. Before each experiment, a mercury droplet, approximately 5 μl in volume, was also instilled in the apparatus. Metallic mercury does not pass through this filter paper. In the presence of metallic mercury, variable inhibition

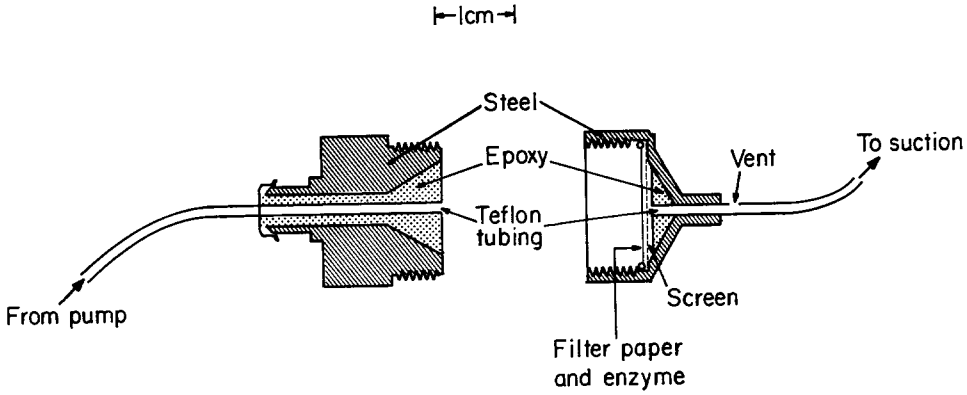

FIGURE 1. Diagram of apparatus. The total lengths of the inflow and outflow tubes were 300 cm and 100 cm, respectively.

of the enzyme was observed, but this was completely overcome by adding 0.1 mM dithiothreitol to all solutions. The inflow tube was usually about 300 cm long and contained the first solution. In its proximal end, another droplet of mercury was instilled. It was then connected to a syringe containing the second solution. In some cases, the second solution was contained between two mercury droplets in the tube and the third solution was in the syringe. A constant speed pump drove the syringe. The rate of flow could be altered by varying the size of the syringe as well as the speed of the pump.

Vibration of the filter holder, suspended in a constant temperature bath with the outflow nozzle protruding, was accomplished mechanically to improve mixing in the upstream chamber (with the aid of the mercury droplet). The experiment was started by turning on the pump. As the first mercury droplet approached the filter, we applied suction to the end of the outflow tube, using a syringe. With practice, the rate of air inflow could be adjusted to achieve fairly uniform interruption of the outflow stream by bubbles. Before and after the application of suction, unwanted effluent ran out the vent.

The contents of the outflow tube were analyzed in aliquots varying from 10 to 50 μl. These were obtained either by cutting the tube into segments, and weighing each segment before and after expelling its contents with air, or by slowly pumping the contents into a succession of micropipettes or tubes. In both methods, we kept a cumulative record of the volume of the aliquots. Cumulated volume could then be equated with elapsed time, knowing the rate of flow. In most of the experiments, flow was 102 μl per sec. Thus 3–10 samples per sec were analyzed.

Analytical methods were as follows: ATP (or ATP + ADP) was measured by absorbance at 260 mμ. Sodium and potassium were determined by atomic absorption spectrophotometry. ^{24}Na was measured in a gamma scintillation counter equipped with a well. ^{45}Ca could not be measured reliably in the presence of ATP by using the scintillation mixtures in common use. The following procedure was found to yield reproducible and stable results: to 5 ml of a mixture of citric acid, 2% (w/v), and 1 M aqueous tris (hydroxymethyl) amino methane, 2% (v/v), dissolved in methanol, was added 10–50 μl of sample, followed by 10 ml of diphenyloxazole 0.4% (w/v) and 1,4-*bis*-2(5-phenyloxazolyl)-benzene, 0.02% (w/v) in toluene. A small amount of quenching was induced by the aqueous medium (less than 5%) and was corrected for by counting at two energy levels, both higher than the maximal energy of tritium. A third channel at lower energy provided values from which tritium concentrations could be calculated. Usually 10^6 counts were accumulated for each sample. The standard deviation of replicate ^{45}Ca samples was always less than 1% and averaged 0.4%.

Most published methods for phosphate were too insensitive or were unsatisfactory for measuring small amounts of phosphate in the presence of large amounts of ATP. The method of Baginski, Foa, and Zak (3) was adapted to a micro scale by using a total reaction volume of 70 μl. In a Zeiss PMQII spectrophotometer (Carl Zeiss, Inc., New York), with cells 10 × 2 mm, it was possible to measure absorbance on 60 μl aliquots. Under these conditions 1 nmole of phosphate yielded an optical density of 0.45 at 860 mμ. Absorbance obeyed Beer's law and was stable for many hours. Freshly prepared solutions of ATP contained less than 0.002 mole of phosphate per mole when measured by this method. Protein was measured by the method of Lowry et al. (4).

RESULTS

Flow Characteristics of the Apparatus

The washout curve of tritiated water, shown on a semilogarithmic scale in Fig. 2, provides a complete description of the rate at which the fluid in the apparatus exchanges with the medium, during perfusion at 102 μl per sec. We assume here that all of the water contained in the apparatus is exchangeable. Within 2 sec, tritium concentration in the effluent is reduced to 4%. The shape of this curve varied somewhat from one experiment to another. In view of the complex and partly unknown geometry of the aqueous channels, complete mathematical interpretation of such curves is not feasible. However the maximal (negative) value of the first derivative of the curve,

0.7 sec^{-1}, gives the flow per unit volume in the most rapidly exchanging portion of the protein matrix (5), which evidently comprises at least 80% of the total volume.

The characteristic pattern of outflow of a solute released from particles on the filter was also examined. Ion exchange particles of comparable size were prepared by grinding "Chelex 100" resin (Bio-Rad Laboratories, Richmond,

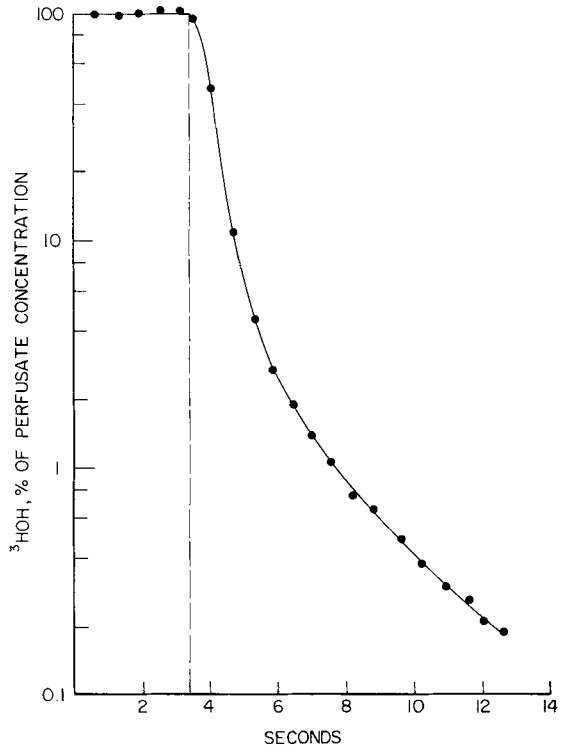

FIGURE 2. Washout curve of tritiated water from the apparatus. Two buffered salt solutions were used, the first also containing tritiated water. 1 mg of enzyme protein was perfused at 102 μl per sec with the two solutions, successively. Tritium measurements made on aliquots of 40–60 μl plotted as percentage of perfusate concentration on a logarithmic scale against time from an arbitary zero. The dashed line, positioned visually, represents the arrival of the second solution.

Cal.) in a mortar, filtering the suspension through paper of 0.8 μ pore size, and then through the apparatus shown in Fig. 1. The particles trapped on the filter could then be equilibrated with medium calcium by prolonged perfusion. When a second solution containing the same calcium concentration in HCl 1 N was suddenly injected (as described in Methods), a transient increment in effluent calcium concentration was observed.

Release of calcium from the microsomal particles was slower. Although

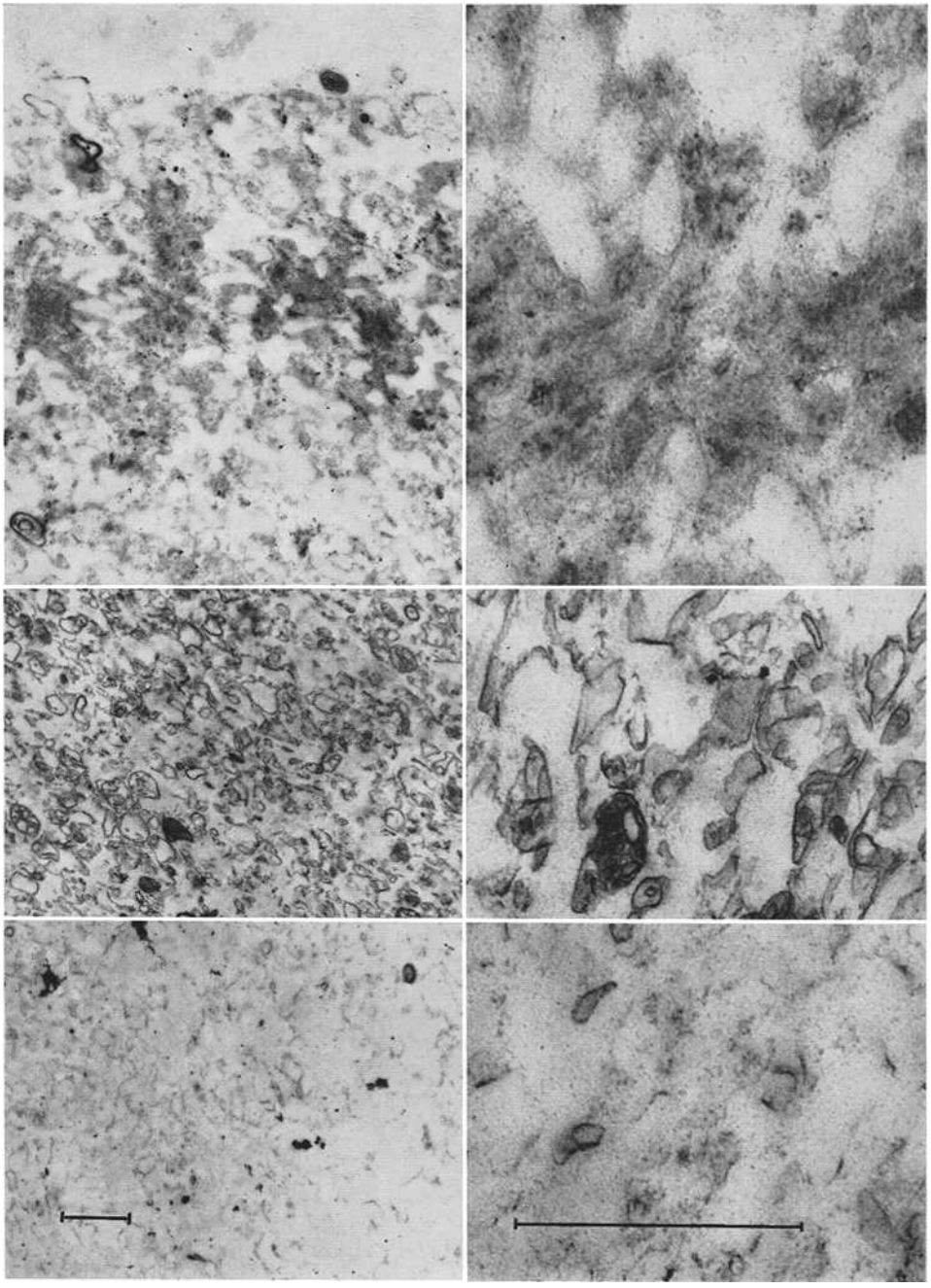

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FIGURE 3

prompt release of some calcium (about 80 pmole per mg of protein) could be detected when the ionic calcium concentration and/or the pH of the medium was suddenly lowered, continued release was detectable for at least 8 sec. Counting of the sediment eluted from the paper after perfusion with the complete medium (see below) including ^{45}Ca, 6×10^{-6} M, followed by prolonged perfusion with the same medium free of calcium, revealed less than 50 pmole of calcium per mg of protein remaining on the filter.

The distribution of particulate matter within the filter is illustrated in Fig. 3. Membrane fragments and ribosomes are concentrated in a surface layer and also in a deeper layer in the meshes of the filter.

Enzymatic Activity Measured by Incubation in Flasks

The properties of this enzyme were similar to those reported by others (2, 6–14). The rate of hydrolysis of ATP in a solution containing 3 mM ATP, 6 mM $MgCl_2$, 20 mM Tris buffer (pH 7.2), and 0.04 mg/ml of enzyme protein was 10–20 μmole per hr per mg of protein. When 100 mM NaCl and 20 mM KCl were also present, activity was two- to fivefold greater (Table I). In these experiments, the incubation time was usually 10 min, but frequent sampling established that the rate of hydrolysis was somewhat more rapid during the first few minutes. Calcium was inhibitory even at 0.3 mM (Table I). Maximal stimulation by sodium ions in the presence of 20 mM KCl occurred at a concentration of 40–50 mM, as shown in Table II.

Enzymatic Activity Measured by Perfusion

During constant perfusion, phosphate output remained constant for at least 1 min (Table III). The rate of hydrolysis of ATP was about 20% slower during perfusion than during incubation (Table IV), when ATP was present in excess. This difference may reflect (*a*) different activities of filtrable and nonfiltrable protein, (*b*) portions of the enzyme entrapped in the filter which

FIGURE 3. Electron microscopic appearance of microsomal fraction (~0.3 mg of protein) on Millipore filter. Upper left: material accumulated on the surface of the filter. Upper right: higher magnification of this zone (scales given by 1 μ bars at bottom). This zone can be shown by protein staining and light microscopy to contain more than half of the total protein, and is about 10 μ deep. The electron-translucent network represents the meshes of the filter. The finely granular material has a ribosome-like appearance, and can be stained with toluidin blue. Middle photographs: material trapped near the center of the filter (~50 μ from the surface). Polymorphous membrane fragments are seen, but few if any mitochondira. Lower photographs: ~100 μ from the upper surface (~35 μ from the lower surface). Scattered delicate membrane fragments are seen. Fixed in 2% OsO_4 in isotonic barbital buffer, pH 7.4; embedded in Araldite® (Cargille Laboratories, Ocean Grove, N. J.); stained with lead citrate; photographed with an RCA EMU III F electron microscope (Radio Corp. of America, Camden, N. J.). Courtesy of Dr. Eduard Gfeller.

are not perfused by the medium, or (*c*) an effect of pressure on activity. The possibility that diffusion is limiting seems extremely improbable. If the criteria of Taylor (15) are applied to flow through pores 0.22 μ in diameter, the existence of radial diffusion gradients in the flowing stream can be readily dismissed. Since the particles are smaller than 0.8 μ in diameter, the rate of diffusion in or out, estimated by any reasonable assumptions from the equations for diffusion from a sphere (16), is very fast compared to the rate of flow.

On the other hand, when flow is reduced, phosphate output diminishes (Table IV), not because the rate of reaction is flow dependent but because

TABLE I

ATP HYDROLYSIS (μmole Pi per mg protein per hr) DURING INCUBATION IN THE PRESENCE OF VARYING CALCIUM CONCENTRATION

	[Ca], mM			
	0	0.3	1.0	3.0
Mg	10.2	12.8	9.3	7.7
Mg + Na + K	56.7	44.6	31.2	19.4

The reaction mixture contained 3 mM Tris-ATP, 6 mM $MgCl_2$, 20 mM Tris buffer (pH 7.2), and 0.04 mg/ml of protein, with or without 100 mM NaCl, 20 mM KCl, plus $CaCl_2$ additions as shown. Incubation was for 10 min at 37°C. The reaction was stopped by the addition of TCA.

TABLE II

ATP HYDROLYSIS AS A FUNCTION OF SODIUM CONCENTRATION

[Na], mM	0	10	20	30	40	50	80	100
Pi formed, *μmole per mg per hr*	9.2	14.7	17.8	20.2	21.9	23.0	23.3	22.8

The reaction mixture contained 3 mM Tris-ATP, 20 mM KCl, 20 mM Tris buffer (pH 7.4), and 0.05 mg per ml of microsomal protein. Incubation was for 10 min at 37°C. The reaction was stopped with TCA.

the effluent ATP concentration, which is presented to the most distal enzyme fragments, is now less than that required for maximal activity. This is illustrated in Table V, in which phosphate output and per cent hydrolysis of ATP are presented at varying ATP concentrations in the perfusate. Here flow was more rapid (72 μl per sec), but nevertheless, the fraction of ATP hydrolyzed fell at the lowest ATP concentration, apparently because of depletion of substrate in some channels. The other three observations conform to Michaelis-Menten kinetics (with a K_M for ATP of 3.1 mM), and indicate that reaction velocity at the lowest ATP concentration should have been 73% higher than observed.

Sudden addition of ATP was achieved by replacing a solution devoid of ATP with the complete mixture, containing in addition tritiated water. As shown in Fig. 4, effluent concentrations of adenine nucleotides and tritium, expressed as percentages of perfusate concentrations, were virtually superimposable. This permitted the use of ^{3}HOH as a marker for ATP in subsequent experiments, but the result itself is of some interest. If substantial fractions of the inflowing ATP were taken up by the enzyme preparation during wash-in or released during washout, tritium and ATP curves would have differed. It should be noted that the per cent hydrolysis of ATP under

TABLE III

ATP HYDROLYSIS DURING CONSTANT PERFUSION OF BRAIN MICROSOMAL PROTEIN

Time, *sec*	5	10	18	22	26	30	34	38	42	46	50	54	62	68
Pi output, *μmole per mg per hr*	43	43	45	43	44	44	41	40	44	42	44	43	42	41

The perfusion medium was the same as in Table I, including 100 mM NaCl, 0.1 mM $CaCl_2$. The filter contained 0.8 mg protein and was perfused at 11 μl per sec at 37°C.

TABLE IV

ATP HYDROLYSIS DURING PERFUSION AT VARYING RATES

Perfusion rate	Pi output
μl per sec	*μmoles per mg per hr, % of control*
11	49, 54
29	79, 89
79	81, 80

Values from two experiments, expressed as percentage of rate observed during incubation, after correction for protein lost during filtration.
The perfusion medium was the same as in Table I, including 100 mM NaCl, and 20 mM KCl. The filter contained 0.48 mg of protein and was perfused for 1 minute, at 37°C.

the conditions of this experiment was about 2%, so that absorbance at 260 mμ measures almost exclusively ATP.

Phosphate release began immediately on ATP wash-in, as shown in Fig. 5, but decayed more slowly than ATP during washout. However, the molar concentration of ATP is higher than that of Pi even after several seconds of washout. Whether the slow decay of Pi concentration is attributable to continued hydrolysis of the remaining ATP in the solution (as reflected in the washout curve) or to release from intermediates accumulated by the enzyme is uncertain. The total "excess" phosphate, calculated simply as the difference in area between the two washout curves shown in the figure, amounted to approximately 3 nmole.

Calcium Binding during Activation by ATP

In these experiments, radioactive calcium was added to both solutions at the same concentration (2×10^{-6} M) and the first solution (ATP-free) was perfused for several minutes in order for the enzyme preparation to achieve equilibrium with the ambient calcium. The arrival of ATP was marked by the disappearance of tritiated water instead of its appearance. When expressed

TABLE V

ATP HYDROLYSIS DURING PERFUSION WITH MEDIA OF VARYING ATP CONCENTRATIONS

[ATP]	Pi formed	ATP hydrolyzed
mM	*μmole per mg per hr*	%
0.1	8.9	23.7
0.3	31.6	28.1
1.0	47.8	12.7
3.0	58.8	5.2

All media contained 100 mM NaCl, 20 mM KCl, 20 mM Tris buffer (pH 7.4), 6 mM $MgCl_2$. The filter contained 1 mg of microsomal protein, and was perfused for 30 sec at 72 μl per sec at 37°C.

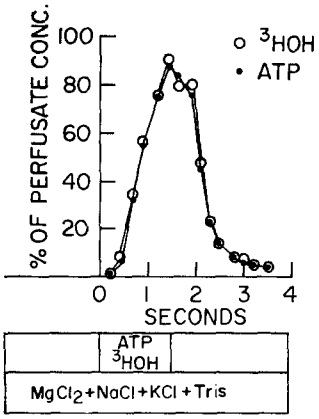

FIGURE 4. Tritiated water as a tracer for ATP. Three solutions were perfused successively, without interrupting flow. The second contained 3 mM ATP and tritiated water; all three contained 6 mM $MgCl_2$ 100 mM, NaCl, 20 mM KCl, and 20 mM Tris buffer (pH 7.2). Radioactivity and OD at 260 mμ plotted as percentages of concentrations in the second solution.

as a fraction of perfusate concentration, the tritium washout curve is obviously complementary to the wash-in curve of nontritiated water and thus of ATP. The medium contained 6 mM $MgCl_2$, 20 mM Tris buffer (pH 7.0), and either 0.12 M NaCl or 0.12 M tetramethylammonium chloride.

A representative experiment is shown in Fig. 6. The insert shows the area, corresponding to approximately 50 pmole of calcium per mg of protein, calculated from the calcium concentration, the flow, and the quantity of protein on the filter. Little or no change in effluent calcium concentration

occurred. The same result was obtained when tetramethylammonium was used instead of sodium. These experiments show that activation by ATP is accompanied by the uptake or release of less than 5 pmole of calcium per mg protein under these conditions.

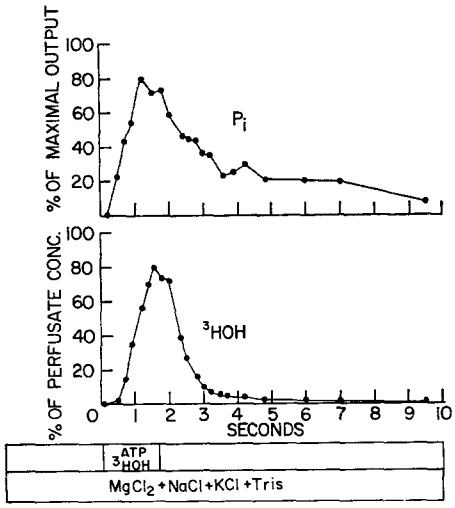

FIGURE 5. Phosphate production during and after a pulse of ATP. Same solutions as in Fig. 3. Tritium used as a measure of ATP concentration. Note that phosphate output falls more slowly than ATP concentration during washout.

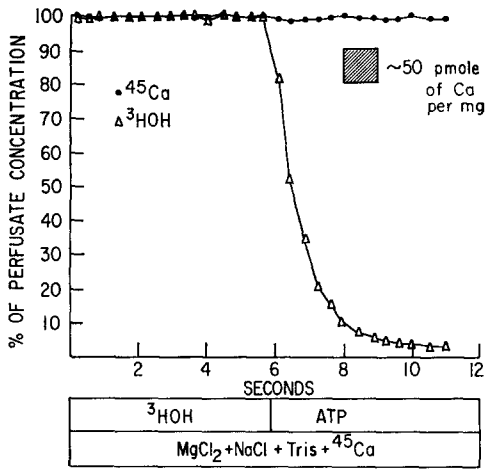

FIGURE 6. Absence of calcium uptake during activation by ATP. Two solutions containing 2 × 10^{-6} M labeled calcium were employed, as shown. The first contained tritiated water and the second contained 3 mM ATP. The appearance of ATP is thus marked by the disappearance of tritium. Calcium concentration in the effluent showed little or no change.

These results contrast sharply with the findings of Lieberman et al. (17) and Otsuka et al. (18), who independently found ATP-dependent calcium uptake by microsomal preparations from nervous tissue amounting to 5–40 nmole per mg. Several possible explanations for this discrepancy may be invoked. First, the period of incubation in the previously reported experiments was 10–15 min. Lieberman et al. (17) also gives the time course of calcium

uptake with measurements as early as 5 sec, but in this instance control observations without ATP are not included. When our preparation was exposed to calcium at very low perfusion rates in the presence or absence of ATP, uptake was readily demonstrable. As noted above, no measurements of changes in calcium binding such as the experiment shown in Fig. 6, were attempted until at least 1 min of equilibration with the medium calcium had elapsed. Second, phosphate was accumulating in the reported experiments but it was removed as fast as it formed in ours. It has been demonstrated that phosphate promotes calcium uptake in muscle microsomes (19, 20). Third, storage at −20°C or the use of deoxycholate in the initial preparation of the microsomes may have greatly reduced calcium-accumulating ability, as others (17, 21) have noted.

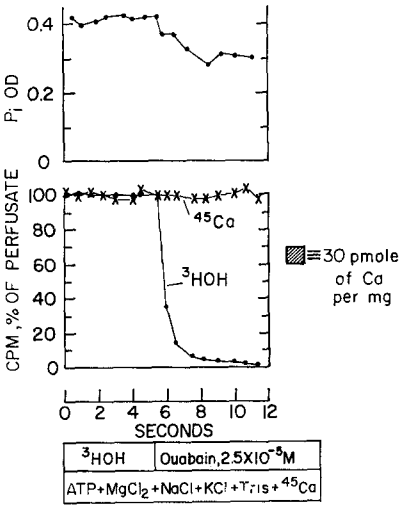

FIGURE 7. Effect of ouabain on enzymatic activity and bound calcium. The arrival of ouabain is marked by the disappearance of tritium. Phosphate output begins to fall immediately, but not as steeply as in Fig. 3, for example. No detectable release of uptake of calcium is seen.

Ouabain Inhibition

The response to perfusion with the complete medium plus ouabain, 2.5×10^{-5} M, was studied in eight experiments, one of which is shown in Fig. 7. Enzyme inhibition began immediately, i.e. within 0.3 sec, but did not reach the maximal level attainable with this concentration of glycoside for at least 10 sec. The rate of attainment of maximal inhibition was higher at greater concentrations of glycoside. The degree of maximal inhibition we observed by perfusion was comparable to that observed in flasks.

The slow development of ouabain inhibition could not be explained by delayed diffusion of the drug within aqueous channels. This was shown by comparing the distribution of ouabain and labeled sodium administered simultaneously in a pulse (Fig. 8). Here ^{24}Na was used simply as a marker for aqueous channels. The use of tritiated water was precluded because the drug

was also labeled with tritium. As shown in the figure, only a minimal displacement of the ouabain curve to the right of the sodium curve could be detected.

Comparison of the areas under the curves for ^{24}Na and labeled ouabain in three experiments showed that virtually all of the administered drug was recovered (assuming recovery of labeled sodium to be complete). The mean transit time of ouabain was, on the average, 0.1 sec longer than the mean transit time of sodium.

Thus the slow development of inhibition must be attributable to slow uptake of the drug by the enzyme, a slow reaction in the enzyme initiated by the drug, or both. We were unable to reactivate the enzyme by perfusion with drug-free media, once inhibition had fully developed.

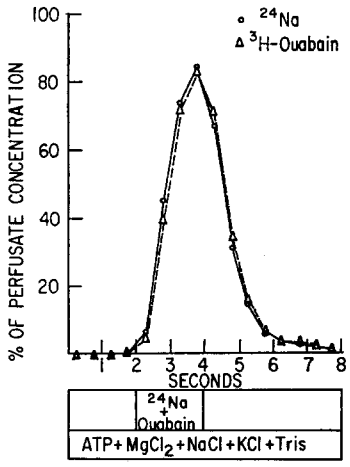

FIGURE 8. Outflow curves of labeled ouabain and labeled sodium administered simultaneously in a pulse. Only a minimal displacement of the ouabain curve to the right of the sodium curve can be detected.

Seven experiments were performed to test the hypothesis (10, 11) that potassium counteracts ouabain inhibition by displacing the drug from sites of competitive binding. Two solutions were used. Tritiated ouabain (New England Nuclear Corp., Boston, Mass.) was added to both at the same concentration, which was either 5×10^{-7} M (five experiments) or 5×10^{-5} M (two experiments). Potassium was absent from either the first or second solution and was either 2 or 20 mM in the second. Sodium was present in both solutions, and the total of sodium plus potassium was held constant. No convincing uptake or release of ouabain coincident with the change of solutions occurred; the maximal quantity of drug instantaneously released or taken up which could have been overlooked was 2 pmole per mg of protein in 5×10^{-7} M ouabain. Matsui and Schwartz (23) report that binding of digoxin by cardiac sarcosomes in 20 pmole per mg lower when potassium is present in addition to ATP, magnesium, and sodium. Our findings are consistent with studies in

erythrocytes (24, 25) which indicate that cardiac glycosides once bound to the enzyme are not readily removed, but they fail to provide a model for the antagonistic effects of added potassium on a number of glycoside-induced responses, such as the agumentation of cardiac contractility (22).

In heart muscle (26–28) and in kidney (29), cardiac glycoside action may involve changes in membrane-bound calcium. The results shown in Fig. 7 demonstrate that calcium release does not accompany the onset of inhibition of ATP-splitting activity by ouabain in this preparation. The amount of calcium released (if any) is clearly less than 10 pmole per mg protein. A more

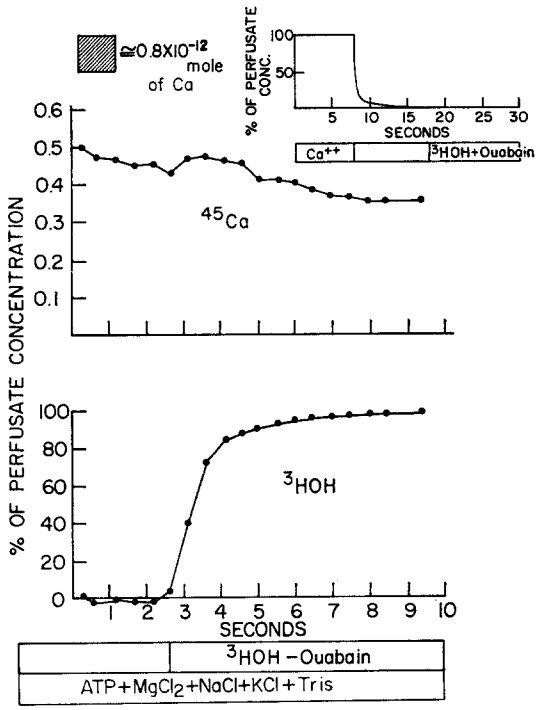

FIGURE 9. Calcium release by ouabain at very low calcium concentration. As shown diagrammatically in the insert, calcium (8×10^{-6} M) was present only in the first solution, perfused for several seconds. After 10 sec washout, calcium concentration had fallen to 0.5% (4×10^{-8} M). The third solution contained ouabain (5×10^{-5} M) and tritiated water. A slight rise in effluent calcium concentration occurred just after ouabain arrival, but the quantity of calcium released is miniscule (<1 pmole per mg) and comparable to random fluctuations in similar experiments without ouabain.

sensitive test was applied by washing out most of the radioactive calcium, after first equilibrating the microsomes with a concentration of 10^{-6} M (Fig. 9). Under these circumstances ouabain addition displaced less than 1 pmole of calcium per mg. The rise in concentration was too small to have statistical significance and comparable to random fluctuations noted in control experiments without ouabain.

Activation by Sodium and Potassium

We performed several experiments in which a pulse of solution containing 100 mM NaCl and 20 mM KCl interrupted perfusion with 120 mM KCl in addition to 3 mM Tris-ATP, 6 mM $MgCl_2$, and 20 mM Tris buffer. Phosphate

concentration rose promptly on the arrival of sodium and declined to the control value on washing out sodium. The speed of onset of the increase in activity is illustrated in Fig. 10, where measurements made 10 times per sec are depicted. No appreciable delay could be detected. A similar response was seen when a pulse of the same composition interrupted perfusion with a

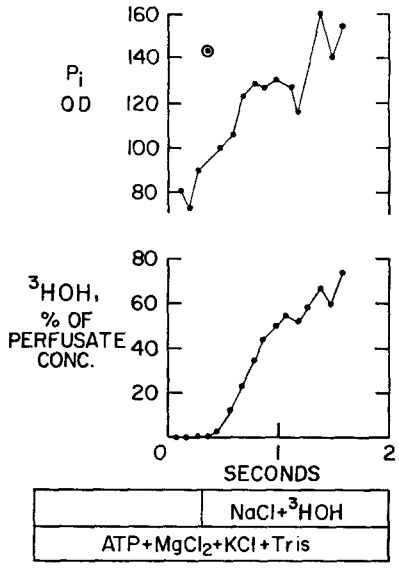

FIGURE 10. Phosphate output during activation by sodium. The first solution contained 120 mM KCl, the second 20 mM KCl, 100 mM NaCl, and tritiated water; both contained 3 mM Tris-ATP, 6 mM $MgCl_2$, and 20 mM Tris buffer (pH 7.4). Magnesium-dependent ATPase activity is represented by the phosphate output during perfusion with the first solution. The arrival of sodium is accompanied by a simultaneous increase in phosphate production, measured 10 times per sec. A single aberrant value is noted.

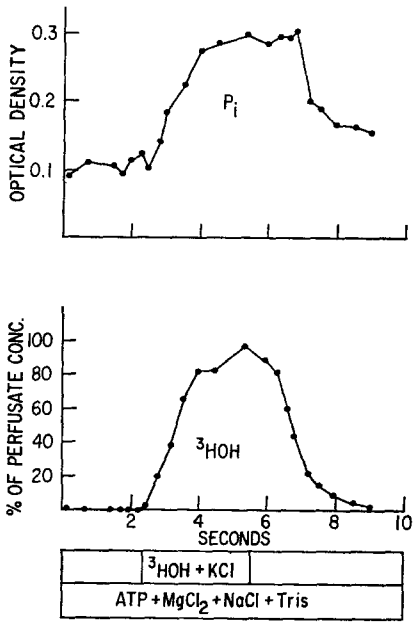

FIGURE 11. Phosphate output during activation by potassium. The first and third solutions contained 120 mM NaCl, the second 100 mM NaCl, 20 mM KCl, other constituents as noted. Phosphate production rises transiently.

potassium-free medium (Fig. 11). As before, the sum of sodium plus potassium was held constant. Experiments on other tissues have shown a rapid decline in the amount of phosphorylated intermediate present when potassium is added to a medium containing sodium and ATP (1). Therefore we tried to see if this

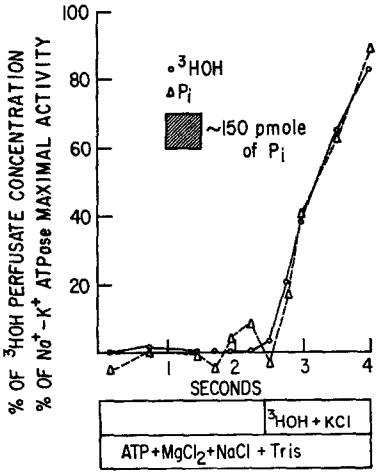

FIGURE 12. Same data as in Fig. 10 plotted together as percentages of maximal response. The insert shows the area which would correspond to release of an extra 150 pmole of phosphate per mg of protein. No such release could be detected.

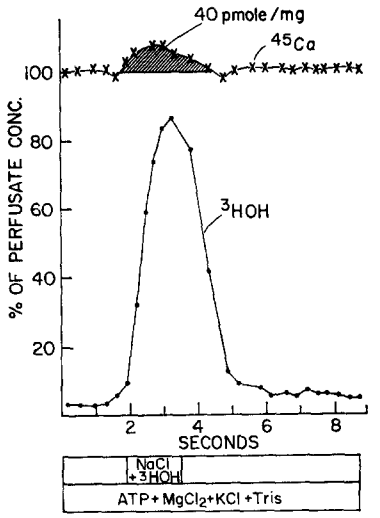

FIGURE 13. Calcium release during activation by sodium. The shaded area represents 40 pmole of calcium per mg of protein. This release occurs simultaneously with the arrival of sodium (marked by tritium), as does the rise in phosphate output (Fig. 10). Temperature 37°C.

phosphate increment could be detected in the effluent. When the data shown in Fig. 10 are replotted as shown in Fig. 12, no excess phosphate production can be detected during activation by potassium. However, a delay of approximately 0.3 sec accompanied by excess phosphate of 50 pmole per mg might yield the same results.

Calcium Release during Activation by Sodium

An experiment identical to that shown in Fig. 10 except for the presence of radioactive calcium at a uniform concentration of 0.037 mM is shown in Fig. 13. Activation by sodium was accompanied by the unequivocal release of calcium in four experiments (Table VI).

In most cases, uptake was also detectable on washing out sodium, but was not as rapid as calcium release. When the same experiment was conducted at 0°C (Fig. 14), no calcium release could be detected. Sodium- and potassium-stimulated enzymatic activity, assayed by incubation for one hour at 0°C, was reduced to less than 1% of the control rate, and magnesium-dependent activity to 2.7%. Potassium activation, as in the experiment shown in Fig. 11,

TABLE VI

CALCIUM RELEASE COINCIDENT WITH SODIUM ADDITION IN INDIVIDUAL EXPERIMENTS AT 37°C AND 0°C

Expt. no.	Temp.	[Ca]	[ATP]	[Ouabain]	Calcium release
	°C	μM	mM	μM	pmole per mg protein
A1	37	3.7	3	0	46
A2	37	4.7	3	0	62
A3	37	5.6	3	0	51
A4	37	5.6	3	0	33
B1	37	4.7	3	25	50
B2	37	5.6	3	100	42
C1	37	4.7	0	0	38
					46±4 (SEM)
D1	0	4.7	3	0	0
D2	0	5.6	3	0	0

Experimental conditions as in Figs. 10 and 13.

was also accompanied by no measurable change in effluent calcium concentration (Fig. 15). Repetition of the experiment shown in Fig. 13 in the absence of ATP or the presence of ouabain yielded nearly identical results (Table VI).

Thus activation by sodium, but not by potassium, is accompanied by a simultaneous release of calcium averaging 46 pmole per mg. The data suggest that this displacement of calcium is from the site at which sodium combines with the enzyme. This competition does not require the presence of ATP but is obliterated at 0°C, where the enzyme is almost completely inactive.

DISCUSSION

The method described here permits changes in ATPase activity to be correlated with uptake or release of cations or cofactors at time intervals of less

than a second. In terms of the estimated turnover number of the enzyme at 37° C (1), these time intervals are long indeed. Nevertheless, events which appear to be simultaneous by this technique can be clearly distinguished from some slower phenomena. For example, the rate of inactivation by ouabain

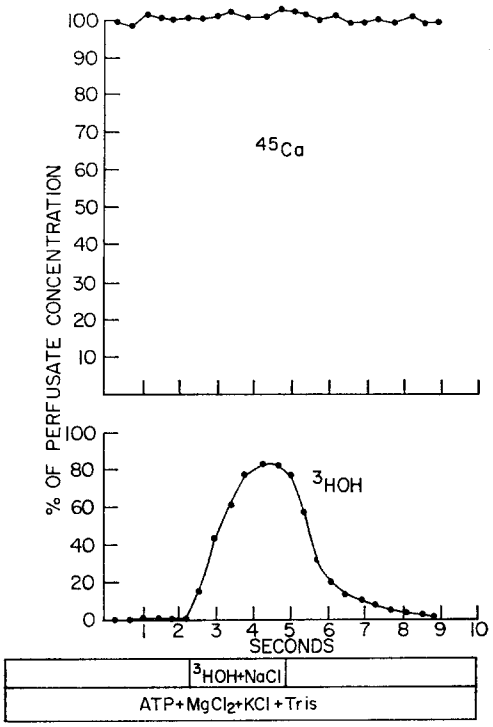

FIGURE 14. Response of bound calcium to a pulse of sodium at 0°C. Same solutions as in Figs. 10 and 13. No calcium release is noted.

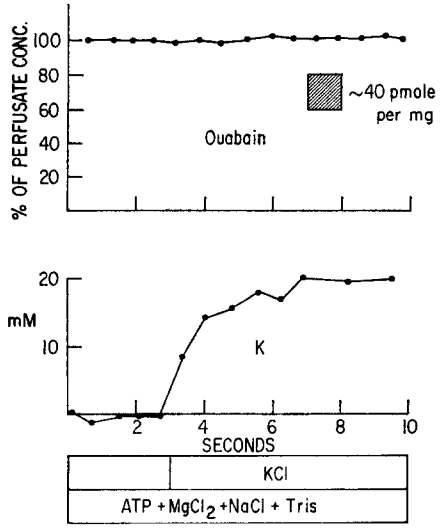

FIGURE 15. Response of bound calcium during activation by potassium. Solutions as in Fig. 11, plus 0.0056 mM $^{45}CaCl_2$. No calcium release can be detected.

was much slower than the rate of activation or inactivation on adding and removing ATP, sodium, or potassium. This finding in itself appears to exclude any postulated mechanism of action of the drug which merely requires its presence at the sites where these agents act, without further chemical reactions.

The time resolution of this technique is limited by the sensitivity of the analytical methods. With smaller amounts of enzyme protein and higher flow rates, changes in cation binding at shorter intervals can be determined. However, the amounts of cation taken up or released must be correspondingly larger in order to be detectable. In the present work, resolution was limited by the sensitivity of our phosphate method; furthermore, the quantity of calcium released by sodium was only a few times greater than the minimum detectable. Thus higher perfusion rates would have been undesirable on two counts.

A general expression of the sensitivity of this technique can be derived by dividing the minimal detectable amount of solute instantaneously released (or taken up) by the ambient concentration at which this amount can be detected. The resulting value, in microliters, is an expression of the minimal detectable change in the volume of distribution of solute within the enzyme preparation, and should be independent of the solute used or its concentration, providing analytical precision is constant. We estimate this minimal volume to be about 5 μl per mg when analytical precision is 1%.

When compared with rapid reaction techniques employed in the study of biochemical reactions (31), this method is quite slow, but it offers the advantages that it requires only simple and inexpensive apparatus, and that any analytically determinable component of the reaction can be assayed at simultaneous times.

As a measure of cation binding to subcellular structures, this method has additional desirable features, when compared with techniques in common use. Ultracentrifugation suffers from the long time required to achieve separation, and from the problem of correcting for trapped supernatant in the sediment. When both sediment and supernatant are analyzed for the solute in question as well as for water content, a correction can be applied, but the calculations are tedious (32). Some have overcome this problem by washing the sediment with media free of the solute (33). In this case, the results yield estimates of the solute bound in an irreversible manner, which seems on a priori grounds least likely to be involved in transport. Filtration through micropore filters has been employed by many workers (17, 19, 20, 27, 28, 34–36), analyzing either the filtrate or the material remaining on the filter paper. In preliminary experiments, we found that both the rate of flow and the pressure gradient across the filter independently affect the apparent binding of calcium to microsomes from various tissues. For this reason we chose a technique in

which flow and pressure are constant during each experiment. In addition, this method measures only changes in the quantity of cation reversibly bound.

The most significant aspect of the results is the release of calcium by sodium. Although our data do not establish that calcium is being displaced from the enzyme, rather than from nonspecific binding sites in the preparation, they point strongly to this conclusion. The absence of an effect of potassium ions indicates a degree of chemical specificity unknown in ligands found in living tissues, with the exception of membrane sites believed to be involved in sodium and potassium transport (37). The absence of calcium displacement at 0°C, where sodium-stimulated activity is also absent, presumably reflects a conformational change in the enzyme at this temperature. The almost complete loss of this enzymatic activity at temperatures near zero has been previously noted in microsomal preparations from brain (10, 12, 14) and kidney (38). The presence of calcium displacement in the absence of ATP or in the presence of ouabain neither supports nor weakens the inference we have drawn, because neither of these agents acting alone induces instantaneous changes in bound calcium in this preparation. Quantitatively, the amount of calcium displaced is similar in magnitude to the number of active sites per mg estimated from the maximal quantity of ^{32}P from terminally labeled ^{32}ATP which is incorporated into a phosphorylated intermediate (1, 10, 30). Furthermore, as Medzihradsky et al. have recently shown, a large fraction of the protein in preparations of this type may be part of the enzyme complex (39).

Several lines of evidence have suggested an interdependence between sodium and calcium in transporting systems, which can be reviewed here only in outline. (*a*) Correlation between the rates of transepithelial movement of sodium and calcium in renal tubule (40, 41), gut (42), gall bladder (43), and amphibian urinary bladder (44). In proximal small intestine, however, uphill transport of calcium appears to be independent of sodium movement (45). (*b*) Correlation between transmembrane movements of calcium and sodium, manifested by steady-state parallelism between their intracellular concentrations among various organs and species (46), by accumulation of both ions intracellularly in some pathologic states (47), and by influx during excitation (48). (*c*) Mechanical responses of contractile tissues proportional to the ratio [Ca]/[Na] or $[Ca]/[Na]^2$ (24, 48). (*d*) Electrical responses of excitable tissues dependent on one of these ratios (48). (*e*) Inhibition of transepithelial (29, 49–51) or transmembrane (48, 52) movement of both cations by cardiac glycosides. In erythrocytes, however, it appears that ouabain does not inhibit active calcium efflux (53). (*f*) Counteraction by sodium of calcium-induced inhibition of ATPase in microsomes from heart (54), but not from kidney cortex (55). It should also be noted that the presence of each of these cations is usually required for the transport of the other, but this cannot be

cited as more than suggestive evidence for interdependent transport mechanisms.

In addition to these physiological observations, there now exists some direct evidence for competitive binding of these two cations to subcellular structures presumed to be involved in ion transport. For example, the ATP-dependent binding of calcium by skeletal muscle microsomes at 0°C was inhibited by sodium or potassium (56). Very recently, Palmer and Posey (35) have reported that calcium uptake by cardiac grana in the presence of ATP is inhibited by sodium, but not by potassium. This response occurred within a few seconds and the amount of calcium displaced by sodium was of the order of 2000 pmole per mg. Similarly, Lieberman, Palmer, and Collins (17) observed ATP-dependent binding of about 5000 pmole of calcium per mg of protein in microsomes prepared from crab nerve, and prompt release of a portion of this bound calcium in the presence of sodium. Sanui and Pace (57) found that high concentrations of calcium displaced sodium from liver microsomes, and Carvalho (33) reported competition between H^+, Ca^{2+}, Mg^{2+}, Na^+, and K^+ for binding by muscle microsomes. On the other hand, Katz and Repke (58) found calcium binding by cardiac microsomes to be facilitated by either sodium or potassium, especially in the presence of ATP. Carsten (28) found little effect of sodium as compared with sucrose in a similar system. Calcium binding by erythrocyte ghosts exhibits only a slight increase on replacing sodium with tetraethylammonium ions (59).

In the present work, the amount of calcium displaced by sodium was considerably smaller than could have been detected by the methods employed in these other reports, and the time intervals examined were shorter. Slower uptake of calcium would not have been detected even if the cumulative amounts involved were much greater.

The most cogent objection to linked transport of calcium and sodium ions mediated by an ATP-hydrolyzing enzyme is the inhibitory effect which calcium usually exerts on the sodium- and potassium-stimulated ATPase (2, 7, 8, 19, 55, 56). One possible explanation is that calcium inhibition may occur elsewhere on the enzyme surface, at a site insulated from the intra- or inter-cellular transport pathways. Disruption of the cell may expose this site to ambient calcium concentrations which it never sees in the intact cell. Calcium stimulation of ATP hydrolysis, observed under certain conditions in erythrocyte ghosts (60–62), and in microsomes from heart (63), intestine (64), and kidney (55, 65) might be detectable more generally if this secondary site inhibition could be prevented. Sachs, Rose, and Hirschowitz (66) have presented evidence that the phosphorylated intermediate in brain microsomes is an acyl phosphate, and have reported that calcium inhibits acetyl phosphatase in this tissue. The breakdown of this intermediate may constitute a secondary site of calcium inhibition. Possibly related is the ob-

servation that external calcium inhibits potassium influx into erythrocytes (67), though not in reconstituted ghosts (60).

ATP-dependent calcium uptake, not shown to be related to sodium, has been observed in muscle (19, 21, 28, 34, 68–70) and more recently in brain (8, 18). According to Ohnishi and Ebashi (69), this reaction in muscle proceeds with great rapidity (<0.03 sec). However, the absence of a stoichiometric relationship between ATP hydrolysis and calcium uptake led these authors to conclude that energy-dependent calcium transport was not involved. Likewise, Lieberman, Palmer, and Collins (17) suggest that the calcium released by sodium from crab nerve may be distinct from that moiety bound in association with ATP.

No calcium release coincident with the onset of ouabain inhibition could be detected. In cardiac and skeletal muscle (22) as well as erythrocyte membranes (59, 71), similar negative or equivocal results have been reported, but more recently an inhibitory effect of ouabain in low concentration on calcium uptake in heart sarcosomes has been found (26–28, 72), and ouabain administered in vivo has been shown to alter the microsomal binding of calcium in the kidney within a few minutes (29). The amounts involved are again much greater than the probable concentration of active transport sites, and the rates of these reactions may be much slower than those under study here.

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