

Calcium Uptake and Associated Adenosine Triphosphatase Activity of Isolated Platelet Membranes

LOIS S. ROBBLEE, DAVID SHEPRO, and
FRANK A. BELAMARICH

From the Boston University Biology Department and School of Medicine,
Boston, Massachusetts 02215

ABSTRACT A platelet subcellular fraction, sedimenting between 14,000 and 40,000 *g* and consisting primarily of membrane vesicles, accumulates up to 200–400 nmoles calcium/mg protein in the presence of ATP and oxalate. Steady-state levels of calcium accumulation are attained in 40–60 min. Calcium uptake requires adenosine triphosphate (ATP), is enhanced by oxalate, and is accompanied by the release of inorganic phosphate. Calcium accumulation and phosphate release require magnesium and are inhibited by Salyrgan (10 μ M) and adenosine diphosphate (ADP) (1 mM), but not by ouabain (0.1 mM). The ATPase activity is stimulated by low concentrations of calcium (5–10 μ M) and is inhibited by 2 mM EGTA. Electron microscopic histochemistry using lead nitrate to precipitate released phosphate results in lead precipitates localized primarily at the inner surface of membrane vesicles. These results provide evidence for a membrane ATPase that is stimulated by low concentrations of calcium and may be involved in the transport of calcium across the membrane. It is postulated that the observed calcium uptake activity is an *in vitro* manifestation of a calcium extrusion pump in the intact platelet.

INTRODUCTION

Platelets normally circulate in the blood as discrete, nonadhesive “cells,” yet they rapidly become sticky and adhere to each other and to the blood vessel wall in response to injury of the blood vessel. Irreversible aggregation of platelets is an early step in hemostasis. An initial event in the chain of reactions leading to irreversible aggregation is a release reaction in which platelets discharge their contents into the plasma milieu. A vast literature exists on the substances that promote release and/or aggregation, but little is known about platelet control mechanisms that prevent these phenomena from occurring except when a stimulus is applied. The requirements for calcium in release and aggregation, plus the analogy of these reactions to secretory (Holmsen,

Day, and Stormorken, 1969) or contractile events (Grette, 1962; White, 1971), suggest that calcium may have a role in platelets comparable to its role in stimulus-secretion coupling (Douglas, 1968) or stimulus-contraction coupling (Ebashi and Endo, 1968).

In 1963, Grette reported that a 14,000 *g* supernatant of platelet homogenates inhibited the superprecipitation of the platelet contractile protein, thrombosthenin, and that the inhibition could be reversed by the addition of calcium ions. Because this activity resembled that of the "relaxing factor" of muscle (Marsh, 1952; Mueller, 1960), Grette proposed the idea that a platelet relaxing factor was involved in maintaining the platelets in an unactivated state. Subsequently, Statland, Heagan, and White (1969) demonstrated that a membrane component of the 14,000 *g* supernatant had the ability to take up calcium in the presence of ATP and suggested that this activity might represent a calcium extrusion pump similar to that of the sarcoplasmic reticulum of muscle.

Studies have been undertaken to examine the properties and conditions of calcium uptake in a platelet membrane preparation in order to determine whether a transport mechanism is involved and whether the transport might function as a calcium extrusion pump. The present communication describes the *in vitro* requirements for calcium uptake by a platelet membrane fraction and presents evidence for a membrane ATPase involved in the transport of calcium across the membrane.

MATERIALS AND METHODS

Materials

All chemicals were analytical grade. ATP,¹ ADP, CTP, GTP, and UTP, obtained as the sodium salts from Sigma Chemical Co., St. Louis, Mo., were treated with Dowex 50-x8 and adjusted to pH 6.7-6.9 with NaOH (Carsten, 1969). MOPS (3-(*N*-morpholino) propane-sulfonic acid) was obtained from Polysciences, Inc., Warrington, Pa. EGTA (Eastman Kodak Co., Rochester, N. Y.) was prepared in 0.1 M Tris and the pH adjusted to 7.5 with HCl. Glutaraldehyde was obtained from Ladd Research Industries, Inc., Burlington, Vt., as a 70% solution and diluted with buffer to a 6% stock solution. Epon 812 embedding materials were also obtained from Ladd Research Industries. All solutions were prepared and stored in Pyrex glassware (Corning Glass Works, Corning, N. Y.), and reactions were carried out in glassware that had been treated with Siliclad (Clay-Adams, Inc., New York).

Isolation of Platelets

Calf blood, obtained at the slaughterhouse, was collected directly into polyethylene bags containing 100 ml of 3.8% sodium citrate, care being taken to avoid collecting

¹ The following abbreviations are used in this paper: EGTA, ethylene glycol bis (β -aminoethyl ether)*N,N'*-tetraacetic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.

the initial flow of blood. If the volume of blood plus anticoagulant exceeded 1 liter, additional citrate was added to maintain the citrate at a $\frac{1}{10}$ volume concentration. The blood was mixed by slow inversion of the bags after which they were placed on ice. Blood from each calf was kept separate so that platelet condition and number could be assessed. Those with a low platelet number, microscopic evidence of fibrin formation, or activated platelets, e.g. with pseudopod formation or microaggregates, were discarded. All subsequent operations were carried out at 4°–5°C. The citrated blood was centrifuged in 500-ml polycarbonate bottles in the GS-3 head of the Sorvall RC-2B centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) at 1200 *g* (2700 rpm) for 6 min. The platelet-rich plasma (PRP) was siphoned off from the sedimented red cells, pooled, and centrifuged at 1800 *g* for 20 min. Platelet pellets were washed three times by resuspending in 20–30 vol of citrated 0.9% saline ($\frac{1}{10}$ vol of 3.8% citrate). Residual red blood cells were removed from the platelet suspension by repeated differential centrifugation at 150 *g* for 3 min. Washed platelets were resuspended in 25 ml Tris-buffered saline (1 vol 0.1 M Tris-HCl, pH 7.0 to 4 vol 0.9% saline) containing 0.5% glucose and stored overnight at 5°C. Preliminary experiments indicated that the overnight storage of platelets at 5°C had no appreciable effect on the calcium uptake activity of subsequently prepared subcellular fractions.

Single units of human blood collected in $\frac{1}{10}$ vol of 3.8% sodium citrate were obtained from Blood Research Institute, Boston, Mass. Within 2 h after collection, the blood was centrifuged in 50-ml polyethylene tubes in the Sorvall GLC centrifuge at 1500 *g* for 20 min to obtain platelet pellets. The red cells sedimented in the initial centrifugation were resuspended by gentle inversion in a volume of citrated saline equal to the volume of PRP that had been removed. The resuspended red cells were centrifuged again at 1000 *g* for 3 min. The supernatant contained platelets that initially had sedimented with the red cells. The supernatant was removed by Pasteur pipette, care being taken to avoid the buffy coat cells, and was centrifuged at 1800 *g* for 20 min. The resulting platelet pellet was resuspended in citrated saline and combined with platelets derived from the PRP. This procedure was found to nearly double the final yield of platelets. The remaining procedures of washing the platelets and removing residual red cells was the same as for calf platelets.

Preparation of Platelet Fractions

The following day the platelets were centrifuged in a graduated conical centrifuge tube in a GLC centrifuge at 1400 *g* for 30 min. The pellets were resuspended in 3 vol of homogenizing medium (30 mM KCl, 5 mM MgCl₂, 10 mM potassium oxalate, and 20 mM Tris-HCl, pH 7.0) (Grette, 1963). 1 ml portions were homogenized in a smooth-walled glass tube with a motor-driven teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa., Cat. No. 42888, Size AA) at 2400 rpm. The homogenizer tube was submerged in an ice bath throughout the homogenization period, and to avoid excessive heat buildup, the homogenization was carried out in three periods of 1½ min each, interrupted by intervals of 2–3 min. After each portion was homogenized, the tube was rinsed with 2 ml of homogenizing medium and the washings combined with the cell homogenate.

The platelet homogenate was centrifuged at 14,000 *g* for 10 min producing pellet fraction I and supernatant fraction IA. The supernatant fraction IA was centrifuged

at 40,000 *g* for 1 h yielding pellet fraction II and supernatant fraction III. Each pellet fraction, I and II, was resuspended in a volume of homogenizing medium equal to that used to homogenize the original platelets. Exceptions to this are indicated in the legends to the figures. The various steps of the isolation and fractionation procedure are shown in Fig. 1. The fractions were kept on ice until used. Preliminary experiments indicated that both calcium uptake activity and ATPase activity decreased

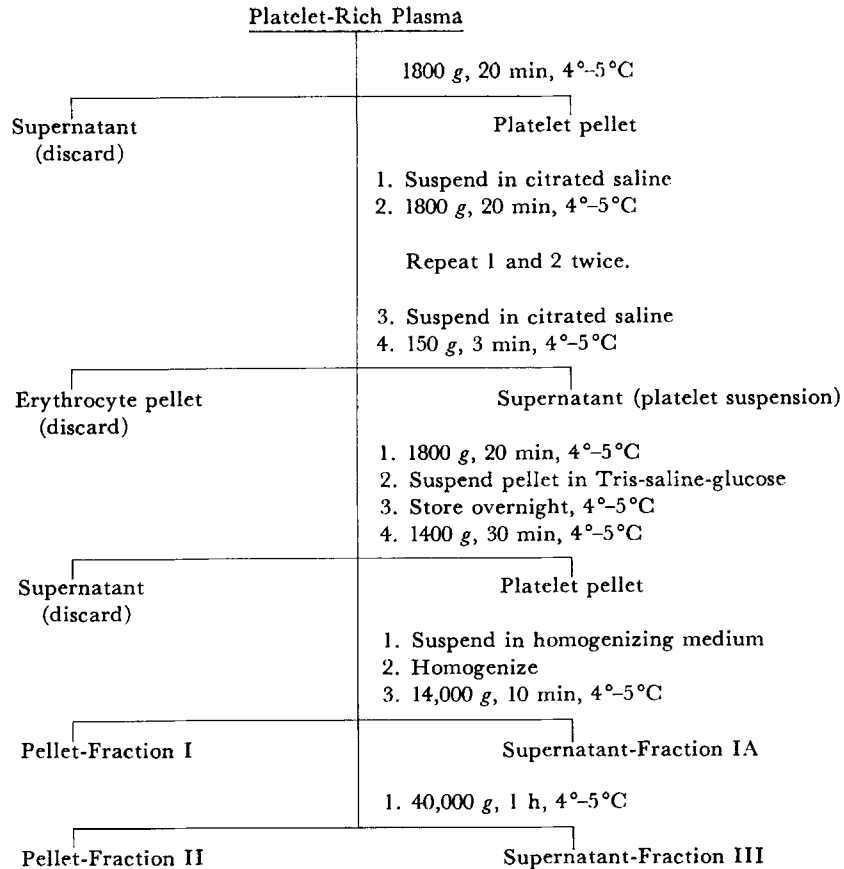


FIGURE 1. Diagram of isolation procedure.

with storage, therefore all experiments were done the same day that the fractions were prepared.

Protein content of platelet fractions was determined by the method of Lowry et al. (1951).

Measurement of Calcium Uptake Activity

The standard incubation medium for measurement of calcium uptake activity contained 100 mM KCl, 5 mM MgCl₂, 2 mM ATP, 20 mM Tris-HCl (pH 7.0), and 50-58 μM CaCl₂ of which 0.52 μM was ⁴⁵CaCl₂. Total reaction volumes ranged from 1 to 5 ml depending upon the experiment with each milliliter of complete reaction

mixture containing 0.1 ml of platelet fraction. Unless stated otherwise in the legends to the figures, complete reaction mixtures contain 1 mM oxalate which is introduced with the platelet fraction (from the homogenizing medium). Because the activity of the preparations deteriorated rapidly at 37°C, all reactions were carried out at ambient temperature (22–24°C) except where indicated and were started by the addition of the platelet fraction to the medium. Continuous stirring was maintained throughout the incubation period. At various time intervals, 0.5-ml portions were filtered on a Millipore Microfiltration Apparatus (Millipore Corp., Bedford, Mass.) using Type HA filters, 0.45 μm pore size (Martonosi and Feretos, 1964). The filters were washed with 50 ml of cold 0.9% saline while on the filtration apparatus, air-dried, and placed in a scintillation counter. To determine the specific activity of ^{45}Ca , a 0.02 ml portion of each reaction mixture was placed on a filter, dried, and the radioactivity determined as for the experimental samples. Specific activity (sp act), expressed as counts per minute per nanomole calcium was calculated as: $\text{sp act} = \text{cpm}/0.02 \text{ ml}/\text{calcium concentration of the reaction mixture}$.

Measurement of ATPase Activity

The reaction mixture for the measurement of ATPase activity was the same as for calcium uptake but with $^{45}\text{CaCl}_2$ omitted. Variations in this medium are indicated in the legends to the figures. ATPase activity was determined by measuring the liberation of inorganic phosphate (P_i). The reaction was carried out at 22°–24°C with continuous stirring. P_i was determined by the following modification (Matsushita and Raacke, 1968; Raacke, personal communication, 1970) of the method of Delsal and Manhoury (1958): at the end of the incubation period, 0.2 ml of the reaction mixture was added to 4 ml of ice-cold molybdate-acetate reagent and mixed rapidly on a vortex mixer; 90 s later 0.2 ml of cold stannous chloride was added with rapid mixing and 3 min later the optical density was measured at 735 nm against distilled water. Appropriate reagent blanks were carried through the same procedure and subtracted from the optical density reading of the sample. The amount of P_i is determined from a standard curve prepared from solutions of varying concentrations of KH_2PO_4 . The molybdate-acetate reagent is prepared fresh from 43 parts acetate buffer (45 g sodium acetate: 3 H_2O in 1 liter 2 N acetic acid), 1 part 37% formaldehyde, and 5 parts of 5% ammonium molybdate:4 H_2O . Stannous chloride is prepared as a stock solution of 5.935 g $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 25 ml of concentrated HCl and is diluted 1:100 with distilled water just before use.

Electron Microscopy

The pellet fractions were resuspended in homogenizing medium and fixed for electron microscopy by adding an equal volume of 6% glutaraldehyde in 0.1 M cacodylate-HCl buffer, pH 7.2. After 1 h at room temperature, the fixed suspensions were centrifuged at 14,000 g for 10 min (fraction I) or at 40,000 g for 1 h (fraction II). The glutaraldehyde-fixed pellets were washed with several changes of cold cacodylate-HCl buffer, postfixed in cold 1% OsO_4 in 0.1 M cacodylate-HCl buffer, stained en bloc with 0.5% uranyl acetate (Farquahar and Palade, 1965), dehydrated through a graded ethanol series, and embedded in Epon 812 (Luft, 1961). Thin sections cut on

an LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.) were double-stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in an RCA EMU-3C or JEM-100B (JEOL U.S.A., Inc., Medford, Mass.) electron microscope.

Enzyme Histochemistry

For the histochemical demonstration of ATPase activity, the membrane fraction II was used exclusively. After the 40,000 *g* centrifugation, the intact pellet was scooped out of the conical centrifuge tube and cut into small blocks of approximately 1 mm size. These blocks were carried through the histochemical incubation and subsequent fixation and embedding as if they were tissue blocks.

The incubation medium for ATPase histochemistry was identical to that used for biochemical determination of ATPase activity except for the addition of 1 mM $\text{Pb}(\text{NO}_3)_2$. In preliminary measurements the ATPase activity of membrane fractions suspended in this medium was about half of that obtained in the absence of lead. In spite of the inhibition, there was still 0.2 $\mu\text{mole P}_i$ released/mg protein per 30 min. Tice and Engel (1966) reported that a minimum of 0.1 $\mu\text{mole P}_i$ /mg protein was adequate to produce an observable precipitate of lead phosphate in a similar system. Small blocks of the platelet membrane pellet were incubated in the ATPase medium for 1 h at room temperature. Control samples were incubated for 1 h at room temperature in the ATPase medium containing 10 μM Salyrgan (Winthrop Laboratories, New York), or in ice-cold ATPase medium for 10 min. After the enzyme incubation, the blocks were rinsed twice with 20 mM Tris-HCl (pH 7.0), fixed for 2 h in 3% cacodylate-buffered glutaraldehyde (pH 7.0), postfixed in 1% OsO_4 , dehydrated in a graded ethanol series, and embedded in Epon 812.

RESULTS

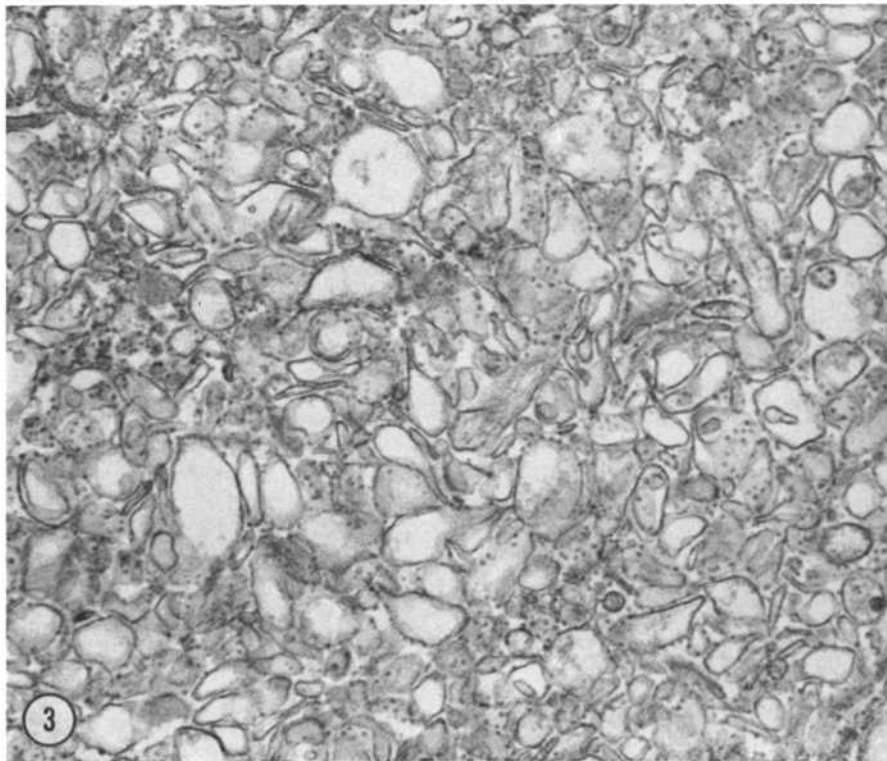
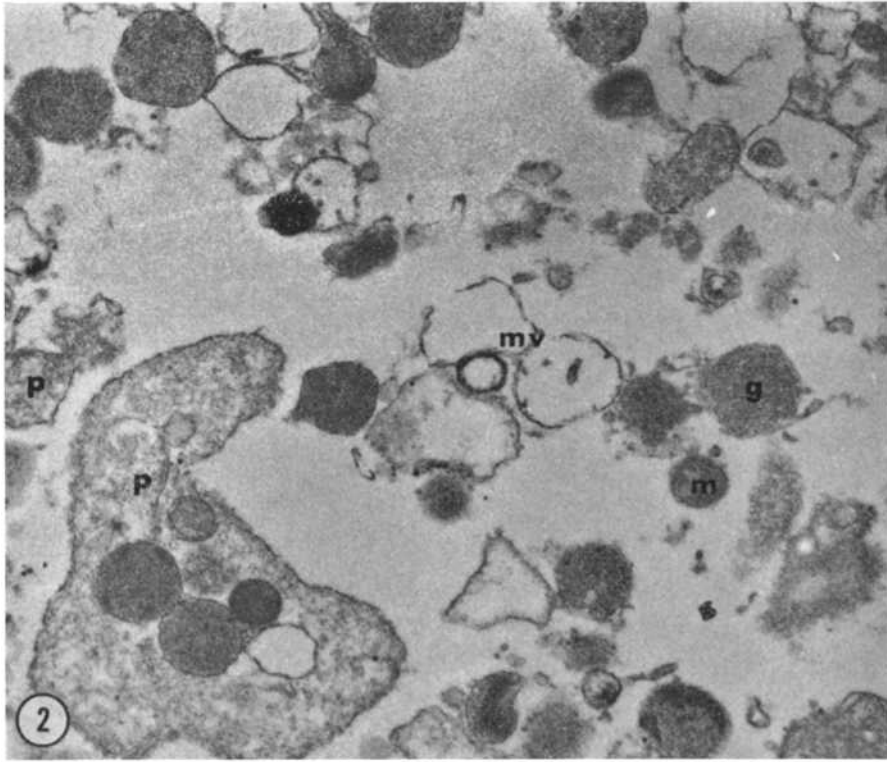
Electron Microscopy of Platelet Fractions

Fraction I contains unbroken platelets, platelet fragments, mitochondria, granules, and coarse membrane fragments (Fig. 2). Fraction II consists primarily of membranous vesicles and 200–300 Å particles believed to be glycogen (Fig. 3). The homogeneity of this fraction was observed to vary from one preparation to another so that a few mitochondria and granules are observed occasionally.

Calcium Uptake Activity of Platelet Fractions

The distribution of calcium uptake activity in the major fractions is shown in Table I. Where calcium uptake activity was measured in the complete homogenate, an enrichment of specific calcium uptake activity is observed in both pellet fractions I and II. The supernatant fraction III has negligible activity.

The morphological homogeneity of fraction II coupled with the higher calcium uptake activity indicated that it is the more suitable fraction for studies of the requirements for calcium uptake. Consequently, only fraction II was used for many of the remaining experiments.



The Effect of Oxalate on Calcium Uptake

Calcium uptake was measured in the presence of varying concentrations of oxalate. For this experiment, the membrane fraction II was washed and resuspended in oxalate-free homogenizing medium. As seen in Fig. 4, oxalate in

TABLE I
DISTRIBUTION OF CALCIUM UPTAKE ACTIVITY

Samples were incubated in the standard incubation medium containing 5 mM potassium oxalate for 60 min at 23°C. Calcium uptake activity is expressed as nanomoles calcium accumulated per milligram protein.

Experiment	Complete homogenate	Fraction			
		I	IA	II	III
1. Calf	52.6	170.5	11.8	215.0	0.87
2. Calf		95.0		342.0	0.76
3. Human		60.2	4.4	79.1	
4. Human		16.4		87.8	

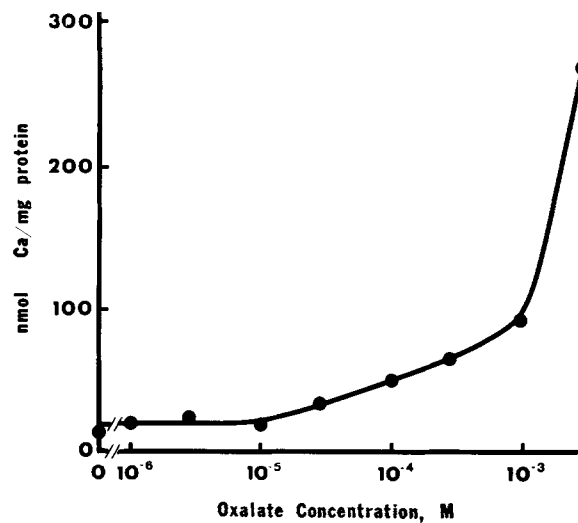


FIGURE 4. The effect of oxalate concentration on calcium uptake activity. Fraction II was resuspended in oxalate-free homogenizing medium and incubated for 30 min in the standard calcium uptake medium containing potassium oxalate in the concentrations indicated on the abscissa. Protein concentrations of the complete reaction mixtures were 0.093 mg/ml.

FIGURE 2. *opposite* Electron micrograph of fraction I. This fraction, which sediments at 14,000 *g*, contains unbroken platelets and platelet fragments (*p*), free granules (*g*) and mitochondria (*m*), and membrane vesicles and fragments (*mv*). Stained en bloc with uranyl acetate. Section stained with uranyl acetate and lead citrate. $\times 26,500$.

FIGURE 3. Electron micrograph of fraction II. This fraction, which sediments at 40,000 *g*, consists primarily of membrane vesicles and glycogen-like particles. Stained en bloc with uranyl acetate. Section stained with uranyl acetate and lead citrate. $\times 26,500$.

concentrations of 1–10 μM has little effect on calcium uptake. From 10 μM to 1 mM oxalate, the total amount of calcium accumulated increases gradually. When the oxalate concentration is above 1 mM, a sharp increase in calcium accumulation is observed. This is characteristic of sarcoplasmic reticulum type of calcium uptake (Hasselbach, 1964).

The time-course of calcium uptake is also influenced by the presence of oxalate in the medium. As seen in Fig. 5, the steady-state level of calcium accumulation is reached within 5 min in the absence of oxalate. In the presence of oxalate, calcium accumulation continues for 60 min or more. A similar

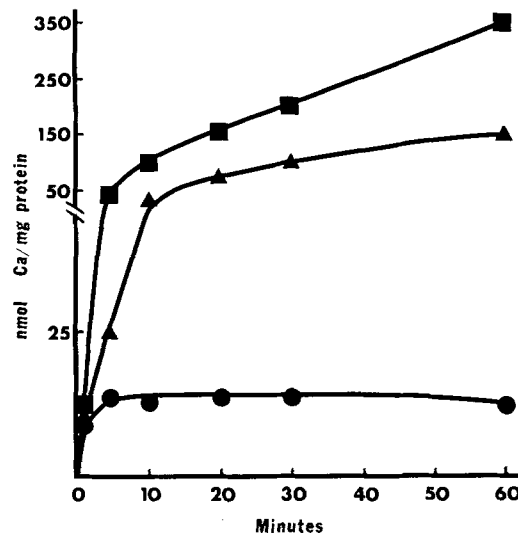


FIGURE 5. The effect of oxalate on time to reach steady state. Fraction II was resuspended in oxalate-free homogenizing medium and incubated in the standard calcium uptake medium without oxalate (\bullet); or with oxalate at 1 mM (\blacktriangle) or 5 mM (\blacksquare) final concentration. Protein concentrations of the complete reaction mixtures were 0.081 mg/ml.

enhancement of the calcium uptake activity by oxalate is observed in the complete homogenate and in fraction I, although the increase is not as great as that seen in fraction II (Fig. 6).

Nucleotide Requirement for Calcium Uptake

A nucleotide triphosphate is required for significant calcium uptake activity. The nucleotide requirement appears to be specific for ATP since calcium uptake activity is substantially lower in the presence of the triphosphates GTP, CTP, or UTP and the diphosphate, ADP (Fig. 7, Table II). ATP-dependent calcium uptake activity is the same with ATP concentrations ranging from 0.5 to 4 mM. ADP in equimolar concentration with ATP is inhibitory (Table II).

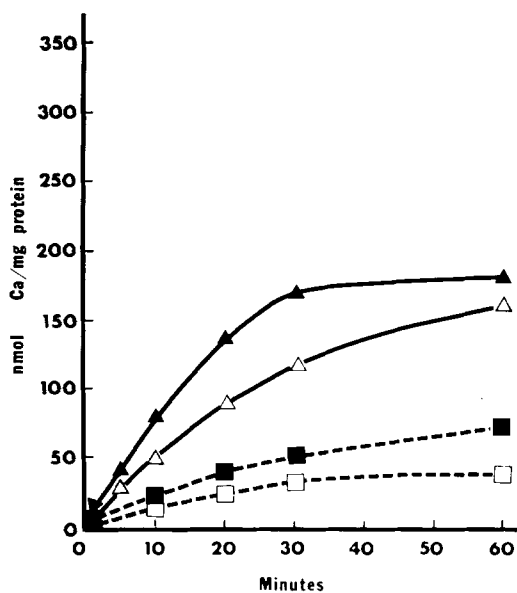


FIGURE 6. The effect of oxalate on calcium uptake activity. Time-course of calcium uptake by: complete homogenate (□, ■); fraction I (△, ▲). Open symbols, 1 mM oxalate; closed symbols, 5 mM oxalate. Protein concentrations of the complete reaction mixtures were 0.510 mg/ml for the complete homogenate and 0.230 mg/ml for fraction I.

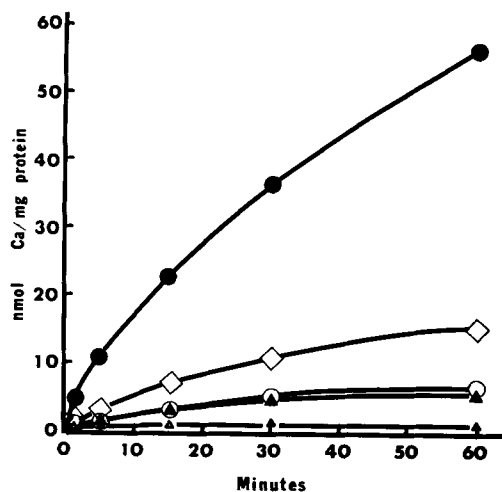


FIGURE 7. The effect of nucleotide triphosphates (NTP) on calcium uptake activity. Fraction II was incubated in the standard calcium uptake medium containing 2 mM ATP (●); 2 mM UTP (□); 2 mM GTP (○); 2 mM CTP (▲); or no NTP (△). Protein concentrations of the complete reaction mixtures were 0.074 mg/ml.

ATPase Activity Associated with Calcium Uptake

ATPase activity was measured under the same conditions as calcium uptake but with ⁴⁵CaCl₂ omitted. EGTA at 2 mM concentration was used to chelate

any extraneous calcium in order to determine whether a calcium-activated ATPase might be present. Table III shows the results of these experiments. EGTA reduces the ATPase activity significantly, suggesting the presence of a calcium-activated ATPase. The parallel effects of Salyrgan on calcium uptake and ATPase activity support the concept of an ATPase being involved in calcium uptake. Sodium azide has less effect on either calcium uptake or ATPase activity indicating a small mitochondrial participation. The ATPase

TABLE II
EFFECT OF ADP ON CALCIUM UPTAKE

Samples were incubated in calcium uptake medium containing the indicated nucleotide for 30 min at 23°C. Protein concentrations of the complete reaction mixtures were 0.040 mg/ml.

Nucleotide	Ca uptake (nmoles/mg)
1 mM ATP	87.0
2 mM ATP	91.9
1 mM ATP + 1 mM ADP	14.1
2 mM ADP	15.9

TABLE III
ATPase ACTIVITY ASSOCIATED WITH CALCIUM UPTAKE

Samples were incubated for 30 min at 23°C in the standard incubation media as described in Methods. Inhibitors were added to the medium so that final concentrations in the complete reaction mixture were: Salyrgan, 10 μ M; sodium azide, 0.5 mM; EGTA, 2.0 mM. Calcium (Ca) uptake is expressed as nanomoles Ca per milligram protein. Inorganic phosphate released (P_i) is expressed as micromoles P_i per milligram protein. Protein concentrations in the complete reaction mixtures were: 1) 0.300 mg/ml; 2) 0.113 mg/ml; and 3) 0.093 mg/ml.

Sample	P_i	Inhibition %	Ca uptake	Inhibition %
1. Fraction I	0.407		5.6	
a. + Salyrgan	0.187	54	1.6	71
b. + Azide	0.367	10	4.3	23
c. + EGTA	0.227	44		
2. Fraction II	0.460		24.3	
a. + Salyrgan	0.274	40	7.1	71
b. + Azide	0.496	0	14.8	39
c. + EGTA	0.274	40		
3. Fraction II	0.540		52.3	
a. + Salyrgan	0.086	84	10.7	79
b. + Azide	0.387	28	53.6	0
c. + EGTA	0.054	90		

activity is inhibited less than 15% by 0.1 mM ouabain indicating that the contribution of the Mg^{++} -dependent, (Na^+ and K^+)-stimulated ATPase to the observed ATPase activity is small. ADP at 1 mM concentration produces 40% inhibition of the ATPase activity.

Enzyme Histochemistry

Samples incubated for demonstration of ATPase activity are heavily labeled with dense black deposits of lead phosphate (Fig. 8). The precipitates are confined to the vesicle lumens, sometimes filling the entire vesicle. In vesicles that are not filled completely, the precipitate is associated with the inner surface of the membrane and is not "floating free" within the lumen. No precipitates are observed in the spaces between vesicles. The inhibition of ATPase activity by Salyrgan is demonstrated by the marked reduction in lead phosphate deposition when Salyrgan is included in the incubation medium (Fig. 9). No deposits are observed in samples incubated in ice-cold ATPase medium.

Effect of Calcium Concentration on Calcium Uptake and ATPase Activity

The total amount of calcium accumulated by fraction II increases with increasing medium calcium concentration (Fig. 10). The ATPase activity also increases with an apparent activation produced by the addition of small increments of calcium. A sharp increase in the amount of inorganic phosphate liberated is observed with the addition of 5–10 μM calcium to the reaction medium. (Fig. 11).

Effect of Magnesium on Calcium Uptake and ATPase Activity

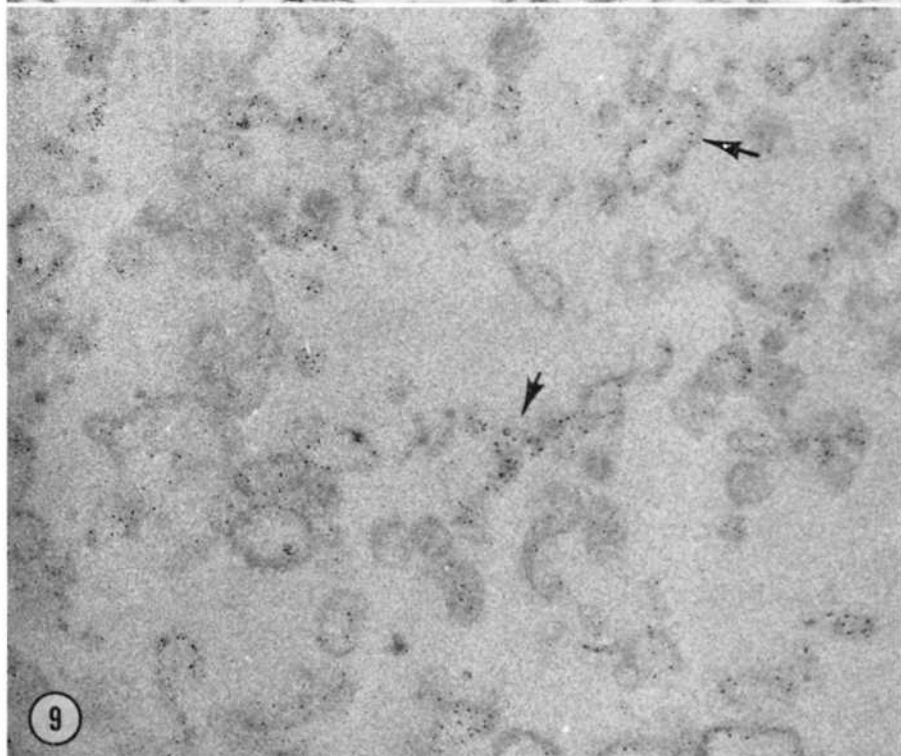
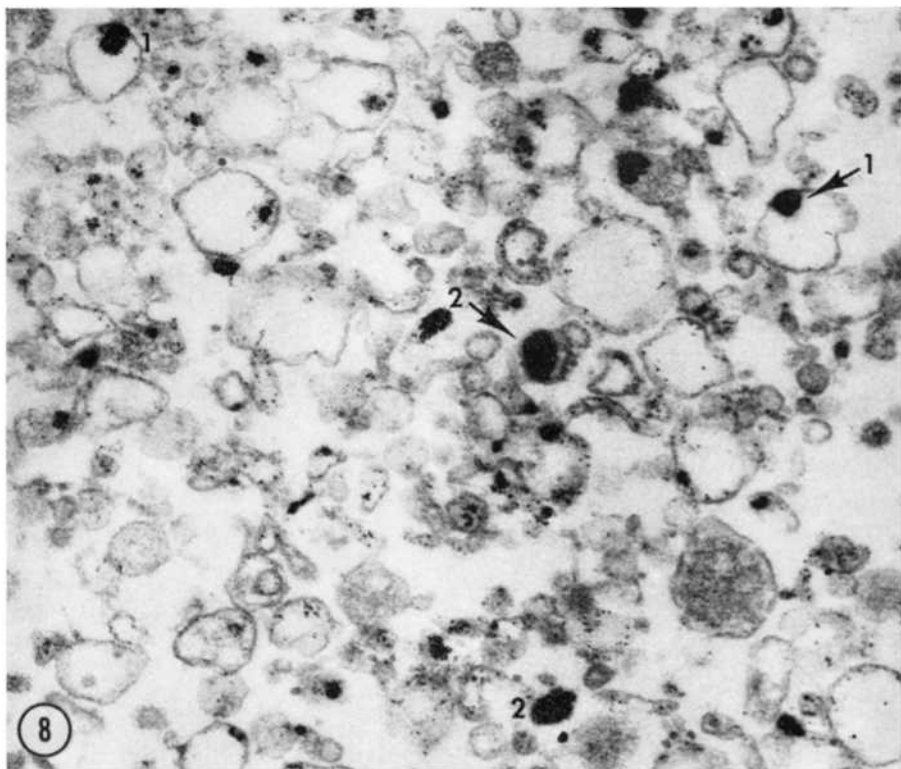
The effect of magnesium concentration on calcium uptake and ATPase activity is shown in Fig. 12. Both activities are maximal at a magnesium concentration of 5.0–7.5 mM. At 0 and 10 mM magnesium there is no measurable ATPase activity although there is some calcium uptake.

Effect of Temperature on Calcium Uptake Activity

The rate of calcium uptake is temperature dependent. From the data in Fig. 13, the activation energy is approximately 13 kcal/mol. At 37°C there is less total calcium uptake at steady state than at 25°C. This difference may be the result of breakdown of the system during prolonged incubation at the higher temperature.

Effect of pH on Calcium Uptake and ATPase Activity

Calcium uptake and ATPase activities were determined over a pH range from 5.0–8.5 using the zwitterionic buffer MOPS (3-(*N*-morpholino) propane-sulfonic acid, pK 7.2) rather than Tris (pK 8.1). Both buffers gave the same results in preliminary experiments at pH 7.0. Fig. 14 shows the effect of vary-



ing the pH of the reaction medium on calcium uptake and ATPase activities. Calcium uptake has a sharp peak at pH 7.0. This is in contrast to the ATPase activity which seems to reach a plateau between pH 6.5 and 7.5 and then increases sharply above pH 7.5. It is not known whether the difference in the two activities at high pH is the result of membranes becoming leaky and unable to retain accumulated calcium, or is the result of other phosphatases in the preparation.

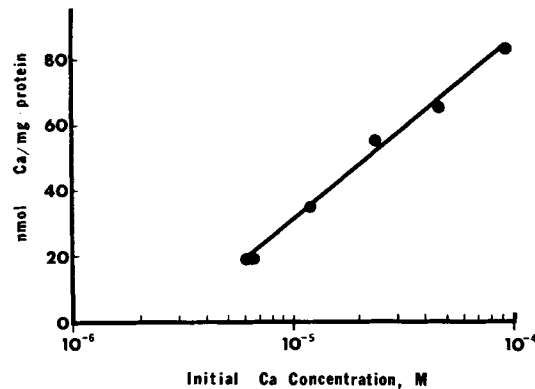


FIGURE 10. The effect of calcium concentration on calcium uptake activity. Fraction II was incubated for 30 min in the standard calcium uptake medium containing a range of calcium concentrations. The concentrations indicated on the abscissa represent the total calcium concentration of the complete reaction mixture at the start of the reaction. Protein concentrations of the complete mixture were 0.263 mg/ml.

DISCUSSION

The results presented here demonstrate that platelet membrane structures accumulate calcium by an ATP-dependent process. Although granules and mitochondria are occasionally found in fraction II, membrane vesicles are the principal component. The enrichment of calcium uptake activity in fraction II indicates that calcium uptake is a membrane-associated activity. The activity observed in fraction I, which contains all types of subcellular structures, is probably the result of its membrane constituent. The participation of membrane structures rather than mitochondria in the calcium uptake by both fractions is demonstrated by the enhancement of calcium uptake activity by

FIGURE 8. *opposite* Electron micrograph of membrane fraction after incubation in standard ATPase medium. Section stained with uranyl acetate and lead citrate to illustrate morphology. Dense precipitates of lead phosphate reaction product are attached to the inner surface of vesicles (1) and sometimes fill the entire lumen (2). × 43,700.

FIGURE 9. Electron micrograph of membrane fraction after incubation in ATPase medium containing Salyrgan. The only lead phosphate reaction product evident is a fine stippling associated with membranes (arrows). Unstained section. × 61,200.

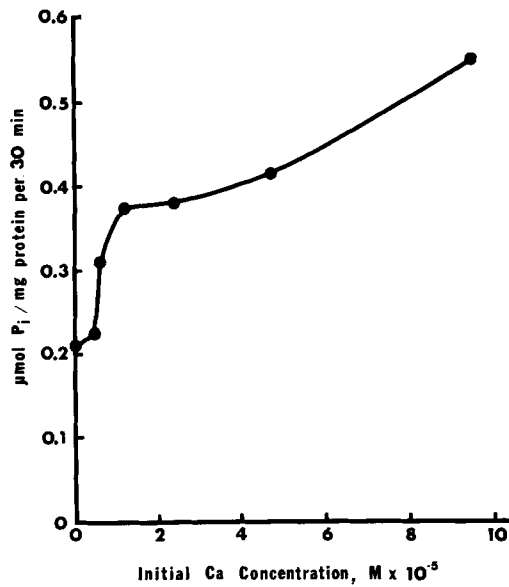


FIGURE 11. The effect of calcium concentration on ATPase activity. Fraction II was incubated for 30 min in the standard ATPase medium containing a range of calcium concentrations. The concentrations indicated on the abscissa represent the total calcium concentration of the complete reaction mixture at the start of the reaction. Protein concentrations of the complete mixtures were 0.263 mg/ml.

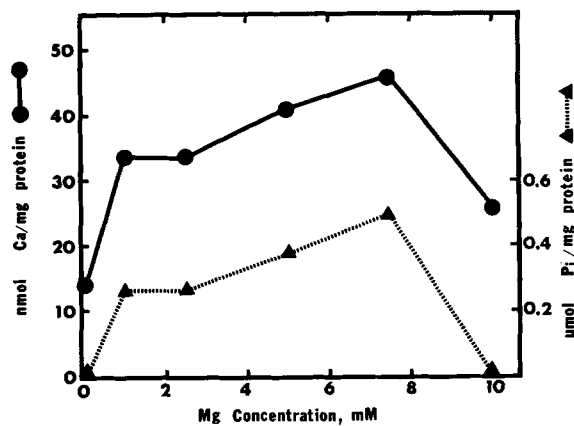


FIGURE 12. The effect of magnesium concentration on calcium uptake (●) and ATPase (▲) activity. Fraction II was resuspended in magnesium-free homogenizing medium and was incubated for 30 min in the standard calcium uptake or ATPase medium containing final concentrations of magnesium as indicated on the abscissa. Protein concentrations of the complete reaction mixtures were 0.041 mg/ml.

oxalate and by the greater inhibition of activity with Salyrgan in low concentration than with sodium azide.

ATP-dependent calcium uptake is accompanied by the liberation of in-

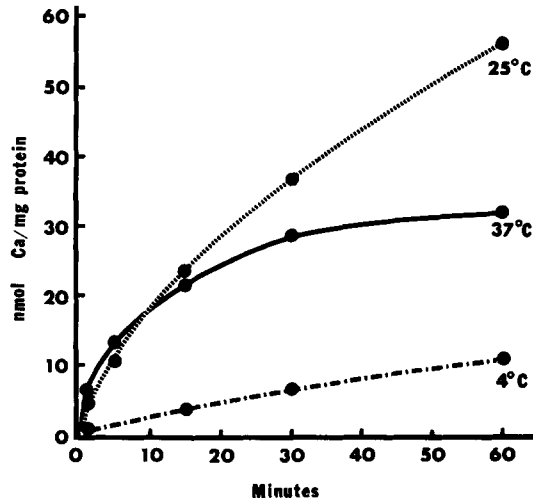


FIGURE 13. The effect of temperature on calcium uptake activity. Fraction II was incubated in the standard calcium uptake medium at the temperatures indicated. The protein concentrations of the complete reaction mixtures were 0.074 mg/ml.

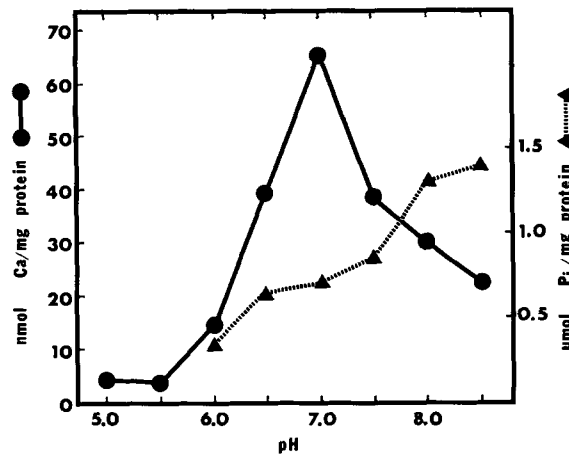


FIGURE 14. The effect of pH on calcium uptake (●) and ATPase activity (▲). Fraction II was incubated for 30 min in standard calcium uptake medium prepared with MOPS buffer at the pH indicated on the abscissa. Protein concentrations of the complete reaction mixtures were 0.101 mg/ml.

organic phosphate. Magnesium ion is required for optimum levels of both activities. The marked increase in phosphate liberation with the addition of low concentrations of calcium is evidence for the presence of a calcium-activated ATPase. This evidence, coupled with the observation that calcium uptake is reduced to very low levels under conditions that inhibit the ATPase activity, suggests that the ATPase activity is associated with calcium uptake.

The demonstration of ATPase activity by enzyme histochemistry provides further evidence for a membrane function. Unfortunately, EGTA cannot be used to identify the ATPase activity histochemically as a calcium-activated enzyme because of the high affinity of EGTA for lead. However, the marked inhibition of histochemical activity by Salyrgan suggests that the same ATPase enzyme is being demonstrated by both biochemical and histochemical methods. It seems reasonable to conclude that the biochemical activity associated with calcium uptake is identical with the membrane-associated activity observed histochemically.

The calcium uptake activity observed here may be the result of a binding of calcium to the membrane, or it may be the result of a transport process in which calcium is moved across the membrane into the vesicle lumen, or it may represent a combination of both. In the absence of ATP, approximately 1 nmol calcium/mg vesicle protein is taken up (Fig. 7). Assuming an approximate vesicle volume of 10 μ l/mg protein (Weber, Herz, and Reiss, 1966), the concentration of calcium within a vesicle lumen equals 100 μ M, which is somewhat higher but in the same order of magnitude as the external medium concentration of 50 μ M. With the addition of ATP there is a 10-fold increase in calcium uptake to 10 nmol/mg protein in the absence of oxalate, and over 200-fold increase to 250–350 nmol/mg protein with 5 mM oxalate in the medium (Figs. 4 and 5). Using the same calculation as before, these uptake values correspond to vesicular calcium concentrations of 1 mM and 25–35 mM, respectively. If the vesicles are freely permeable to calcium, then an equilibrium concentration within the vesicles of 50 μ M would be expected. Amounts in excess of this might be due to binding of calcium by sites on the membrane and is probably the case in the ATP-free system. The presence of ATP might induce a preferential binding of calcium as has been described for the sarcoplasmic reticulum by Carvalho and Leo (1967), or it might actively promote the translocation of calcium across the membrane into the lumen where it could exist as the free ion or be bound to the inner aspect of the membrane (Weber, Herz, and Reiss, 1966; Vanderkooi and Martonosi, 1971). The present experiments cannot completely distinguish between these possibilities, but the enhancement of calcium uptake by oxalate indicates that at least some of the accumulated calcium is available to combine with oxalate. If the free calcium concentration is above 0.5 mM in the presence of 5 mM magnesium, ATP, and oxalate, precipitation of calcium oxalate will occur (Hasselbach and Makinose, 1961). The above calculations indicate that there is sufficient intravesicular calcium to precipitate with oxalate even if there is as much as 50% error in the values used.

ATP-dependent, oxalate-enhanced calcium uptake is a property of microsomal fractions derived from skeletal muscle (Martonosi and Feretos, 1964), cardiac muscle (Weber, 1966), and uterine muscle (Carsten, 1969), and a

similar activity has been demonstrated in red cell membrane fragments (Cha, Shin, and Lee, 1971). Microsomes prepared from brain tissue also accumulate calcium in an ATP-dependent process, but this uptake is not enhanced by oxalate (Ohtsuka, Ohtsuki, and Ebashi, 1965; Diamond and Goldberg, 1971). In all types of muscle microsomes, calcium uptake is a rapid process and reaches steady state within a few minutes. It is generally accepted that the calcium-accumulating membranes isolated from muscle are derived from the sarcotubular system of the intact muscle (Porter, 1961), and that calcium uptake by isolated sarcotubular membranes is the *in vitro* manifestation of the *in vivo* process by which the sarcotubular system removes calcium from the sarcoplasm thus producing relaxation (Weber, Herz, and Reiss, 1963). A rapid rate of calcium uptake is required for correlation of calcium uptake with the speed of relaxation-contraction cycles and the sarcoplasmic reticulum appears to be specialized to fulfill the rate requirements (Ebashi and Endo, 1968).

In contrast, the calcium uptake observed in red cell membrane fragments is a slow reaction reaching steady state in about 40 min (Cha, Shin, and Lee, 1971). A calcium-extrusion pump has been demonstrated in red cells (Schatzmann, 1966; Schatzmann and Vincenzi, 1969) and it is believed that the calcium uptake observed in red cell membrane fragments results from membrane inversions occurring during preparation of the membrane fragments (Steck et al., 1970). Inversions produce inside-out membrane vesicles so that an extrusion pump in intact cells acts like an inward, accumulating pump in inverted membrane vesicles. The slow rate of calcium uptake in red cell membrane fragments compared with the rapid rate in muscle microsomes may reflect differences in function of the calcium pumps, the red cell pump having a homeostatic function of maintaining a low intracellular calcium concentration.

Because of the morphological complexity of a platelet compared to a red cell, it is not possible to define precisely a location for the membranes involved in calcium uptake. In addition to a plasma membrane, platelets have an intracellular membrane system comprising the surface-connected tubules and the dense tubules (Behnke, 1967). It is not known whether the plasma membrane forms vesicles. In the absence of evidence to the contrary, it is assumed that the platelet membrane preparations used here contain a mixture of inside-out and right-side-out vesicles and also that these vesicles are derived from both internal and external membranes. Since transport is a vectorial process which proceeds actively in only one direction, then only those vesicles which are oriented with transporting sites facing the medium can be expected to be active in calcium uptake. Thus far there is no evidence as to which way the active vesicles are oriented and one can only speculate about the direction of calcium transport in the intact platelet.

The similarity of the platelet release reaction to secretory processes (Grette, 1962; Holmsen, Day, and Stormorken, 1969) and the requirement for calcium in both release (Grette, 1962; Mustard and Packham, 1970; Kinlough-Rathbone and Mustard, 1971) and secretion (Banks, 1971; Rahaminoff, 1971), suggests that calcium may act in platelets as a stimulus-secretion coupling agent (Douglas, 1968). It is reasonable to postulate that some mechanism is required to keep the free calcium in the cytoplasm at a low level until a stimulus for release occurs. The calcium uptake activity of platelet membrane fractions may be an *in vitro* expression of a calcium extrusion pump in the intact cell, just as calcium uptake activities of microsomal fractions of muscle, secretory and nervous tissues, and membrane fragments of red cells represent calcium extrusion pumps in their respective tissues (Ebashi and Endo, 1968; Banks, 1971; Diamond and Goldberg, 1971; Cha, Shin, and Lee, 1971). The calcium extrusion pumps of muscle and secretory tissues play an important role in the removal of calcium after the contractile or secretory response. In platelets, however, the secretory response of release is generally followed by irreversible aggregation that ultimately leads to the destruction of the cell. A calcium pump would not be required to function as it does in muscle or secretory cells, to rapidly return the cells to a "relaxed" or "nonsecreting" state. The slow rate of calcium uptake by platelet membranes is more in keeping with a homeostatic function of maintaining a constant, low concentration of free intracellular calcium.

This work was supported by grants from the National Institutes of Health (HE-10002-4, HE-05411-13) and Office of Naval Research (105-592).

Received for publication 25 September 1972.

REFERENCES

- BANKS, P. 1971. Involvement of calcium in the secretion of catecholamines. *In* Calcium and Cellular Function. A. W. Cuthbert, editor. St. Martin's Press, Inc., New York, 148.
- BEHNKE, O. 1967. Electron microscopic observations on the membrane systems of the rat blood platelet. *Anat. Rec.* **158**:121.
- CARSTEN, M. E. 1969. Role of calcium binding by the sarcoplasmic reticulum in the contraction and relaxation of uterine smooth muscle. *J. Gen. Physiol.* **53**:414.
- CARVALHO, A. P., and B. LEO. 1967. Effects of ATP on the interaction of Ca^{++} , Mg^{++} , and K^{+} with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. Gen. Physiol.* **50**:1327.
- CHA, Y. N., B. C. SHIN, and K. S. LEE. 1971. Active uptake of Ca^{++} and Ca^{++} -activated Mg^{++} -ATPase in red cell membrane fragments. *J. Gen. Physiol.* **57**:202.
- DELSAL, J. L., and H. MANHOURI. 1958. Étude comparative des dosages colorimétriques due phosphore. IV. Dosage de l'orthophosphate en présence d'esters phosphoriques (nouvelle méthodes). *Bull. Soc. Chim. Biol.* **40**:1623.
- DIAMOND, I., and A. L. GOLDBERG. 1971. Uptake and release of ^{45}Ca by brain microsomes, synaptosomes and synaptic vesicles. *J. Neurochem.* **18**:1419.
- DOUGLAS, W. W. 1968. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* **34**:451.
- EBASHI, S., and M. ENDO. 1968. Calcium ion and muscle contraction. *Prog. Biophys. Mol. Biol.* **18**:123.

- FARQUHAR, M. G., and G. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:253.
- GRETTE, K. 1962. Studies on the mechanism of thrombin-catalyzed hemostatic reactions in blood platelets. *Acta Physiol. Scand. Suppl.* **195**:5.
- GRETTE, K. 1963. Relaxing factor in extracts of blood platelets and its function in the cells. *Nature (Lond.)*. **198**:488.
- HASSELBACH, W. 1964. Relaxing factor and the relaxation of muscle. *Prog. Biophys. Mol. Biol.* **14**:167.
- HASSELBACH, W., and M. MAKINOSE. 1961. Die Calcium-pumpe der "Erschlaffungsgrana" des Muskels und ihre Abhängigkeit von der ATP-Spaltung. *Biochem. Z.* **333**:518.
- HOLMSEN, H., H. J. DAY, and H. STORMORKEN. 1969. The blood platelet release reaction. *Scand. J. Haematol. Suppl.* **8**:1.
- KINLOUGH-RATHBONE, R. L., and J. F. MUSTARD. 1971. The role of divalent cations in the platelet release reaction. *Fed. Proc.* **30**:41A.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
- LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
- MARSH, B. B. 1952. The effects of adenosine triphosphate on the fibre volume of a muscle homogenate. *Biochim. Biophys. Acta.* **9**:247.
- MARTONOSI, A., and R. FERETOS. 1964. Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments. *J. Biol. Chem.* **239**:648.
- MATSUSHITA, S., and I. D. RAACKE. 1968. Purification of nucleoside triphosphatases from pea seedling ribosomes. *Biochim. Biophys. Acta.* **166**:707.
- MUELLER, H. 1960. The action of relaxing factor on actomyosin. *Biochim. Biophys. Acta.* **39**:93.
- MUSTARD, J. F., and M. A. PACKHAM. 1970. Factors influencing platelet function: adhesion, release and aggregation. *Pharmacol. Rev.* **22**:97.
- OHTSUKA, M., I. OHTSUKI, and S. EBASHI. 1965. ATP-dependent Ca binding of brain microsomes. *J. Biochem. (Tokyo)*. **58**:188.
- PORTER, K. R. 1961. The sarcoplasmic reticulum. Its recent history and present status. *J. Biochem. Biophys. Cytol.* **10** (Suppl.):219.
- RAHAMINOFF, R. 1971. Role of calcium ions in neuromuscular transmission. In *Calcium and Cellular Function*. A. W. Cuthbert, editor. St. Martin's Press, Inc., New York. 131.
- REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.
- SCHATZMANN, H. J. 1966. ATP-dependent Ca^{++} extrusion from human red cells. *Experientia (Basel)*. **22**:364.
- SCHATZMANN, H. J., and F. F. VINCENZI. 1969. Calcium movements across the membrane of human red cells. *J. Physiol. (Lond.)*. **201**:369.
- STATLAND, B. E., B. M. HEAGAN, and J. G. WHITE. 1969. Uptake of calcium by platelet relaxing factor. *Nature (Lond.)*. **223**:521.
- STECK, T. L., R. S. WEINSTEIN, J. H. STRAUSS, and D. F. H. WALLACH. 1970. Inside-out red cell membrane vesicles: preparation and purification. *Science (Wash. D. C.)*. **168**:255.
- TICE, L. W., and A. G. ENGEL. 1966. Cytochemistry of phosphatases of the sarcoplasmic reticulum. II. *In situ* localization of the Mg-dependent enzyme. *J. Cell Biol.* **31**:489.
- VANDERKOOI, J. M., and A. MARTONOSI. 1971. Sarcoplasmic reticulum. XIII. Changes in the fluorescence of 8-anilino-1-naphthalene sulfonate during Ca^{++} transport. *Arch. Biochem. Biophys.* **144**:99.
- WEBER, A. 1966. Energized calcium transport and relaxing factors. In *Current Topics in Bioenergetics*. D. R. Sanadi, editor. Academic Press, Inc., New York. 203.
- WEBER, A., R. HERZ, and I. REISS. 1963. On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. *J. Gen. Physiol.* **46**:679.
- WEBER, A., R. HERZ, and I. REISS. 1966. Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem. Z.* **345**:329.
- WHITE, J. G. 1971. Platelet morphology. In *The Circulating Platelet*. S. A. Johnson, editor. Academic Press, Inc., New York. 45.