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ADENOSINE TRIPHOSPHATE-LINKED CONCENTRATION OF CALCIUM IONS IN A PARTICULATE FRACTION OF RABBIT MUSCLE

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ABSTRACT

ATPase and ATP-dependent calcium ion concentration was studied with a membrane fraction isolated from homogenized rabbit skeletal muscle by differential centrifugation. Electron micrographs of the fraction indicate that it consists mainly of resealed tubules and vesicles of the endoplasmic reticulum. The up-to-1400-fold concentration of calcium in this fraction might be explained by proposing the existence of an energy-requiring system for the transport of calcium ions into the tubules or vesicles.

Ebashi *et al.* (1, 3, 8) isolated a particulate fraction from muscle which was identified as a component of the relaxation system. This fraction was similar to the magnesium-dependent ATPase-containing particles[1] of Kielley and Meyerhof (6, 7) and, like these, had high ATPase activity. In addition, in preliminary experiments, Ebashi (2, 2a) had observed a retention of calcium ion with this fraction. The metabolic effects found here became even more interesting when it appeared that, by their ultracentrifugal characteristics, what had been termed particles are, indeed, fragments of the endoplasmic, or, in this case, sarcoplasmic reticulum of Porter and Palade (14).

We will describe in this paper the calcium ion concentration function, its dependence on ATP, and its relation to the ATPase effect. Elsewhere, Ebashi (2) has already discussed in part the possible interrelationship between the relaxing activity and the various metabolic effects shown by this fraction. This work was done about 3 years ago but, due to various circumstances, publication has been delayed. In the meantime, Hasselbach and his coworkers (5, 10) have reported on observations similar to the ones described here.

[1] The following abbreviations are used: AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; PEP, phosphoenolpyruvic acid; PP, pyrophosphate; ~P, energy-rich phosphate.

METHODS

Preparation of the Particulate Fraction

The procedures are essentially the same as those described in the previous paper (1) but slight modifications have been made. The hind leg and back muscles of a rabbit were ground in a meat grinder and 200 gm of the minced muscle were added to 600 ml of 0.05 M $KHCO_3$ solution. They were then homogenized in a Waring blendor for 1 minute, centrifuged at 10,400 *g* for 20 minutes in the small Lourdes rotor, after which the supernatant fraction was passed through glass wool to remove the lipid layer, and recentrifuged at 38,000 *g* for 1 hour in the Spinco rotor no. 21. The supernatant was discarded and the pellet dispersed in 100 ml of 0.03 M $KHCO_3$ in a Potter-Elvehjem tissue grinder; the resuspended material was centrifuged at 8,200 *g* for 20 minutes in the small Lourdes rotor and the sediment discarded. This supernatant was again centrifuged for 1 hour at 38,000 *g* in the Spinco rotor no. 30. The precipitate

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was suspended in an amount of 0.03 M $KHCO_3$ sufficient to make a 4 to 6 per cent solution by dry weight. Unless otherwise noted, this preparation was used in all experiments.

In cases where further purification is needed the preparation may be diluted 20-fold with 0.03 M $KHCO_3$, centrifuged at 8,200 *g* for 10 minutes, and the supernatant recentrifuged at 38,000 *g* for 45 minutes. The procedure may be repeated to obtain a more purified preparation.

The amount of membrane fraction is given in dry weight. The amount of sediment was generally expressed in wet weight, arbitrarily using five times the dry weight value. Such an approximate weight value was adopted and used for estimating concentrations inside the particulate fraction.

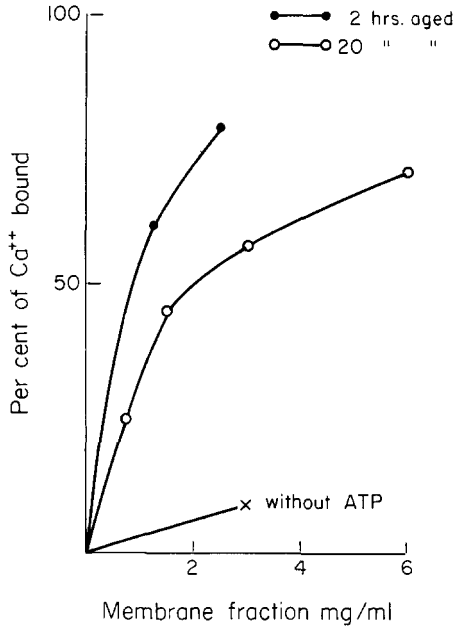


FIGURE 1

ATP-dependent calcium ion binding with various concentrations of normal and aged membrane fraction. See Methods for further details.

Standard Assay for Calcium Ion Binding

Each ml of solution contained, 2 μmoles of ATP, 10 μmoles of $MgCl_2$, 144 μmoles of KCl, 20 μmoles of tris-maleate buffer (pH 6.8), generally 0.01 to 0.1 μmole of $Ca^{45}Cl_2$, and a specified amount of sediment. The total volume was usually 12 ml. Unless otherwise noted, all solutions were kept at below 2°C. The mixture was transferred to Spinco tubes and the reaction was initiated by adding the ATP just before inserting the tube into the centrifuge; for the determination of calcium ion binding, a very fast reaction, the mixture was spun without prior incubation for 15 minutes at 100,000 *g* using rotor no. 40. During spinning, the reaction goes to completion. Both the precipitate and supernatants were then analyzed for Ca^{45} radioactivity.

Assay for ATP-ADP Exchange and ATPase

Each ml of reaction mixture contained: 2.5 μmoles of ATP, 2.5 μmoles of uniformly labeled C^{14}-ADP, 100 μmoles of KCl, 4 μmoles of $MgCl_2$, 30 μmoles of tris-maleate buffer (pH 6.8), and a specified amount of membrane fraction; the final volume was 0.6 ml. The reaction was initiated by adding the ATP-C^{14}-ADP mixture; it was stopped by shaking with 0.1 ml of a 1:1 mixture of chloroform and ether. After removal of the chloroform and ether by aeration, 0.05

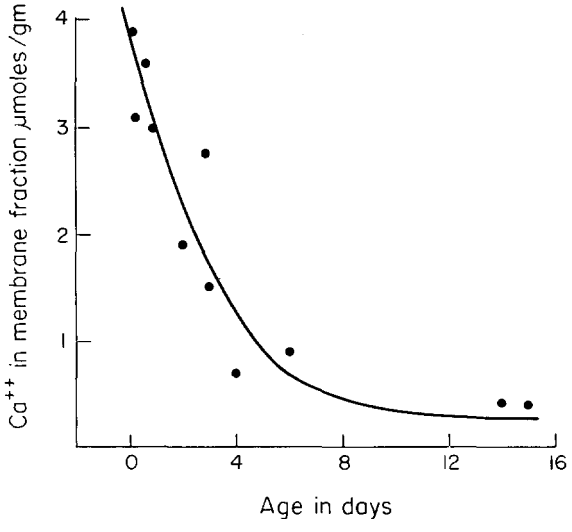


FIGURE 2

Effect of aging at 0°C on calcium ion binding. These data were obtained from various samples prepared at different times. Calcium concentration, 0.01 μmoles per ml; pH 6.8; membrane fraction concentration, 0.7 to 3.0 mg per ml. See Methods for further details.

ml was usually used for assay. The nucleotides were separated by paper electrophoresis according to Markham and Smith (9).

ATPase was determined by measuring inorganic phosphate in the remaining part of the reaction mixture by the method of Fiske and Subbarow (4). Myokinase was determined by the method of Noda and Kuby (11).

RESULTS

Calcium Ion Accumulation in Membrane Fragments

Following up earlier observations by Ebashi (2) on a retention of calcium ion by the membrane fraction, it was discovered that the addition of

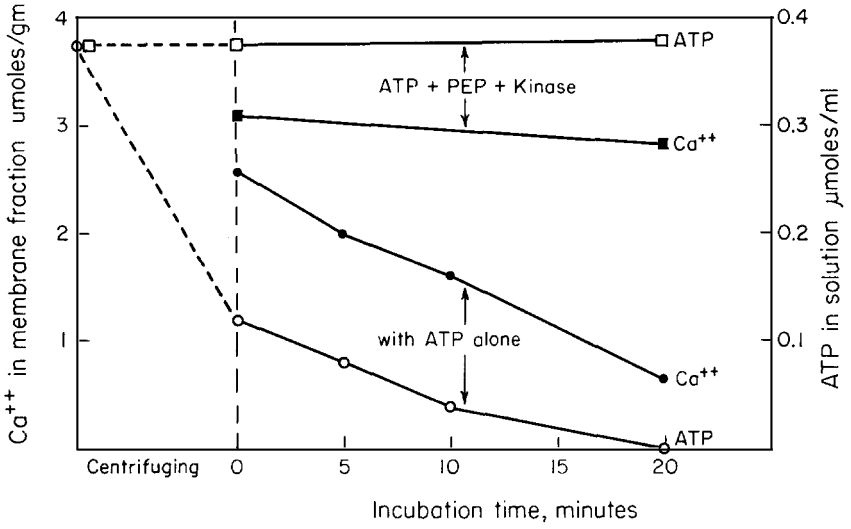


FIGURE 3

Dependence of calcium ion binding on maintenance of ATP concentration. When used to maintain ATP levels, 2 μmoles per ml of PEP and 50 units per ml of pyruvate kinase were added. Reaction mixtures contained 0.8 mg per ml of membrane fraction, were incubated at 15 °C for the periods indicated in the figure, and centrifuged for assay of calcium ion binding as described under Methods. On the left of the zero line of the figure indicating the time before centrifugation, the amount of added ATP is noted. During the 15 minutes' centrifugation at 0 °C in the Spinco, necessary to separate the membrane fraction for calcium determination, the ATP concentration, if not regenerated by the PEP system, falls to the value noted on the zero line. After 20 minutes, ATP in this series of assays has practically completely disappeared and, correspondingly, calcium has been released, during the incubation, from the membrane fraction. If ATP is maintained, however, by the PEP system, calcium is steadily kept in the membrane-vesicle fraction as shown by the upper two lines.

Assay for ATP Binding

Procedures were essentially the same as those for calcium ion binding, but uniformly labeled C^{14}-ATP was used in place of Ca^{45} as well as 2 μmoles of PEP and about 140 units of pyruvate kinase per ml.

Materials

Myokinase was obtained from Boehringer & Soehne, Mannheim, Germany, and C^{14}-ATP and ADP were obtained from Schwarz Bio-Research Inc., Orangeburg, New York. Ca^{45} was obtained from Oak Ridge National Laboratory.

ATP greatly stimulated this effect. It will appear from the following experiments that calcium ion accumulation is essentially an ATP-linked reaction. Fig. 1 shows the quantitative relation between calcium ion binding and amounts of membrane fraction. With fresh preparations about 80 per cent of the calcium ion in solution was bound maximally. This is equivalent to 1400-fold concentration of calcium ion in the granules as compared with the surrounding fluid. ADP showed a slight effect, which may be due to the presence of myokinase. When ADP was added to

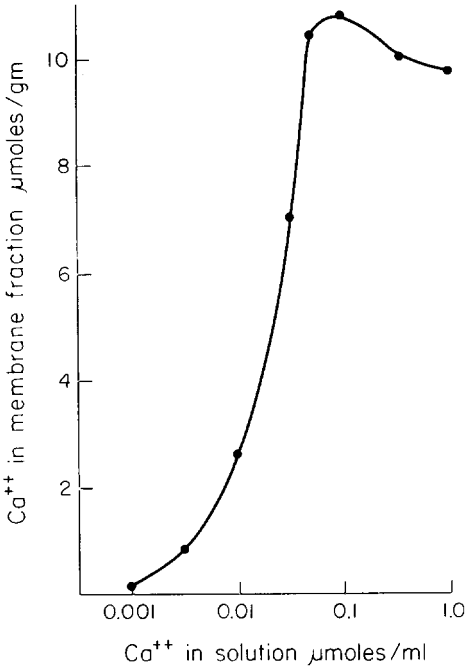


FIGURE 4

Calcium ion binding of membrane fraction as a function of calcium concentration in solution. Each ml of solution contained 2.0 mg of membrane fraction. See Methods for further details.

ATP in equimolar amounts it caused a 30 per cent inhibition. AMP and PP were without effect.

The pH optimum for the calcium ion concentrating reaction is 6.5. As shown by the lower curve in Fig. 1, part of the concentrating ability is lost on aging. This loss appears more clearly in Fig. 2. The relative ease of deterioration parallels that of the relaxing effect, but contrasts with the ATP-splitting reaction which is rather insensitive and may even slightly increase with similar treatment.

In the following experiments a dependence upon a constant supply of ATP for holding calcium ion in the membrane fraction will be demonstrated. As shown in Fig. 3, two parallel sets of tubes were used; in one set ATP was allowed to decompose, in the other its concentration was maintained by PEP + pyruvate kinase + ATP, acting as an energy-rich phosphate feeder system. The tubes were incubated at 15°C for varying periods of time before spinning for 15 minutes in the Spinco at 0°C. Due to reactions taking place during this spin, the samples showed an already marked decrease in ATP at "zero" time. On further incubation, the amount of bound calcium ion decreased parallel with the decrease in ATP concentration in those tubes where the ~P-

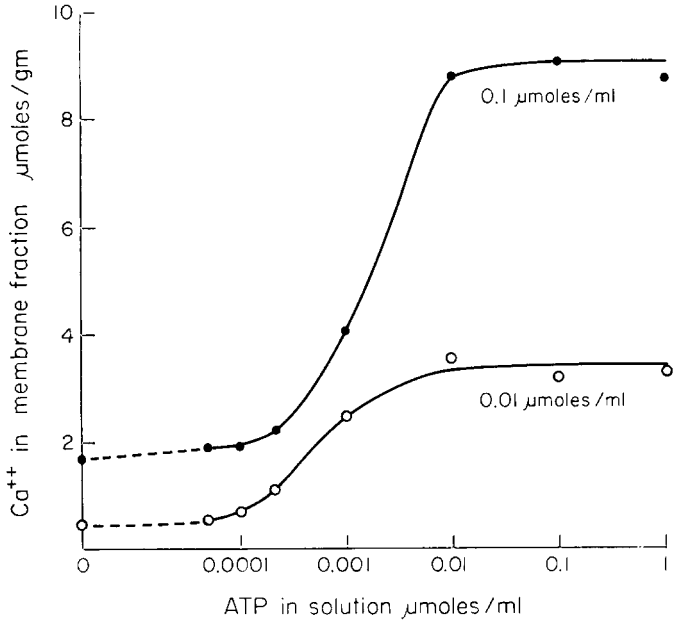


FIGURE 5

Calcium ion binding of membrane fraction as a function of ATP concentration in solution. Each ml of solution contained 2 μmoles of PEP, 140 units of pyruvate kinase, 1.0 mg of membrane fraction and either 0.1 or 0.01 μmoles per ml of calcium ion. See Methods for further details.

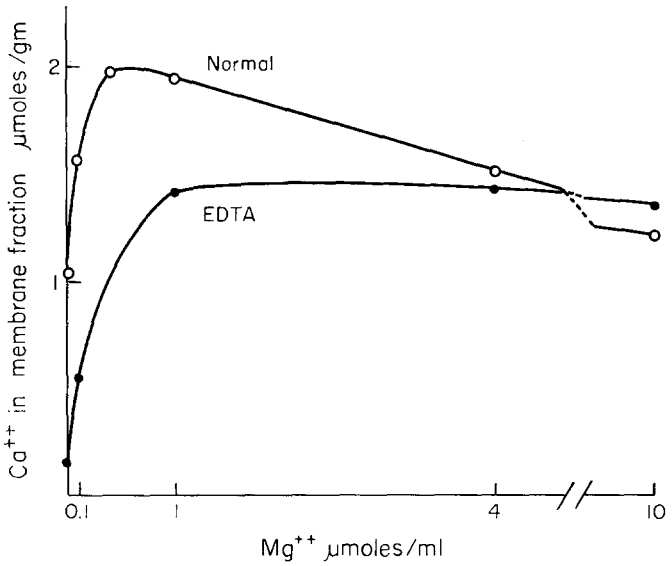


FIGURE 6

Dependence of calcium ion binding on magnesium concentration. In the lower curve the usual preparation was diluted by 0.03 M $KHCO_3$ containing 5 μmoles of EDTA per ml and then centrifuged. Reaction mixtures contained 0.7 mg of membrane fraction per ml in the usual preparation, and 0.8 mg in the EDTA-washed preparation. See Methods for further details.

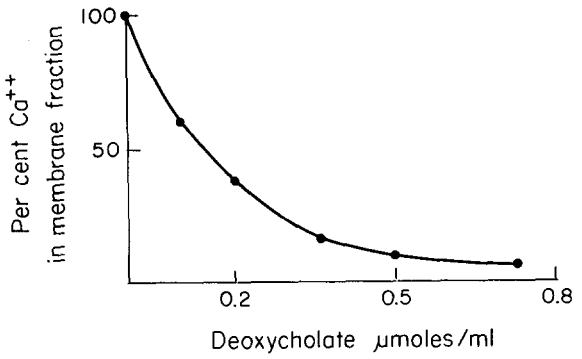


FIGURE 7

Inhibition of calcium ion accumulation by deoxycholate. 1.5 mg per ml of membrane fraction was used. See Methods for further details.

feeder system was omitted. With the feeder system present, however, the amount of calcium ion reached its maximal level during the 15 minutes' spin at 0°C and was maintained at this level with further incubation. Furthermore, addition of ~P-feeder system after decomposition of ATP lifted the calcium ion concentration again to nearly maximum level. Thus, when the ATP level is maintained, the calcium ion remains bound and, when it falls, calcium ion is released reversibly.

The effect of calcium ion concentration on fixation is shown in Fig. 4. The system appears to be saturated near 10^{-4} M; higher concentrations are slightly inhibitory. The level of ATP that would effect a maximal calcium ion binding could be determined, in spite of the ATPase activity in the preparation, by maintaining a steady-state level of ATP with a ~P-feeder system. Fig. 5 shows that the saturation level of ATP is 10^{-5} M and, furthermore, that it is unchanged with a variation of calcium ion concentration. The calcium ion concentration eventually attained in the membrane fraction exceeds the steady-state ATP concentration in the surrounding fluid by a factor of 1000 or more. It is, therefore, a dynamic transfer in or across a membrane, and may be interpreted as an ATP-dependent osmotic

concentration of calcium ion. As will be described later, electron micrographs indicate the fraction to contain vesicles of endoplasmic reticulum, some of which appear to be closed, possibly representing resealed tube fragments.

As would be expected for an ATP-linked reaction, the calcium ion binding is dependent upon magnesium, as shown in Fig. 6; this requirement appears most clearly with an EDTA-washed preparation. It was shown in Fig. 2 that the calcium ion concentration function diminishes and eventually disappears on aging. Similarly, deoxycholate inhibits, as seen in Fig. 7; it may be significant that both aging and deoxycholate also inhibit relaxation in contrast to ATPase.

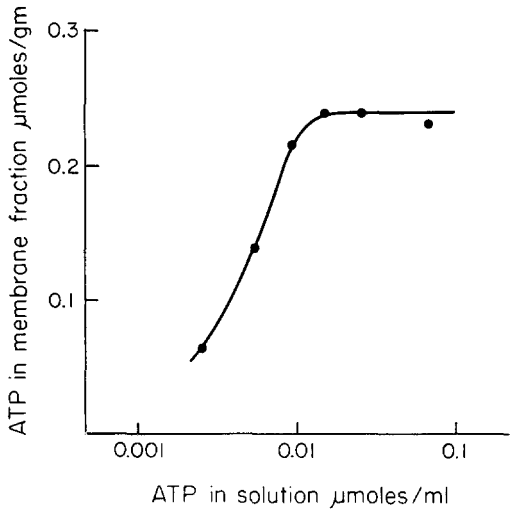


FIGURE 8

ATP binding of membrane fraction as a function of ATP concentration in solution. The solution contained 2.5 mg per ml of membrane fraction. See Methods for further details.

ATP-Binding

If C^{14}-ATP is incubated with the preparation, including a ~P-feeder system, some excess of ATP is found in the membrane fraction (Fig. 8), the concentration of ATP being 20-fold over that in the surrounding medium. As shown in Fig. 8, above 0.01 μmoles per ml the ATP-binding levels off and does not respond to further increase in ATP concentration. Comparison of ATP binding with that of calcium ion shows that about 50 to 70 moles of calcium ion may be held in steady-state per mole of ATP bound possibly to the membrane.

Ebashi had already studied the ATPase in this preparation (1). We find now that, in addition to the ATPase, the preparation also catalyzes a rather rapid exchange between ATP and ADP and that the two effects seem to be closely connected with each other. Fig. 9, for example, shows that the pH optimum of the two reactions overlaps. Both are dependent upon the addition of magnesium and, here again, the effect of mag-

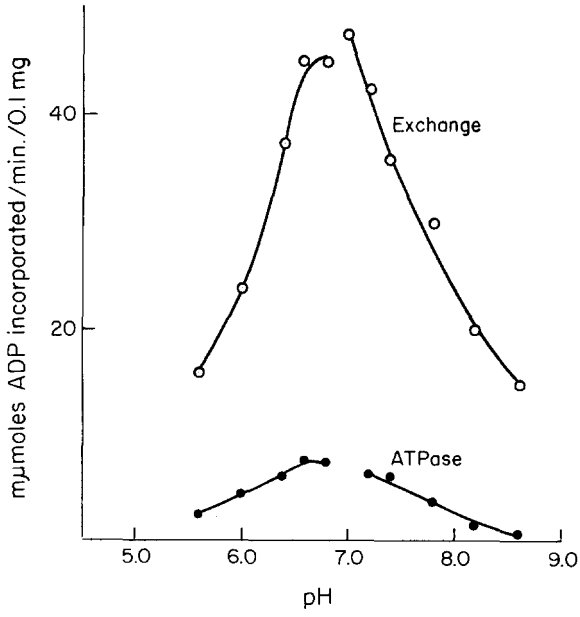


FIGURE 9

ATP-ADP exchange reaction and ATPase of membrane fraction as a function of pH. Each ml of reaction mixture contained 0.1 mg of membrane fraction. Tris-maleate buffer was used below pH 6.8 and tris buffer above pH 7.0. See Methods for further details.

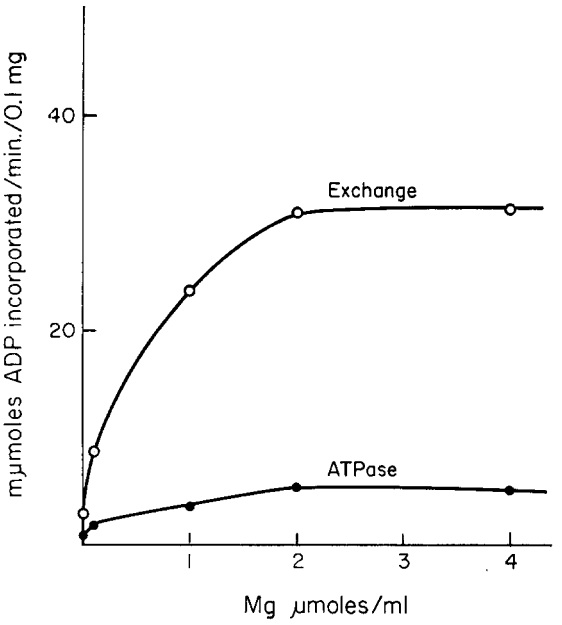


FIGURE 10
Effect of varying concentrations of magnesium ion on the ATP-ADP exchange reaction and ATPase of the particulate fraction. 0.1 mg per ml of membrane fraction was used in each case. See Methods for details.

nesium concentration is parallel with both reactions, as shown in Fig. 10. Deoxycholate at lower concentrations causes a stimulation and only at relatively high concentrations eventually inhibits both reactions. This effect is shown in Fig. 11, and it contrasts with the strong inhibition by deoxycholate of the calcium ion concentration and the relaxation effects. It may be significant that calcium ion inhibits both ATPase and exchange and that the inhibition is reversed by deoxycholate. This is illustrated by the experiments presented in Fig. 12(*A* and *B*).

The catalysis of ATP-ADP exchange may be interpreted as indicating a reversible phosphorylation of the membrane: ATP + membrane $\rightleftarrows$ ADP + membrane-P. However, in attempts to prove a transfer of phosphate to the membrane by using terminally labelled ATP^{32}, no convincing phosphate binding by the membrane fraction was observed.

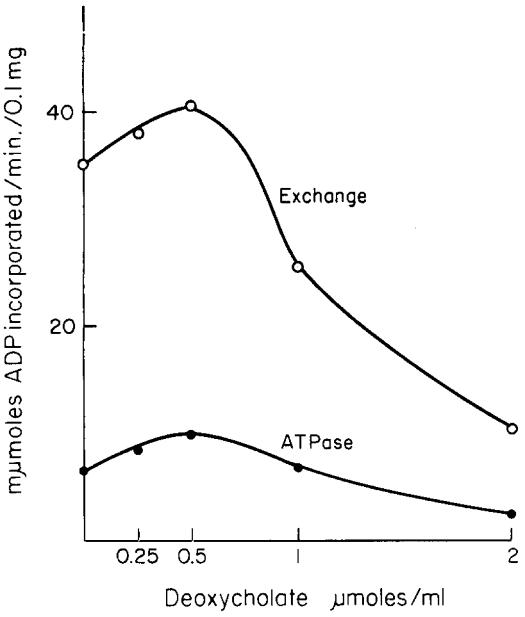


FIGURE 11
Effect of deoxycholate on the ATP-ADP exchange reaction and ATPase of the membrane fraction. Each reaction mixture contained 0.1 mg of membrane fraction per ml. See Methods for details.

ELECTRON MICROGRAPHS

Methods

Pellets of the particulate fraction obtained as described above were fixed *in situ* (at the bottom of the tube) by overlaying them with a 2 per cent OsO_4 solution in 0.03 M $KHCO_3$. After dehydration in ethanol and embedding in an 80:20 mixture of butyl-methyl methacrylate, the preparations were sectioned parallel to the direction of centrifugation to make possible their systematic survey, from top to bottom, by electron microscopy.

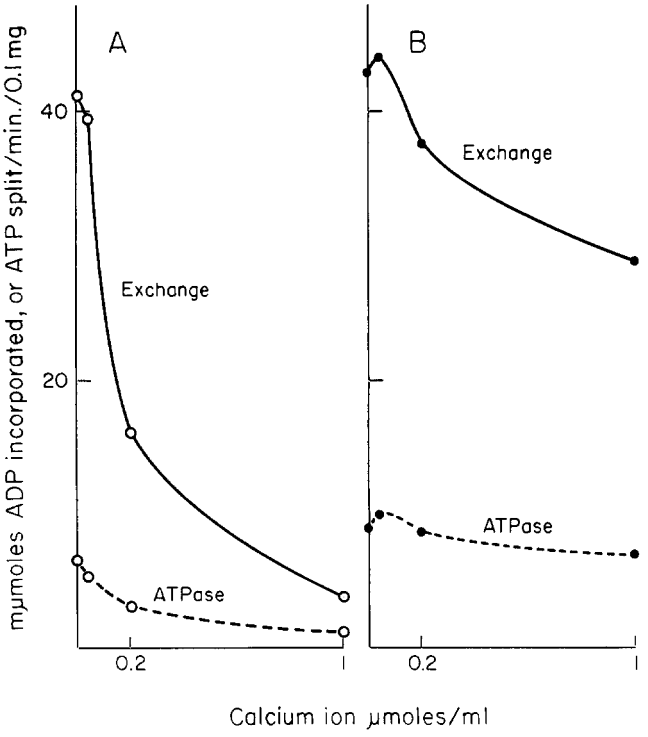


FIGURE 12

Inhibition of ATPase and ATP-ADP exchange by calcium. *A*, effect of increased calcium ion concentration. *B*, antagonism by deoxycholate to calcium ion inhibition.

In both experiments, 0.1 mg per ml of membrane fraction and 0.5 μmoles per ml of deoxycholate were used. See Methods for details.

Findings

The survey showed that the pellets were stratified and heterogeneous, the following components forming three successive and unequal layers: *Top:* (less than 5 per cent of the pellet thickness) dense particles, 10 to 15 mμ in diameter, similar to ribosomes isolated from other sources but more varied in size; *Middle:* (about 10 per cent of the pellet thickness) dense granules ~20 mμ in diameter, reminiscent of the glycogen particles seen in intact muscle fibers; *Bottom:* vesicles (diameter: 60 to 200 mμ) either spherical or flattened (cisternae), and tubules (diameter: 15 to 30 mμ; length: indefinite) mixed with a small amount of 20 mμ particles (Fig. 13).

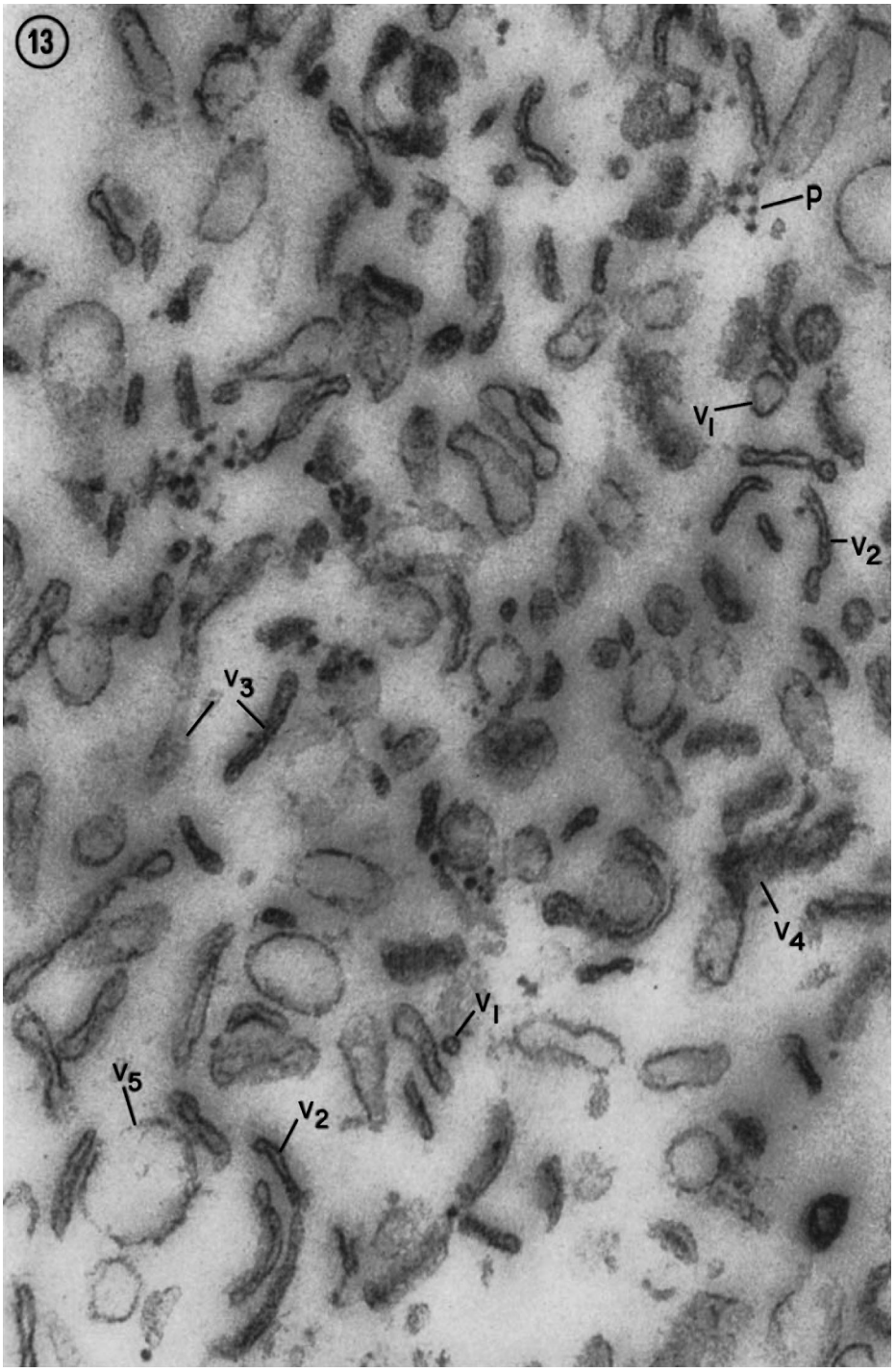
Most vesicles and tubules appeared as closed structures bound by a continuous, well defined,

FIGURE 13

Representative field in the bottom layer of a particulate fraction pellet. Activity for calcium ion concentration and relaxation, good.

The circular profiles (v_1) represent sectioned vesicles or transversely cut tubules; the elongated profiles (v_2) correspond to normally cut cisternae and longitudinally sectioned tubules. Oblique (v_3) or grazing (v_4) sections through vesicles are seen in many places. Most of these elements are bounded by a well defined continuous membrane. Vesicles with membrane defects or discontinuities (v_5) are rare.

Particles (p) of ~ 20 mμ diameter (glycogen?) represent a minor component of the preparation at this level in the pellet. × 80,000.


13
P
V1
V2
V3
V4
V1
V5
V2

smooth surfaced membrane. Broken open elements were rarely encountered.

Since the bottom layer made about 85 per cent of the total thickness of the pellet, the vesicles and tubules mentioned were the dominant structural element of the preparation. Their appearance and dimensions varied to a certain extent with position and degree of packing in the pellet: larger elements were found in the bottom layers of the preparation, and most vesicles appeared flattened in tightly packed sediments. Notwithstanding these variations, the isolated elements were similar in shape and size to the tubules and cisternae of the sarcoplasmic reticulum of the intact muscle fiber. Therefore the preparation appeared to consist mainly of "healed" or "closed"[2] fragments of the sarcoplasmic reticulum, a conclusion strengthened by the occasional encounter of vesicles with the localized membrane thickening (Fig. 14) that characterizes the terminal cisternae *in situ*, or vesicles with the typical grouping shown by the triads of the system in the intact muscle fiber (Fig. 15). The pellets did not contain nuclei, myofilaments, mitochondria, or recognizable mitochondrial fragments.

In preparations aged (Fig. 16) or treated with deoxycholate, which had rather poor activity, the limiting membranes of the vesicular elements were less sharply outlined and frequently discontinuous.

[2] This feature suggests that the fragmentation of the sarcoplasmic reticulum is due to a generalized pinching off process which occurs during tissue homogenization. A similar process has been postulated in the formation of hepatic and pancreatic microsomes from the corresponding endoplasmic reticula (12, 13).

CONCLUSIONS

A particulate fraction from rabbit muscle known to contain ATPase (6, 7) and relaxing factor (1) is found to catalyze an ATP-linked calcium ion concentration effect. This was studied by comparing calcium concentrations in the supernatant and particulate fractions. The reaction is so fast that during 15 minutes' centrifugation in the cooled Spinco it is complete and calcium may be concentrated in the particulate fraction up to 1400-fold. This effect is dependent upon a continuous supply of ATP. A 20-fold concentration of ATP against the surrounding fluid was found in the same fraction; the ratio of calcium ion to ATP is approximately 70 to 1. This seems to leave, as the only interpretation, a dynamic function of ATP in the process of calcium ion accumulation. The reaction appears to be calcium ion–specific since no concentration of monovalent ions was observed with this fraction.

This effect is unstable to aging and to deoxycholate or other detergents in a manner similar to that found for the relaxation effect of the same fraction (1). In contrast, ATPase is either not affected or is rather stimulated by aging or detergents; the same is true for ADP-ATP exchange which was found with this fraction and which appears to run pretty much parallel with the ATPase.

The electron micrographic picture of the fraction consists mainly of membrane-bounded elements, apparently resealed fragments of the endoplasmic reticulum. It is rather attractive to consider the accumulation of calcium ion as being due to an ATP-dependent transport of calcium

FIGURES 14 AND 15

Small fields in the bottom layer of a particulate preparation. Activity for calcium ion concentration and relaxation, good. × 60,000.

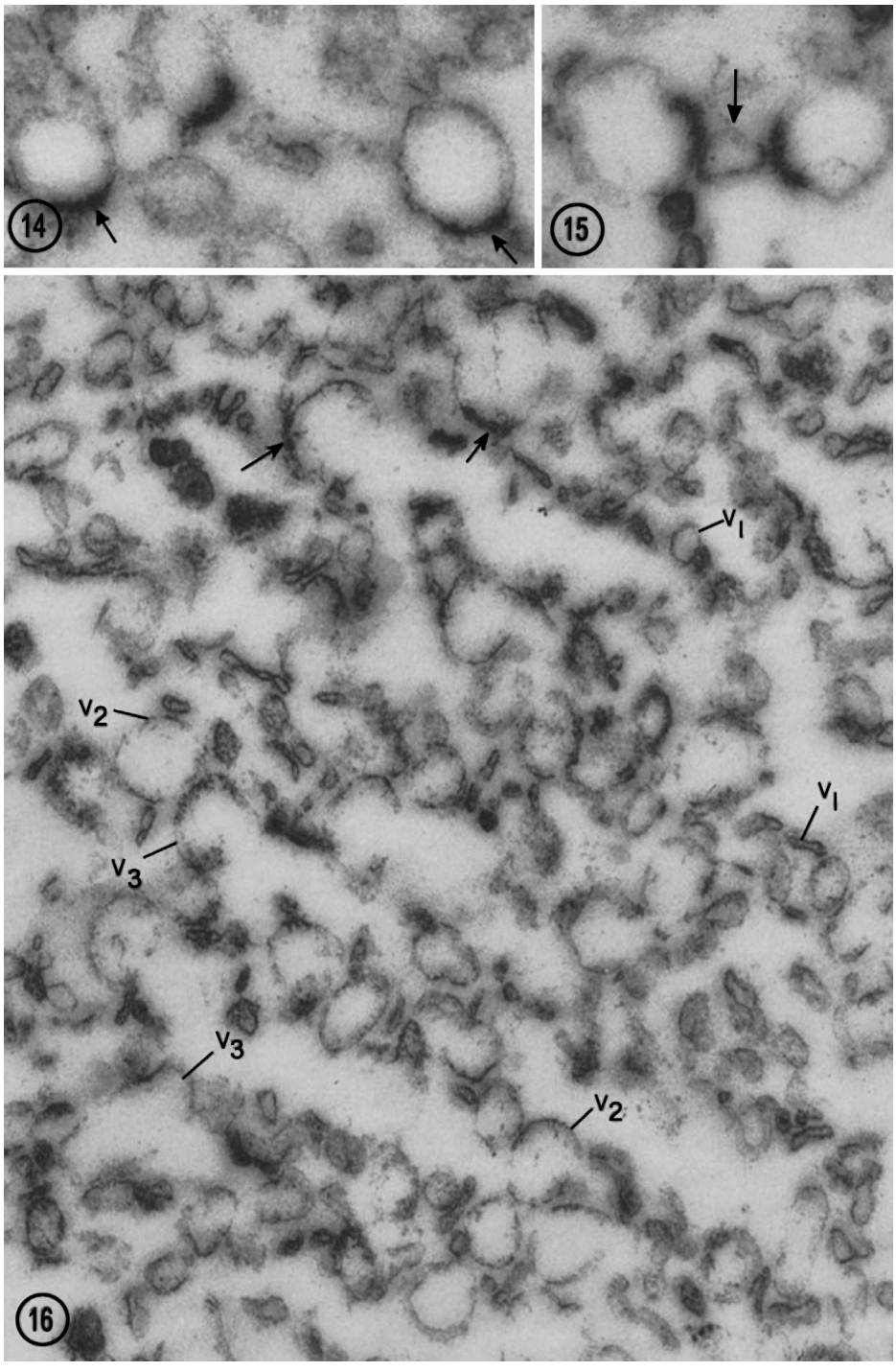
In Fig. 14 the arrows point to the local membrane thickening that characterizes terminal cisternae *in situ*.

In Fig. 15 the arrow marks an intermediate vesicle still located between two terminal cisternae, as in the triads of intact muscle fibers.

FIGURE 16

Bottom layer in a particulate preparation aged for 6 days. Activity rather poor.

Profiles of apparently intact vesicles are still present (v_1) but many elements show limited (v_2) or gross (v_3)discontinuity of their limiting membranes. The arrows point to local membrane thickenings of the type encountered in terminal cisternae *in situ*. × 60,000.


14
15
16
V1
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V1
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ion across the membrane which holds it reversibly inside the vesicles.

We are greatly indebted to Dr. George Palade for his interest and help, and comments on this paper. We are very grateful for permission to include his electron micrographs, which he prepared and annotated.

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