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# CHARACTERIZATION OF DOG CARDIAC MICROSOMES

## USE OF ZONAL CENTRIFUGATION TO FRACTIONATE FRAGMENTED SARCOPLASMIC RETICULUM, $(Na^+ + K^+)$-ACTIVATED ATPase AND MITOCHONDRIAL FRAGMENTS*

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SUMMARY

Cardiac microsomes represent a heterogeneous fraction which contains mitochondrial, plasma membrane and lysosomal enzymes in addition to markers believed to originate in the sarcoplasmic reticulum. The exact composition of this fraction depends on the method of preparation in that prolonged homogenization of ventricular myocardium increases both the yield of microsomal protein and the proportion of the mitochondrial contaminant.

Ultracentrifugation of cardiac microsomes on density gradients made with sucrose alone is of limited value in isolating fragmented sarcoplasmic reticulum. Because of aggregation of the microsomes, zonal ultracentrifugation in sucrose permits isolation of material with only slight enhancement in the activity of markers for the sarcoplasmic reticulum. In the presence of LiBr, used under conditions which inhibit the damaging effects of this salt on the activities studied, aggregation of the microsomal fraction is reduced and density gradient fractionation is more effective.

The fragmented sarcoplasmic reticulum prepared by zonal centrifugation in 0.5 M LiBr contains less than 1/5 the level of mitochondrial enzymes found in the original microsomes while the rate of $Ca^{2+}$ uptake is enhanced 2-fold and the extent of $Ca^{2+}$ uptake is enhanced 4-fold over that in the crude microsomal fraction. The sarcoplasmic reticulum markers were concentrated in a region of the gradient containing approx. 5 % of the original protein that did not correspond to an obvious protein peak.

## INTRODUCTION AND MATERIALS AND METHODS

The microsomal fraction prepared from mammalian hearts has been studied in an attempt to characterize the biochemical properties of the cardiac sarcoplasmic

Abbreviation: EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane.

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reticulum[1–13]. Cardiac microsomes contain vesicular fragments that bind $Ca^{2+}$, and in the presence of oxalate they take up large quantities of this cation to form dense precipitates of calcium oxalate in their interior. This fraction does not, however, represent a pure preparation of sarcoplasmic reticulum fragments, but contains significant amounts of fragments derived from other subcellular organelles, notably mitochondria[6,9,14–16]. In addition, an active ($Na^+ + K^+$)-activated ATPase, presumably derived from the plasma membrane, can be prepared from cardiac microsomes[17–23], and microsomes prepared from heart[24] and other tissues[25] contain fragmented lysosomes and peroxisomes.

High-speed centrifugation in sucrose density gradients has been used to enhance the purity of both fragmented skeletal[26,27] and cardiac[4,9,13] sarcoplasmic reticulum, and zonal centrifugation of skeletal muscle microsomes in sucrose has proved useful in the preparation of fragmented sarcoplasmic reticulum poor in mitrochondrial fragments[28]. In the present report we shall describe the applicability of zonal centrifugation to the preparation of fragmented cardiac sarcoplasmic reticulum. This method permits the isolation of small amounts of fragmented sarcoplasmic reticulum with effective separation from mitochondrial markers, and some separation from the ($Na^+ + K^+$)-activated ATPase that is attributable to the plasma membrane.

*Methods*

Table I lists markers used to indicate the presence of elements derived from different subcellular organelles

ATPase activity was calculated from measurements of $P_i$ liberation, determined by the method of TAUSSKY AND SCHORR[29]. The ($Na^+ + K^+$)-activated ATPase activity was estimated by subtracting the ATPase activity in the presence of 0.12 M NaCl and 5 mM MgATP from that in 0.10 M NaCl, 0.02 M KCl and 5 mM MgATP. In later experiments this marker was measured in the presence of 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA) because $Ca^{2+}$ inhibits the enzyme. This extra activity, seen when both $Na^+$ and $K^+$ were included together, was over 75 % inhibited by 1 $\mu$M ouabain. The azide-insensitive ATPase was calculated as the portion of the ATPase activity determined in 0.12 M NaCl, 5 mM MgATP and 0.1 mM $CaCl_2$ that was present under identical conditions except for the addition of 5 mM $NaN_3$. The $N_3^-$-insensitive $Ca^{2+}$-activated ATPase was calculated by subtracting the ATPase activity measured in 5 mM MgATP, 5 mM $NaN_3$ and 1 mM EGTA from that measured under identical conditions except that 0.1 mM $CaCl_2$ was substituted for the EGTA. The $Ca^{2+}$-ac-

TABLE I

MARKERS FOR SUBCELLULAR ORGANELLES

| *Organelle* | *Marker* |
|---|---|
| Plasma membrane | ($Na^+ + K^+$)-activated ATPase |
| Sarcoplasmic reticulum | $Ca^{2+}$-activated ATPase |
| | Oxalate-facilitated $Ca^{2+}$ uptake |
| Mitochondria | Succinate–cytochrome *c* reductase |
| | Cytochrome oxidase |
| | Azide-sensitive ATPase |
| | Oligomycin-sensitive ATPase |
| Lysosomes | Acid phosphatase |

tivated $N_3^-$-insensitive ATPase activity of these microsomes probably arises largely from the fragmented sarcoplasmic reticulum because the $Ca^{2+}$-activated ATPase associated with oxalate-facilitated $Ca^{2+}$ uptake is not significantly inhibited by these concentrations of $N_3^-$ (ref. 9).

$Ca^{2+}$ uptake was measured by methods previously described[9]. In most experiments, $Ca^{2+}$ uptake was measured at pH 7.0 in solutions containing 2.5 mM oxalate in which free $Ca^{2+}$ concentration was buffered at approx. 10 $\mu$M with 0.125 mM $^{45}$CaEGTA and 0.028 mM EGTA. For this reason, uptakes are less than those in experiments which we reported previously[9] in which the initial $CaCl_2$ concentration was 0.1 mM. At the present time there is some uncertainty as to the binding constant of EGTA for $Ca^{2+}$ in the buffers used in these experiments. We have used values for the dissociation constant of CaEGTA of 0.878 $\mu$M and 2.27 $\mu$M for pH 7.0 and 6.8, respectively[30]. In later experiments $Ca^{2+}$ uptake was measured at pH 6.8 where free $Ca^{2+}$ concentration was buffered as described in APPENDIX. The low initial level of $Ca^{2+}$ concentration would be expected to prevent significant $Ca^{2+}$ uptake by mitochondria as well as to prevent precipitation of calcium oxalate, which has a solubility product of 2.57 nM.

Succinate–cytochrome *c* reductase was determined by the method of RABINOWITZ AND DE BERNARD[31] at 25° in 0.5 mM KCN, 1 % bovine serum albumin, 0.03 mM oxidized cytochrome *c*, 10 mM succinate and 0.12 M $KH_2PO_4$ buffer adjusted to pH 7.4 with KOH. The preliminary incubation of 37°, needed to activate the enzyme in intact mitochondria, was omitted in these studies with microsomes.

Cytochrome oxidase was determined by the methods of COOPERSTEIN AND LAZAROW[32], and SMITH[33] as modified by RABINOWITZ *et al.*[34].

Acid phosphatase activity was determined with $\beta$-glycerol phosphate as substrate by the method of APPELMANS *et al.*[35]. Release of $P_i$ was linear with time during the 1-h period required for quantification of these low activities. Total activity was measured with protein samples suspended in distilled water and homogenized in a Waring blender for 3 min prior to the assay.

Refractive indices were determined in an Abbé refractometer (Zeiss Instrument Co.) and the readings converted to densities by the use of appropriate standards.

Densities were determined at 22° by weighing 10-ml volumetric flasks filled with various sucrose solutions.

Protein concentrations were determined by the biuret method. Bovine serum albumin was the standard.

Reagents. All inorganic reagents were obtained from Mallinckrodt Chemical Works. Sucrose (special enzyme grade) was obtained from Mann Research Laboratories. Tris–ATP was prepared from $Na_2ATP$ (Sigma Chemical Co.) as described earlier[9]. Cytochrome *c*, histidine and imidazole were obtained from Sigma Chemical Co.; succinic acid from Matheson Coleman and Bell Division of the Matheson Co.; $NaN_3$ from British Drug Houses and EGTA from Lamont Laboratories. Distilled water was deionized and redistilled from glass prior to use.

Density gradient ultracentrifugation was performed in an SW 25.1 rotor (Beckman Instruments Co.). Linear gradients were made with a Beckman Density Gradient Former and samples removed after centrifugation through a needle introduced through the top of the tube. The fractions were washed by the methods described below (see Zonal ultracentrifugation).

Samples were applied either to the top of the gradient in 10 % (w/v) sucrose as described previously[9] or in 45 % (w/v) sucrose placed at the bottom of the tube prior to formation of the gradient.

Zonal ultracentrifugation was carried out in a B14 Ti rotor (Beckman Inst. Co.). Gradients were introduced while the rotor was rotating at 3000 rev./min, after which the sample in concentrated sucrose was introduced through a syringe so that it was "below" the denser end of the gradient. In some experiments a more dense "cushion" was introduced by another syringe, though better results were obtained when the "cushion" was omitted. The rotor was then sealed and run at 45000 rev./min for 2 h. Introduction of the sample into the "bottom" of the gradient prevents contamination of the less dense fragmented sarcoplasmic reticulum by denser mitochondrial fragments that might be in transit from a sample introduced at the "top" of the gradient.

At the end of the high-speed centrifugation the rotor was decelerated to 3000 rev./min and the gradient removed from the center of the rotor by the introduction of 60 % (w/v) sucrose to the outside of the rotor. A series of 10-ml samples was collected, the protein concentrations estimated by measurements of absorbance at 280 nm and densities calculated from measurements of refractive index. Selected fractions were then diluted to 30 ml with water and collected by centrifugation for 1 h at 105000 × *g*. The pellets were washed once or twice with 10 % (w/v) sucrose containing 0.1 M Tris at pH 7.0 and stored in buffered 40 % (w/v) sucrose until use.

## RESULTS

### *Preparation of ventricular myocardium*

Hearts were excised from dogs anaesthetized with pentobarbital. Unselected dogs were used in early experiments, but it was found that an occasional heart was fibrotic and yielded microsomes with low biological activities. For this reason, and to avoid problems possibly arising from prior administration of drugs and antibiotics to non-conditioned dogs, hearts were taken only from healthy dogs whose initial immunization and preparation (deworming, *etc.*) had been completed.

After excision, beating hearts were washed in cold water and placed in crushed ice. The coronaries were perfused with the ice cold buffered sucrose used for homogenization (see below) and the epicardial vessels "stripped" in order to remove as much blood as possible. The heart was kept on ice for several minutes to allow uniform cooling. The atria, valves and great vessels were removed along with epicardial fat, and the heart ground in a small electric meat grinder.

### *Homogenization*

Ventricular myocardium was homogenized in a Waring blender equipped with a variable transformer. Blades were kept sharp, and replaced when nicks appeared. Homogenization was begun at low speed (50 V* for 30 sec) followed by intermediate speeds (75 V* for 30 sec then 100 V* for 15 sec) then high speed (120 V*).

Initial studies indicated that prolongation of the period of homogenization at 120 V* increased the yield of protein in the microsomal fraction (Fig. 1A). However, after approx. 30 sec of high speed homogenization (for this 38 g heart) the proportion

* Voltage delivered by variable transformer.

of $N_3^-$-insensitive ATPase decreased (Fig. 1C) as did that of the $N_3^-$-insensitive $Ca^{2+}$-activated ATPase (Fig. 1B), indicating an increasing proportion of mitochondrial fragments. The level of $(Na^+ + K^+)$-activated ATPase also increased as homogenization was prolonged (Fig. 1D) so that the duration of homogenization at high speed was kept below 10 sec per 10 g of myocardium. Because subsequent experience has demonstrated no advantages that could be attributed to the initial low-speed homogenization, it was omitted and the ground muscle subjected immediately to high-speed homogenization.

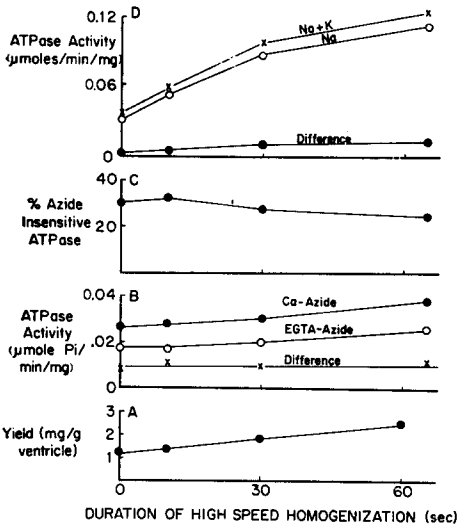
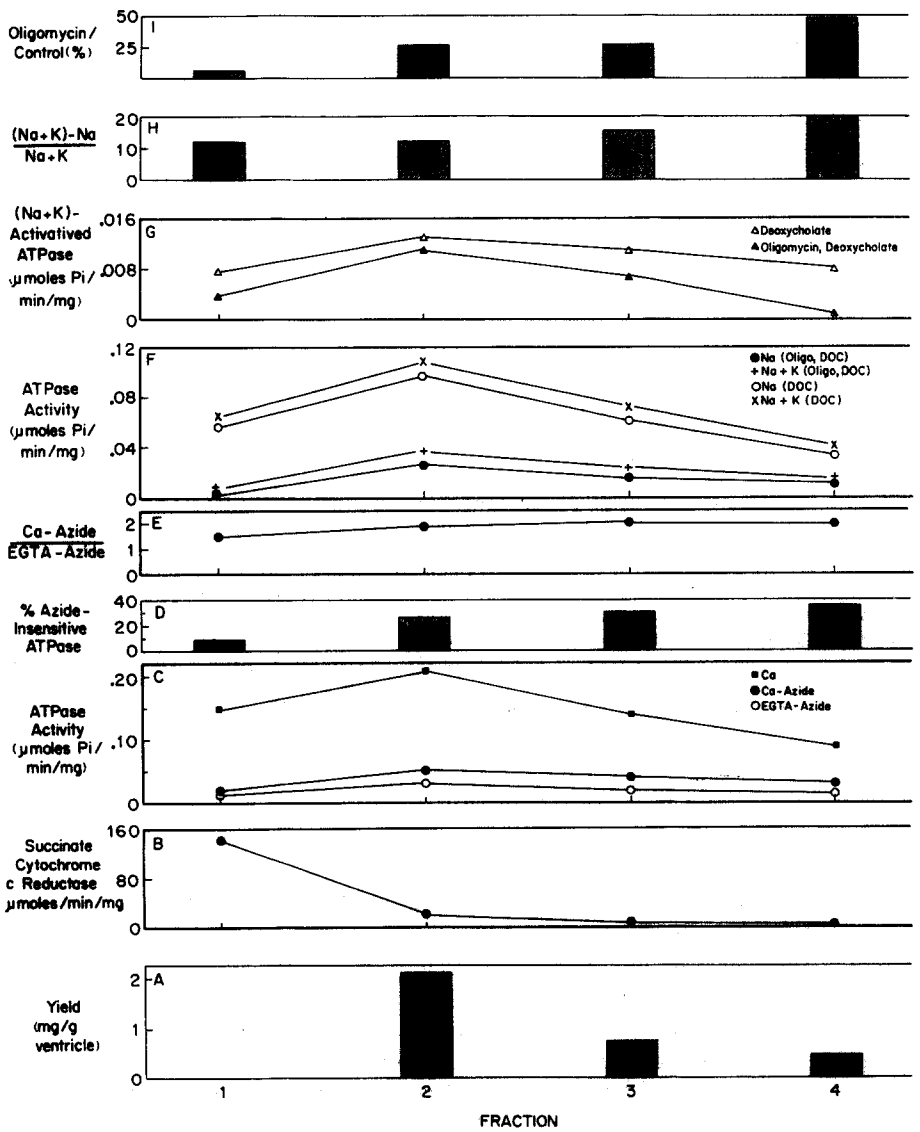



Fig. 1. Effects of duration of high-speed homogenization of ventricular myocardium on properties of unfractionated microsomes. A. Yield of microsomes (mg microsomal protein per g wet wt. of ground ventricle) collected by centrifugation at 145000 × *g* for 90 min after a preliminary centrifugation at 12000 × *g* for 10 min. B. ATPase activities measured in the presence of 0.1 mM $CaCl_2$ (●), or with 1.0 mM EGTA (○) at pH 7.0 with 0.2–0.4 mg/ml microsomal protein, 0.12 M NaCl, 19 mM histidine, 5 mM $NaN_3$ and 5 mM MgATP at 25°. The difference between the activities in $CaCl_2$ and EGTA (×) represents the $N_3^-$-insensitive, $Ca^{2+}$-activated ATPase. C. Fraction of the $Ca^{2+}$-activated ATPase activity that is insensitive to $N_3^-$, measured at pH 7.0 with 0.2–0.4 mg/ml microsomal protein in 0.12 M NaCl, 19 mM histidine, 0.1 mM $CaCl_2$ and 5 mM MgATP at 25°. Reactions were carried out in the presence of absence of 5 mM $NaN_3$. D. $(Na^+ + K^+)$-activated ATPase activity of deoxycholate-treated (0.02%) microsomes. Reactions were carried out at pH 7.0 in 0.12 M NaCl (○), or 0.10 M NaCl + 0.02 M KCl (×) with 0.125–0.250 mg/ml microsomal protein, 19 mM histidine and 5 mM MgATP at 25°. The difference between the ATPase activities in NaCl + KCl and in NaCl alone (●), is also plotted.

*Differential centrifugation*

To prepare microsomes having a maximal $Ca^{2+}$-activated ATPase activity, studies were carried out with fractions collected by centrifugation at various speeds (Fig. 2). The yield of protein decreased as each supernatant was subjected to higher centrifugal forces (Fig. 2A). Succinate–cytochrome *c* reductase (Fig. 2B) was maximal and the proportion of $N_3^-$-insensitive ATPase (Fig. 2D) was minimal in the "mitochondrial" fraction (Fraction 1) collected at 18000 × *g*. Succinate–cytochrome *c* reductase activity decreased and $N_3^-$-insensitive ATPase activity increased markedly in the "heavy microsomes" collected at 30000 × *g* (Fraction 2), but there was little

additional change in these activities when the microsomes were collected at higher centrifugal forces. On the other hand, the level of ATPase activity was less in the lighter fractions (Fig. 2C). The absolute level of the ($Na^+ + K^+$)-activated ATPase seen in 0.02% deoxycholate in the presence or absence of 4 $\mu$g oligomycin per mg of protein (Fig. 2F) was maximal in the "heavy microsomes" (Fig. 2G), but the proportion of this plasma membrane ATPase was highest in the lightest microsomal fraction (Fig. 2H). The oligomycin-insensitive ATPase, like the $N_3^-$-insensitive ATPase, was greatest in the ligher fractions (Fig. 2I). On the basis of these findings it was decided that Fractions 2 and 3 would be appropriate for further purification on sucrose gradients. All subsequent studies were therefore carried out with microsomes collected



by centrifugation at 47000 × *g* for 60 min after prior centrifugation at 15000 × *g* for 30 min.

*Use of LiBr in density gradient centrifugation*

Because preliminary studies with density gradient ultracentrifugation in sucrose indicated that aggregation had occurred between microsomal elements derived from different subcellular organelles (see below), various dispersing agents were used in the density gradients. Both urea (1 M) and cholic acid (0.2 mg/ml) were used in preliminary experiments. While neither greatly reduced $Ca^{2+}$ uptake if the microsomes were kept in concentrated sucrose (ref. 36, see below), our limited experience indicated that these agents had no advantages over LiBr.

Microsomes exposed in isotonic sucrose to structure-disrupting agents such as LiBr rapidly lost their ability to take up $Ca^{2+}$. To preserve activity it was necessary to keep the microsomes in hypertonic sucrose during the periods that they were in contact with LiBr[36]. Because very low concentrations of LiBr in the assay mixtures inhibited both $Ca^{2+}$ uptake and succinate–cytochrome *c* reductase, the salt was washed out by addition of 2 vol. of distilled water to the microsomes in 40 % (w/v) sucrose and LiBr followed by centrifugation at 105000 × *g* for 1 h. After the pellet was homogenized gently in buffered 10 % (w/v) sucrose and centrifuged again at 150000 × *g* for 20 min, the final pellet was suspended in 40 % (w/v) sucrose to preserve activity[36]. Under these conditions, overnight exposure on ice to concentrations of LiBr below 1.0 M did not inhibit the various markers (Fig. 3). Comparison of the yields of protein after exposure to LiBr provided no evidence that higher concentrations of this salt solubilized significant amounts of protein (Fig. 3A). Cytochrome oxidase activity was somewhat more resistant to LiBr than was succinate–cytochrome *c* reductase (Fig. 3B). The ability to take up $Ca^{2+}$ was well preserved, and an increased $Ca^{2+}$ uptake seen after exposure to 0.5–1.0 M LiBr (Fig. 3C) was regularly seen. The proportion of $N_3^-$-

← Fig. 2. Properties of different fractions of homogenized myocardium. Fractions were collected (from the supernatant obtained after centrifugation at 1500 × *g* for 5 min) by centrifugation for 10 min at 18000 × *g* (Fraction 1), 60 min at 30000 × *g* (Fraction 2, starting material was Fraction 1), 60 min at 45000 × *g* (Fraction 3, starting material was Fraction 2), and 60 min at 60000 × *g* (Fraction 4, starting material was Fraction 3). A. Yield of protein. The yield in Fraction 1 was very much higher than those of Fractions 2–4. B. Succinate–cytochrome *c* reductase. Reactions were carried out with approx. 0.05 mg protein per ml. C. ATPase activity measured in (i) 0.1 mM $CaCl_2$ without (■) and (ii) in the presence of 5 mM $NaN_3$ (●), and (iii) in 1.0 mM EGTA and 5 mM $NaN_3$ (○). All reaction mixtures contained 0.25 mg/ml protein, 0.12 M NaCl, 19 mM histidine and 5 mM MgATP at pH 7.0. Reactions were carried out at 25°. D. Fraction of the $Ca^{2+}$-activated ATPase activity that is insensitive to $N_3^-$, determined by dividing (ii) by (i) (Panel C). E. Fraction of $N_3^-$-insensitive ATPase activity that is activated by $Ca^{2+}$, measured by dividing (ii) by (iii) (Panel C). F. $Na^+$ and ($Na^+ + K^+$)-activated ATPase activities measured in the absence (○, ×) and presence (●, +) of 4 μg oligomycin (oligo) per mg protein. Reactions were carried out at pH 7.0 in 0.02 % deoxycholate (DOC), 19 mM histidine and 5 mM MgATP with 0.25 mg/ml protein and 0.12 M NaCl (○, ●) or 0.10 NaCl + 0.12 M KCl (×, +). Measurements were made at 25°. G. ($Na^+ + K^+$)-activated ATPase activities in the absence (△) or presence (▲) of oligomycin. Data obtained by subtracting the values for $Na^+$-activated ATPase from ($Na^+ + K^+$)-activated ATPase in Panel F. H. Proportion of ($Na^+ + K^+$)-activated ATPase activity calculated by subtracting the values for $Na^+$-activated ATPase (○) from ($Na^+ + K^+$)-activated ATPase (×) and dividing this value by the ($Na^+ + K^+$)-activated ATPase (×) (data in Panel F). I. Oligomycin sensitivity of $Na^+$-activated ATPase calculated by dividing the $Na^+$-activated ATPase activity in the presence of oligomycin (●) by the $Na^+$-activated ATPase activity in the absence of oligomycin (○) (data in Panel F).

insensitive ATPase activity was increased by LiBr (Fig. 3E) due to reduction in the $N_3^-$-sensitive ATPase (Fig. 3D), but the $Ca^{2+}$-activated ATPase manifest in $N_3^-$ was not inhibited by < 1.0 M LiBr (Fig. 3F). Although total ($Na^+ + K^+$)-activated ATPase was not significantly altered by LiBr (Fig. 3H), exposure to high concentrations of the salt increased the proportion of this plasma membrane ATPase activity (Fig. 3G) by inhibiting other ATPase (see Fig. 3D).

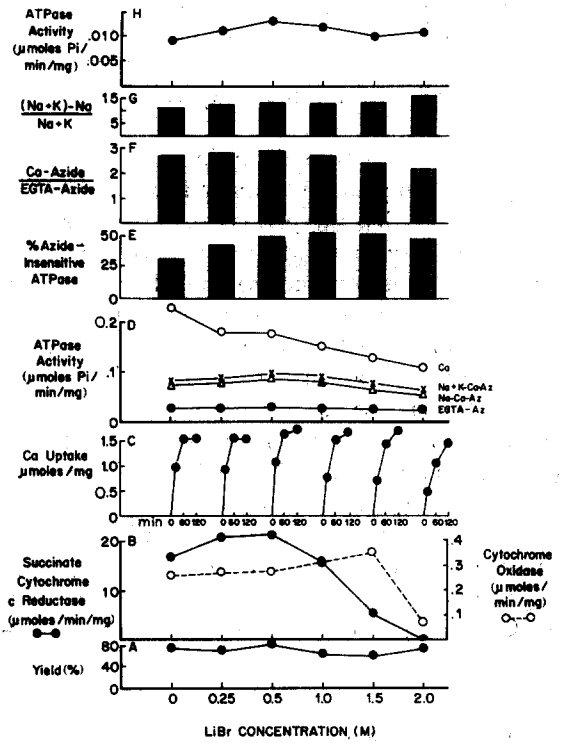



Fig. 3. Effects of LiBr pretreatment on cardiac microsomes. Cardiac microsomes were exposed to various concentrations of LiBr overnight at 0°, after which the microsomes were collected and washed (see METHODS). The following analyses were then carried out: (A) yield (% of starting material); (B) cytochrome oxidase (○) and succinate–cytochrome *c* reductase (●); (C) $Ca^{2+}$ uptake from $1 \cdot 10^{-5}$ M $Ca^{2+}$ at 25° in 0.12 M NaCl, with values plotted for the uptakes at 20, 60, and 120 min; (D) ATPase activity of 0.25 mg/ml protein in the presence of 0.12 M NaCl, 0.1 mM $CaCl_2$ (○), 0.10 M NaCl, 0.02 M KCl, 0.1 mM $CaCl_2$ and 5 mM $NaN_3$ (×), 0.12 M NaCl, 0.1 mM $CaCl_2$ and 5 mM $NaN_3$ (△), and 0.12 M NaCl, 1 mM EGTA and 5 mM $NaN_3$ (●); (E) the proportion of $N_3$-insensitive $Ca^{2+}$-activated ATPase; (F) the proportion of the $N_3^-$-insensitive ATPase that is activated by $Ca^{2+}$; (G) the proportion of $N_3^-$-insensitive ATPase represented by the ($Na^+ + K^+$)-ATPase; and (H) the ($Na^+ + K^+$)-activated ATPase in the presence of $N_3^-$, calculated by subtracting the values for NaCl alone (△, Panel D) from those in the presence of NaCl and KCl (×, Panel D).

*Comparison of density gradients in the presence and absence of LiBr*

Ultracentrifugation of cardiac microsomes in linear sucrose density gradients effected a limited fractionation. Although distinct bands formed after 2 h at 25000 rev./min in the SW 25.1 rotor (Fig. 4) considerable aggregation was noted visually. Aggregation was more striking after zonal ultracentrifugation at 45000 rev./min (see below).

Preliminary studies in which microsomes were suspended in solutions of different sucrose concentration and centrifuged in angle-head rotors indicated that this procedure was of little value in the isolation of mitochondria-free fragmented sarcoplasmic reticulum (Fig. 5). Decreasing sucrose concentration, as expected, increased the proportion of protein recovered in the pellet (Fig. 5A) and reduced somewhat

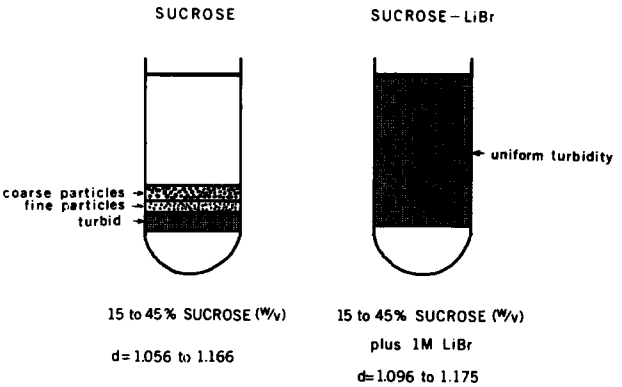



Fig. 4. Comparison of sucrose density gradients with (right) and without (left) 1.0 M LiBr. 3 ml of cardiac microsomes in 45% (w/v) sucrose, containing 16 mg of protein, were applied to the bottom of the gradient, and the rotor accelerated to 25000 rev./min for 2 h. The resulting patterns, observed visually, are diagrammed and the range of sucrose concentrations and gradient densities are shown at the bottom of the diagrams.

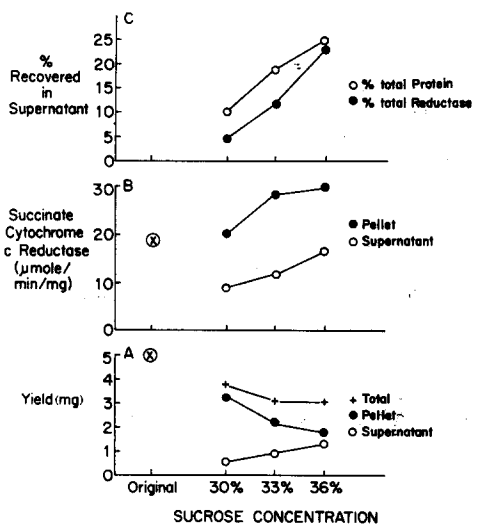



Fig. 5. Effects of centrifugation of cardiac microsomes in various sucrose concentrations on the distribution of succinate–cytochrome *c* reductase. A. Yield of the supernatants (○), pellets (●) and their sums (+) after centrifugation for 1 h in 30, 33 and 36% (w/v) sucrose at 200000 × *g*. Subsequently the supernatant was diluted and protein collected by centrifugation for 1 h at 200000 × *g*. The initial sample washed once in 10% (w/v) sucrose–5 mM histidine at pH 7.4 and collected at 105000 × *g* contained 5 mg of protein (×). B. Specific activity of succinate–cytochrome *c* reductase of the pellet (●) and supernatant (○) fractions compared with that of original microsomes (×). C. Percent recovery of protein (○) and reductase activity (●) in the supernatant fraction.

the specific activity of succinate cytochrome *c* reductase in the supernatant (Fig. 5B)*. From a practical standpoint, however, more effective removal of mitochondrial fragments by sedimentation in the less dense sucrose solutions reduced the yield of protein in the supernatant almost as much as it did the recovery of the mitochondrial marker (Fig. 5C).

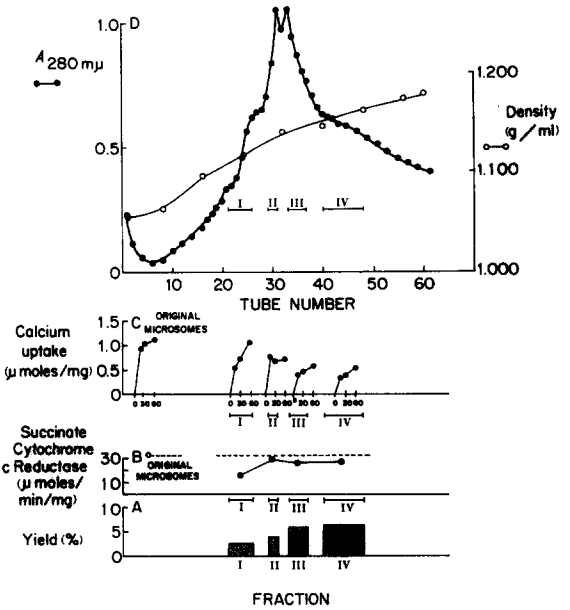



Fig. 6. Zonal centrifugation of cardiac microsomes in sucrose alone. A. Yield in various fractions expressed as percent of initial protein. The width of the different bars indicates the number of tubes pooled to constitute the sample (see Panel D). B. Succinate–cytochrome *c* reductase activities. C. $Ca^{2+}$ uptake from $1 \cdot 10^{-5}$ M $Ca^{2+}$ after 15, 30 and 60 min measured at 25° in reaction mixtures containing 0.025 mg/ml protein, 0.12 M NaCl, 0.04 M histidine, 2.5 mM Tris–oxalate and 5 mM MgATP at pH 6.8. D. Distributions of protein, measured as absorbance at 280 mμ (●) and density, calculated from refractive indices (○).

In spite of the clear banding seen when cardiac microsomes were centrifuged in sucrose density gradients in the swinging-bucket rotor (see Fig. 4), there was relatively little fractionation of either succinate–cytochrome *c* reductase or $Ca^{2+}$ uptake. After zonal centrifugation in sucrose alone (Fig. 6), the level of succinate–cytochrome *c* reductase in the least dense fraction (Fraction 1) was half that of the original microsomes (Fig. 6B) while there was little increase in $Ca^{2+}$ uptake (Fig. 6C, see also ref. 9).

*Zonal centrifugation in the presence of LiBr*

Comparison of sucrose density gradient separations of cardiac microsomes in the presence and absence of LiBr indicated that the salt abolished the gross aggregates seen with sucrose alone. LiBr also caused the turbidity to appear at lower densities

* The apparent paradox in Fig. 5B of the reductase rising in both supernatant and pellet at higher sucrose concentrations is readily explained because the proportion of protein material in the supernatant increased, presumably due to greater mitochondrial contamination, while the pellet which contained less total protein had less microsomal contamination at the higher sucrose concentrations.

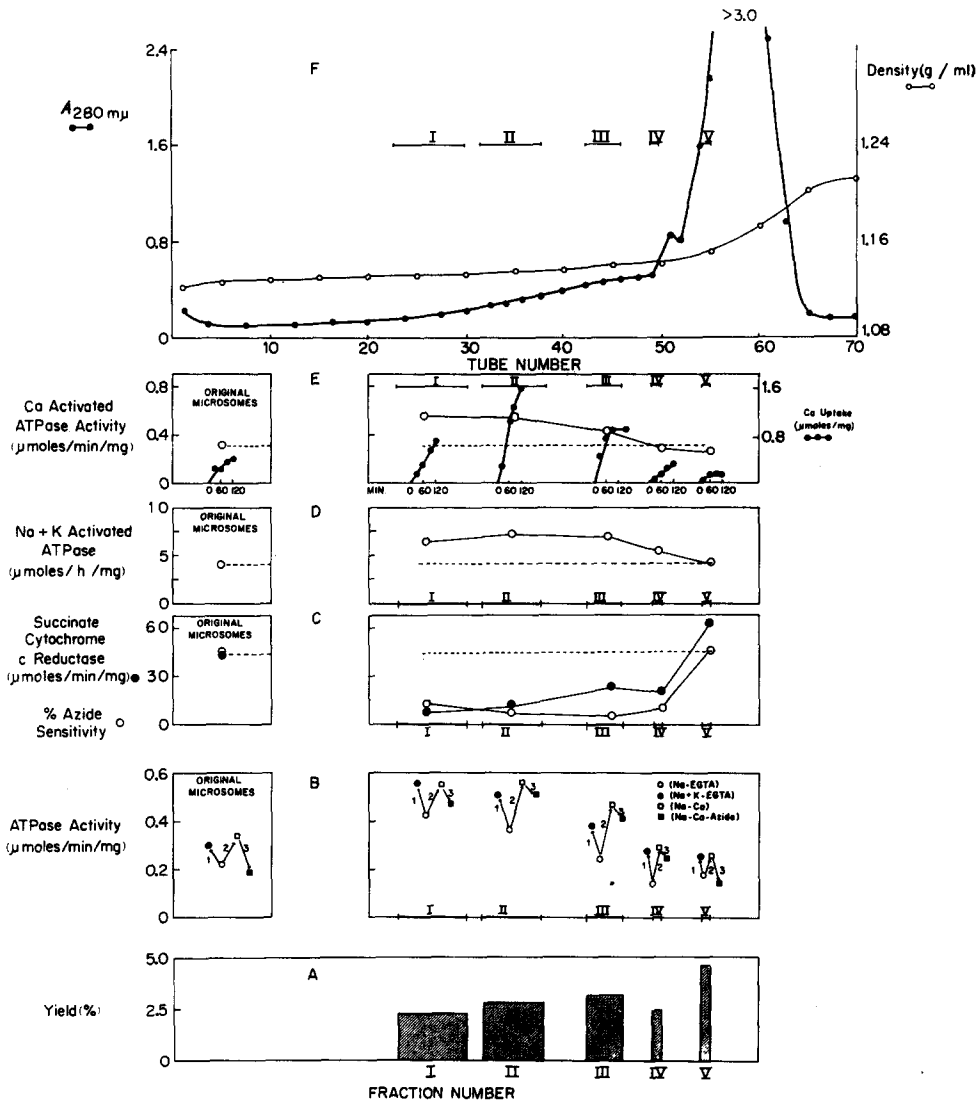



Fig. 7. Zonal centrifugation of cardiac microsomes in sucrose containing 0.5 M LiBr. A. Yields of various fractions expressed as percent of initial protein. The width of the different bars indicates the number of tubes pooled to constitute the sample (Panel F). B. ATPase activities measured in 0.12 M NaCl and $1 \cdot 10^{-8}$ M $Ca^{2+}$ (CaEGTA buffer) (○), in 0.10 M NaCl, 0.02 M KCl and $1 \cdot 10^{-8}$ M $Ca^{2+}$ (●), in 0.12 M NaCl and $1 \cdot 10^{-5}$ M $Ca^{2+}$ (CaEGTA buffer) (□), and in 0.12 M NaCl, $1 \cdot 10^{-5}$ M $Ca^{2+}$ and 5 mM $NaN_3$ (■). All reactions were carried out at 25° with 0.05 mg/ml protein in 3 mM MgATP and 0.04 M imidazole at pH 6.8. C. Succinate–cytochrome *c* reductase (●) measured with approx. 0.05 mg/ml protein, and $N_3^-$ sensitivity (○) calculated from the data in Panel B by dividing the ATPase activity in 0.12 M NaCl, $1 \cdot 10^{-5}$ M $Ca^{2+}$, 5 mM $NaN_3$ (■ in Panel B) by the activity in 0.12 M NaCl, $1 \cdot 10^{-5}$ M $Ca^{2+}$ (□ in Panel B). This corresponds to the arrow labeled 3 in Panel B. D. ($Na^+ + K^+$)-activated ATPase activity calculated by subtracting the $Na^+$-activated ATPase (○ in Panel B) from the ATPase activity in $Na^+ + K^+$ (● in Panel B). This corresponds to the arrow labeled 1 in Panel B. E. $Ca^{2+}$-activated ATPase (○) calculated by subtracting the $Na^+$-activated ATPase at $1 \cdot 10^{-8}$ M $Ca^{2+}$ (○ in Panel B), from the $Ca^{2+}$-activated ATPase in the presence of $Na^+$ (□ in Panel B). This corresponds to the arrow labeled 2 in Panel B. $Ca^{2+}$ uptake measured from $1 \cdot 10^{-5}$ M $Ca^{2+}$ after 30, 60, 90 and 120 min (●) was measured in reaction mixtures containing 0.025 mg/ml protein, 0.12 M NaCl, 2.5 mM Tris–oxalate, 5 mM MgATP and 0.04 M imidazole at pH 6.8. F. Distributions of protein, measured as absorbance at 280 mμ (●) and density, calculated from refractive indices (○).

than when microsomes were centrifuged in sucrose alone (Fig. 4). This salt is effective, therefore, in dispersing the microsomes.

When zonal centrifugation of cardiac microsomes was carried out with 0.5 M LiBr present in the sucrose density gradient, useful separations could be achieved (Fig. 7). The sarcoplasmic reticulum markers were concentrated in an area of the gradient (Fraction II) which represents only 5 % of the total protein although they were not confined to this area (Fig. 7A). The distribution of these markers in the gradient did not correspond to an obvious protein peak (Fig. 7F). The extent of $Ca^{2+}$ uptake, the major marker for sarcoplasmic reticulum (Fig. 7E), was increased several fold in Fraction II. The initial rate of $Ca^{2+}$ uptake was approximately doubled (Fig. 8) and the $Ca^{2+}$-activated ATPase activity was approximately twice as high in Fraction II as in the starting material (Fig. 7E). The markers for mitochondria were concentrated in Fraction V, at the denser end of the gradient*. The levels of succinate–cytochrome *c* reductase and $N_3^-$-sensitive ATPase in Fraction II were approx. 1/5 those of the starting material (Fig. 7C). For the reductase, the value of 7.5 μmoles/min per mg is approx. 3 % that of a crude mitochondrial preparation.

$(Na^+ + K^+)$-activated ATPase was maximal in Fractions I and II (Figs. 7B and 7D). That the plasma membrane marker appeared in a less dense region of the gradient than that in which the sarcoplasmic reticulum markers are found was a common finding in these experiments.

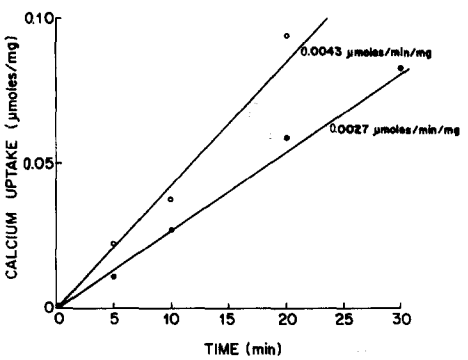



Fig. 8. Comparison of the initial rates of $Ca^{2+}$ uptake by crude microsomes (●) and an active fraction (○) purified by zonal centrifugation in 0.5 M LiBr (corresponding to Fractions I and II in Fig. 7). The reaction mixture contained 0.25 mg protein per ml, 0.12 M NaCl, 2.5 mM Tris–oxalate, 5 mM MgATP, 0.025 mM $^{45}CaCl_2$, 0.189 mM EGTA (free $[Ca^{2+}] = 3 \cdot 10^{-6}$ M) and 0.1 M histidine at pH 6.8.

*Lysosomes*

Relatively little acid phosphatase activity was found in these microsomes. The rate of $P_i$ liberation of the crude microsomes was approx. 0.090 μmole/h per mg protein. Due to lack of adequate quantities of material, assays could not be carried out on fractions from the gradients.

* Only the "top" of this fraction was sampled because previous experience with other gradients showed much higher levels of mitochondrial markers at the denser end of this protein peak. This area of the gradient, which corresponded in position to the applied sample, was visibly pink.

## DISCUSSION

The present findings are in accord with the often expressed view that the cardiac microsomal fraction is a heterogeneous mixture of membrane elements derived from a large number of subcellular membrane structures. An additional content of actomyosin might be present, though brief homogenization of the muscle in sucrose would be expected to minimize such a contamination[37]. While we cannot exclude an actomyosin contamination in the original microsomes, the contractile proteins would not appear in the fractions prepared by zonal ultracentrifugation because they are considerably more dense ($\bar{v}$ = approx. 0.7) than the lipoprotein membranes, and would not float upwards during centrifugation.

The amount of microsomal protein, as would be expected, is increased by prolonged homogenization of the muscle (Fig. 1). As noted previously[38], homogenization in the Waring blender disrupts the mitochondria so that the increased $N_3^-$-sensitive ATPase activity found in the microsomal fraction collected after prolonged homogenization reflects a greater amount of mitochondrial fragments.

Several investigators have found that markers for skeletal sarcoplasmic reticulum are most abundant in the "heavy" microsomes[27,39], a finding that is also noted in the case of the cardiac microsomes (Fig. 2). However, the "heavy" cardiac microsomes also contain significantly more mitochondrial fragments than do the "lighter" microsomes (Fig. 2), as evidenced by higher succinate–cytochrome *c* reductase and greater $N_3^-$ and oligomycin sensitivities of their ATPase activity. The proportion of plasma membrane fragments appears to be higher in the "light" microsomes, especially in the fraction collected between 45000 and 60000 $\times g$.

Although some purification of cardiac sarcoplasmic reticulum can be achieved by centrifugation in sucrose density gradients[4,9,13] and some reduction in the specific activity of succinate–cytochrome *c* reductase can be achieved by high-speed centrifugation in 30% (w/v) sucrose (Fig. 5), these methods fail to remove more than half of the reductase (Figs. 5 and 6) while effecting little enhancement of $Ca^{2+}$ uptake (Fig. 5)[9,*]. This failure to achieve optimal fractionation represents to some extent the overlapping of various markers which have fairly broad distributions in sucrose density gradients (see ref. 25), as well as aggregation of the microsomal particles that is evident on visual inspection of the gradients (Fig. 4). For these reasons it was decided to utilize solubilizing agents to improve the efficiency of separation of the various markers. Preliminary experiments with urea and cholate indicated that these agents had no advantages over LiBr, which has been used extensively in this work.

Exposure of microsomes to concentrations of LiBr up to 1.0 M in isotonic sucrose rapidly impairs the ability to take up $Ca^{2+}$ (ref. 36), whereas the same concentrations of LiBr have no detrimental effects on this activity when the microsomes are suspended in 40% (w/v) sucrose (Fig. 3)[36]. LiBr at concentrations of 0.5–1.0 M also has no effects on cytochrome oxidase, succinate–cytochrome *c* reductase or the ($Na^+ + K^+$)-activated ATPase when microsomes are kept in 40% (w/v) sucrose during exposure to the salt (Fig. 3). While total $Ca^{2+}$-activated ATPase activity is slightly reduced, $<$ 1.0 M LiBr causes no significant attenuation of the $Ca^{2+}$-activated $N_3^-$-insensitive

* The data in this paper and our previous study[9] are not comparable quantitatively because of different initial $Ca^{2+}$ concentrations.

ATPase activity (Fig. 3). These findings indicate the suitability of this salt for fractionation of cardiac microsomes as long as hypertonic sucrose concentrations are maintained[36]. This is possible at all stages of the exposure to LiBr except during zonal ultracentrifugation, when sarcoplasmic reticulum fragments migrate to a density that fails to provide full protection against the structure-disrupting actions of the salt.

Zonal ultracentrifugation in LiBr confirms previous studies that indicate the heterogeneity of cardiac microsomes[14–16]. The markers for sarcoplasmic reticulum are concentrated in an area of the gradient that corresponds to less than 5 % of the initial protein, a finding that is in accord with electron microscopic evidence that the heart's sarcoplasmic reticulum is poorly developed[40–43]. The high cardiac content of mitochondria is reflected in these microsomes by the appearance of high activities of mitochondrial markers in a major protein peak at the denser region of the gradients (Fig. 7). The presence of fragmented plasma membranes, demonstrated by other studies of cardiac microsomes[17–23], is confirmed by the significant ($Na^+ + K^+$)-activated ATPase activity which is concentrated in the less dense region of the gradient. An acid phosphatase attributable to lysosomal membranes could also be identified in these microsomes, although monamine oxidase activity (benzylamine or kynuramine a substrate), a marker for the mitochondrial outer membrane could not be quantified. The slight separation of the plasma membrane marker from the fragmented sarcoplasmic reticulum shown in fig. 7, with the former appearing to be less dense than the latter, was a common finding in these studies.

If, in fact, the fragmented sarcoplasmic reticulum comprises only approx. 5 % of the microsomal protein, effective fractionation should enhance the markers for this cell organelle by a factor of 20. As shown in Fig. 7, the actual enhancement of $Ca^{2+}$-activated ATPase is only 2-fold, the enhancement of total $Ca^{2+}$ uptake is about 4-fold, while that of the rate of uptake is 2–4-fold (see also fig. 8, in which a broader "cut" of the zonal distribution was used to obtain enough material for study). The low enhancement of $Ca^{2+}$-activated ATPase probably reflects the relative non-specificity of this activity in that mitochondrial and other ATPases exhibit $Ca^{2+}$ activation under the conditions of these experiments, as evidenced by the high $Ca^{2+}$-activated ATPase in Fraction V. Furthermore, the $Ca^{2+}$ uptakes seen in Fraction II (Fig. 7) probably reflect some impairment of activity which occurred during zonal purification. This procedure, which takes 2 h with an additional 1 h to unload the rotor and analyze the fractions, requires exposure of the microsomes to LiBr under conditions that tend to damage the sensitive sarcoplasmic reticulum markers, *viz.* in approx. 25 % (w/v) sucrose at temperatures above 0°.

This technique can provide small quanties of highly purified fragmented sarcoplasmic reticulum, but the yield of active material is low. These probably reflect the relatively small proportion of microsomal protein that is derived from the sarcoplasmic reticulum more than defects in the methods used for purification.

## APPENDIX

### *Calculation of free $Ca^{2+}$ concentration*

In these experiments we have attempted to stabilize the concentration of ionized calcium in solutions containing ATP and EGTA, both of which bind strongly to $Ca^{2+}$, as well as $Mg^{2+}$ which binds to ATP. The binding constants for these interactions have

been measured[30,44], and are strongly dependent on pH so that stabilization of free $Ca^{2+}$ concentration requires the careful adjustment and maintenance of pH. In this regard it should be noted that buffers commonly used in studies of this sort are inadequate when significant ATP hydrolysis takes place[45].

In the present study we have used 5 mM MgATP (equimolar concentrations of $MgCl_2$ and Tris–ATP) as a substrate. The ionic species of ATP which binds strongly to both $Ca^{2+}$ and $Mg^{2+}$ is $ATP^{4-}$. At pH 6.8, the relative abundances of $ATP^{3-}$ and $ATP^{4-}$ can be calculated from the dissociation constant of the fourth $H^+$ of the nucleotide, which is $10^{-6.97}$ (ref. 44).

The ratio $[ATP^{4-}]/[ATP^{3-}]$ will be 0.63 so that

$$[ATP^{3-}] = 1.59[ATP^{4-}] \quad (1)$$

In $5 \cdot 10^{-3}$ M MgATP,

$$[ATP^{4-}] + [ATP^{3-}] + [MgATP^{2-}] = 5 \cdot 10^{-3} \quad (2)$$

and

$$[Mg^{2+}] + [MgATP^{2-}] = 5 \cdot 10^{-3} \quad (3)$$

From Eqns. 1 and 2:

$$[ATP^{4-}] + 1.59[ATP^{4-}] + [MgATP^{2-}] = 5 \cdot 10^{-3}$$

so

$$2.59[ATP^{4-}] + [MgATP^{2-}] = 5 \cdot 10^{-3} \quad (4)$$

solving for $[ATP^{4-}]$ (Eqn. 4) gives:

$$[ATP^{4-}] = 1.93 \cdot 10^{-3} - 0.386\,[MgATP^{2-}] \quad (5)$$

Using the binding constant for $Mg^{2+}$ and $ATP^{4-}$ given in ref. 44:

$$\frac{[MgATP^{2-}]}{[Mg^{2+}]\,[ATP^{4-}]} = 8.8 \cdot 10^4 \quad (6)$$

and the equation for total $Mg^{2+}$ (Eqn. 3), the concentration of $MgATP^{2-}$ can be determined from the equation:

$$\frac{[MgATP^{2-}]}{(5 \cdot 10^{-3} - [MgATP^{2-}])\,(1.93 \cdot 10^{-3} - 0.386[MgATP^{2-}])} = 8.8 \cdot 10^4 \quad (7)$$

Solution of the quadratic equation generated from Eqn. 7 gives:

$$[MgATP^{2-}] = 4.63 \cdot 10^{-3} \quad (8)$$

and from Eqn. 2:

$$[ATP^{4-}] + [ATP^{3-}] = 5 \cdot 10^{-3} - 4.63 \cdot 10^{-3}$$

so that the free ATP concentration is $3.7 \cdot 10^{-4}$ M, of which $ATP^{4-}$ is present at a concentration of $1.4 \cdot 10^{-4}$ M (Eqn. 1). Thus, in $5 \cdot 10^{-3}$ M $MgATP^{2-}$ at pH 6.8, the concentration of $ATP^{4-}$ is $1.4 \cdot 10^{-4}$ M. This concentration of $ATP^{4-}$ will be buffered by the $5 \cdot 10^{-3}$ M $MgATP^{2-}$.

To achieve buffered free $Ca^{2+}$ concentrations above $1 \cdot 10^{-6}$ M, it is necessary to add only $CaCl_2$. The binding constant for $CaATP^{2-}$ is given in ref. 30 as:

$$\frac{[CaATP^{2-}]}{[Ca^{2+}][ATP^{4-}]} = 3.15 \cdot 10^4 \quad (9)$$

For an amount of $CaCl_2$ added to give a total $Ca^{2+}$ concentration $= y$: $[Ca^{2+}] + [CaATP^{2-}] = y$, so

$$[Ca^{2+}] = y - [CaATP^{2-}] \quad (10)$$

From Eqn. 9, and the buffered concentration of $ATP^{4-}$ of $1.4 \cdot 10^{-4}$ (see above) the ratio $[CaATP^{2-}]/[Ca^{2+}] = 4.41$, and from Eqn. 10 it follows that:

$$\frac{[CaATP^{2-}]}{y - [CaATP^{2-}]} = 4.41 \quad (11)$$

Solving for $[CaATP^{2-}]$ gives:

$$[CaATP^{2-}] = 0.81\, y \quad (12)$$

Thus, for any addition of $CaCl_2$ to a final concentration $= y$, from Eqns. 10 and 12, the free $Ca^{2+}$ concentration is readily calculated to be $0.19\, y$.

This ATP buffer system is useful to stabilize $Ca^{2+}$ concentrations above $1 \cdot 10^{-6}$ M ($y = 5.26 \cdot 10^{-6}$ M) when $^{45}Ca^{2+}$ uptake or binding is being measured. The level of added $^{45}Ca^{2+}$ at $1 \cdot 10^{-6}$ M will be approx. 5 times the level of unlabeled $Ca^{2+}$ present in the reaction mixtures as a contaminant (see ref. 9) so that at these low $Ca^{2+}$ concentrations significant errors in relating changes in $^{45}Ca^{2+}$ concentration to changes in total $Ca^{2+}$ concentration will appear. Thus, it becomes necessary to add more $^{45}Ca^{2+}$ and to use EGTA buffers to achieve free $Ca^{2+}$ concentrations below $1 \cdot 10^{-6}$ M.

In order to simplify the preparation of $Ca^{2+}$ buffers in solutions containing both $ATP^{4-}$ and EGTA, we arbitrarily chose a concentration of $2.5 \cdot 10^{-5}$ M $^{45}CaCl_2$ for $y$. At pH 6.8, the binding constant for CaEGTA is $4.4 \cdot 10^5$ (ref. 30) so:

$$\frac{[CaEGTA]}{[Ca^{2+}][EGTA]} = 4.4 \cdot 10^5 \quad (13)$$

and

$$[Ca^{2+}] + [CaATP^{2-}] + [CaEGTA] = 2.5 \cdot 10^{-5}$$

This latter equation can be simplified using the relationship between the first two terms expressed in Eqn. 11, where $[CaATP^{2-}] = 4.41\, [Ca^{2+}]$, so that:

$$5.41\, [Ca^{2+}] + [CaEGTA] = 2.5 \cdot 10^{-5} \quad (14)$$

For any addition of EGTA to a total final concentration of $z$,

$$[EGTA] + [CaEGTA] = z$$

Substituting $(z - [CaEGTA])$ for [EGTA] in Eqn. 13 gives:

$$\frac{[CaEGTA]}{[Ca^{2+}](z - [CaEGTA])} = 4.4 \cdot 10^5 \quad (15)$$

From Eqn. 14, the concentration of CaEGTA can be expressed in terms of $[Ca^{2+}]$:

$$[CaEGTA] = 2.5 \cdot 10^{-5} - 5.41\, [Ca^{2+}] \quad (16)$$

so that from Eqns. 15 and 16 an expression can be constructed to contain only the terms $z$ and $[Ca^{2+}]$:

$$\frac{(2.5\cdot10^{-5} - 5.41\,[Ca^{2+}])}{[Ca^{2+}]\,[z - (2.5\cdot10^{-5} - 5.41\,[Ca^{2+}])]} = 4.4\cdot10^{5} \quad (17)$$

Solving Eqn. 17 for $z$ in terms of $[Ca^{2+}]$ gives:

$$z = \frac{2.5\cdot10^{-5} + 5.59\,[Ca^{2+}] - 23.8\cdot10^{-5}\,[Ca^{2+}]^2}{4.4\cdot10^{5}\,[Ca^{2+}]} \quad (18)$$

Substituting desired values for $[Ca^{2+}]$ will give $z$, the amount of [EGTA] to be added to achieve the desired free $Ca^{2+}$ concentration.

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