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# ISOLATION AND CHARACTERIZATION OF CARDIAC SARCOLEMMA

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## Summary

A procedure was developed for the isolation of cardiac sarcolemmal vesicles. These vesicles are enriched about ten-fold (with respect to the tissue homogenate) in $K^+$-stimulated *p*-nitrophenylphosphatase, ($Na^+ + K^+$)-ATPase, 5′-nucleotidase activities and sialic acid content, all of which are believed to be components of the sarcolemma. The sarcolemma of tissue culture cardiac cells were radioiodinated and the distribution of this radioiodine paralleled the distribution of the other membrane markers above. There was very little contamination of the sarcolemmal fraction by sarcoplasmic reticulum (as judged by $Ca^{2+}$-ATPase and glucose-6-phosphatase activities) or inner mitochondrial membranes (as judged by succinate dehydrogenase activity). There may, however, be some contamination by outer mitochondrial membranes (as judged by monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase activities) which have rarely been monitored in cardiac sarcolemmal preparations. The purity of this preparation is good when compared with other cardiac sarcolemmal preparations. This preparation should be very useful in studying the roles of the cardiac sarcolemma (e.g. in excitation contraction coupling and $Ca^{2+}$ binding).

## Introduction

It is well-known that the [$Ca^{2+}$] surrounding the myofilaments controls the strength of muscular contraction [1]. The strength of cardiac muscle contraction depends directly on the extracellular [$Ca^{2+}$] up to about 12 mM [2] and is

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

rapidly decreased upon exposure to lanthanum [3], an ion which does not enter the cells [4]. These findings are in sharp contrast to results from skeletal muscle where alterations in external [$Ca^{2+}$] or additions of lanthanum have small and very slow effects [5,6]. This, in conjunction with much other ultrastructural and physiological data, suggest that the excitation-contraction coupling sequence is very different in cardiac and skeletal muscle and that $Ca^{2+}$ at superficial sites is critical for the control of tension development in cardiac muscle [7]. The exact role which sarcolemmal-bound $Ca^{2+}$ plays in myocardial contraction has not been established. In addition the source of contractile-dependent $Ca^{2+}$ in cardiac muscle is a matter of considerable controversy. A good preparation of isolated cardiac sarcolemma should allow characterization of $Ca^{2+}$ binding and transport in these membranes and should increase our understanding of the roles which the sarcolemma and sarcolemmal-bound $Ca^{2+}$ play in the excitation-contraction coupling sequence in the myocardium.

The wide variety of methods used to isolate cardiac sarcolemma and the variability of results speaks to the difficulties involved in the isolation of these membranes. Primary tools used in preparations include Waring blender [8], Potter-Elvehem type [9] or Polytron homogenization [10], hypotonic shock [11], low speed differential centrifugation [12—14], low or high speed centrifugation plus sucrose density gradient centrifugation [10,15—21], various lengths and strengths of high salt extraction [17] or a tangential gaseous shear [22]. Sucrose density centrifugation has been used to isolate sarcolemma of both high density ($\rho > 40\%$ (w/w) sucrose, greater than 1.18 g/ml) [15—18,21] and low density ($\rho < 32\%$ (w/w) sucrose, less than 1.13 g/ml) [10,19,20]. Also, results with various membrane enzyme markers have been inconsistent. Some groups have purified one enzyme marker with little purification of another marker. Most plasma membranes from other tissues have densities of 1.10—1.15 g/ml [23,24] similar to the 'light' membranes mentioned above. Thus the methods employed in this study were aimed at the isolation of 'light' membranes and used the techniques of Kidwai et al. [10] and Heller and Harary [19] as a starting point.

The purpose of this study was to develop a relatively pure and well-characterized cardiac sarcolemmal preparation which could then be used in correlation with physiological studies to elucidate the role of the sarcolemma in the excitation-contraction coupling sequence in the myocardium.

## Methods and Materials

The methods of Kidwai et al. [10] and Heller and Harary [19] were used as a starting point. Direct application of these methods did not give satisfactory results with respect to purification or yield. The technique developed ($KCl/PP_i$ procedure) is described below.

*$KCL/PP_i$ procedure.* Neonatal (5—8 days old) Sprague-Dawley rats were decapitated and the hearts were quickly removed, cut open along two axes and rinsed in ice-cold homogenizing medium (250 mM sucrose, 10 mM Tris-HCl, pH 8.1, at 4°C). The hearts were then weighed and minced in a small volume of homogenizing medium. The homogenization was carried out using a Polytron

PT 10ST (Brinkman Instruments, Westbury, NY) at a setting of 7 for 8—10 s total, in two bursts. The homogenization volume was 15—20 ml and the initial muscle wet weight was routinely 1—3 g. The homogenate was diluted with homogenizing medium 1 : 20 (wet weight : homogenate volume) and filtered through two layers of 40 mesh wire gauze (Small Parts, Inc. Miami, FL) in the barrel of a large syringe using light positive pressure. A volume of KCl/$PP_i$ solution (3 M KCl, 0.25 M sodium pyrophosphate) was added to the filtrate to a final concentration of 0.3 M KCl and 25 mM $PP_i$. This material was mixed vigorously and immediately centrifuged at 177 000 × *g* for 40 min in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Fullerton, CA). The supernatant was discarded, the pellet was resuspended in homogenizing medium and 20 ml were layered over 6 ml of 36% sucrose. These tubes were centrifuged at 131 000 × *g* for 30 min (in a swinging bucket rotor, SW27). The resulting pellet was discarded and the supernatant including the interface was removed and diluted back to 250 mM sucrose with 10 mM Tris. This material was then centrifuged at 177 000 × *g* for 40 min. The supernatant was discarded and the pellet was resuspended in 42% sucrose and layered under a 27—36% continuous (or discontinuous) sucrose density gradient. Continuous gradients were formed with a conventional type of gradient former in conjunction with a Buchler Auto-Densi-Flow II gradient applicator and remover (Buchler Instruments, Fort Lee, NJ). Discontinuous gradients were made by layering 5-ml portions of 27, 30, 32, 34 and 36% sucrose in a centrifuge tube. The gradients were centrifuged overnight (14—16 h) at 122 000 × *g* in a SW27 rotor. The gradients wre fractionated, using the Buchler gradient applicator and remover in conjunction with a peristaltic pump, and assayed immediately.

*Analytical methods.* $K^+$-stimulated *p*-nitrophenylphosphatase ($K^+$-phosphatase) activity was measured in a medium containing: 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM *p*-nitrophenylphosphate at pH 7.6. Duplicates were also run with 20 mM KCl present and with both 20 mM KCl and 10 mM ouabain present so that the $K^+$-stimulated and ouabain-inhibited portion of the phosphatase activity could be measured. The tubes were preincubated at 37°C for 5 min and *p*-nitrophenylphosphate was added at timed intervals to start the reaction. All enzyme incubations were carried out at 37°C. The reaction was quenched after 15—25 min by the addition of 2.0 ml of 1.0 N NaOH. The *p*-nitrophenol produced was measured by the absorbance at 410 or 420 nm. A standard of 50 μM *p*-nitrophenol was routinely run.

($Na^+ + K^+$)-ATPase activity was measured in a medium containing: 120 mM NaCl, 50 mM Tris, 5 mM sodium azide, 1 mM EDTA, 3.5 mM $MgCl_2$ and 3.0 mM ATP at pH 7.0. Duplicates were also run with 20 mM KCl or 10 mM ouabain and 20 mM KCl present so that the ($Na^+ + K^+$)-stimulated and ouabain-inhibited ATPase, respectively, could be measured. 10 mM ouabain completely inhibited ($Na^+ + K^+$)-ATPase, inhibited 90% of the $K^+$-stimulated phosphatase and did not affect basal activities of these enzymes. Inorganic phosphate released was measured by the technique of Fiske and SubbaRow [26]. $Ca^{2+}$-ATPase activity was measured in a medium containing: 100 mM KCl, 50 mM imidazole, 5 mM sodium azide, 3 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA), 3.5 mM $MgCl_2$, 2 mM potassium oxalate and 3.0 mM ATP at pH 7.5. Duplicates were also run with 3 mM $CaCl_2$ present so

that the $Ca^{2+}$-stimulated portion of the ATPase could be measured. 5′-nucleotidase (5′-AMPase) activity was measured by the procedure of Edwards and Maguire [27] in a medium containing: 30 mM Tris and 2 mM AMP (or phenylphosphate to measure nonspecific phosphatase) at pH 7.5. Alkaline phosphatase activity was measured by the method of Mircheff and Wright [28] in a medium containing: 5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.2 mM $ZnCl_2$ and 50 mM Tris at pH 9.0. Either *p*-nitrophenylphosphate (5 mM) or 5′-AMP (1.5 mM) was the substrate. Glucose-6-phosphatase activity was measured by a modification of the procedure of Nordlie and Arion [29] in a medium containing: 80 mM sodium cacodylate, 4 mM EDTA, 1 mM NaF, 20 mM glucose 6-phosphate at pH 6.5. Inorganic phosphates were determined as above [26].

Succinate dehydrogenase activity was measured by the procedure of Pennington [30]. The reaction mixture contained: 50 mM potassium phosphate buffer, 50 mM sodium succinate, 25 mM sucrose and 0.1% 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride at pH 7.4. Monoamine oxidase activity was measured by the procedure of Wurtman and Axelrod [31] in a medium containing 90 mM sodium phosphate buffer, 1.3 μM [*side-chain*-2-$^{14}$C]tryptamine bisuccinate (0.02 μCi/ml) (New England Nuclear, Boston, MA). Rotenone-insensitive NADH cytochrome *c* reductase activity was measured by the procedure of Sottocasa et al. [32]. The reaction medium contained: 50 mM sodium phosphate, 0.3 mM KCN, 0.1 mM NADH, 0.1 mM cytochrome *c* and 1.5 μM rotenone at pH 7.5.

Sialic acid was determined by the technique of Warren [33] including a 1 h incubation of samples in 0.1 M $H_2SO_4$ at 80°C. Proteins were determined by the method of Lowry et al. [34] with bovine serum albumin standards.

For electron microscopy, fractions were diluted, centrifuged at 150 000 × *g* for 60 min, and the pellets were fixed in 2% gluteraldehyde in 0.1 M cacodylate buffer for 2 h. They were then postfixed in 1% osmium tetroxide for 1 h and dehydrated in graded ethanol, embedded in Epon 812 and sectioned with a diamond knife on a Porter-Blum MT-2 ultramicrotome. Sections were then examined on a Siemens Elmiskop IA electron microscope.

A technique for enzymatic radioiodination of tissue-cultured cell membranes was adapted from several sources [19,35,36]. Intact tissue culture cells (prepared from neonatal rat hearts as previously described [37]) were iodinated with either $^{125}$I or $^{131}$I (New England Nuclear, Boston, MA) by lactoperoxidase with a glucose/glucose oxidase system to generate hydrogen peroxide. A dish of tissue culture cells was incubated with about 10 ml of a medium containing: 4.7 munits/ml lactoperoxidase, 5.0 munits/ml glucose oxidase, 50—70 μCi/ml $^{125}$I or $^{131}$I (as NaI) in normal *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer (136 mM NaCl, 3.6 mM KCl, 1.0 mM $CaCl_2$, 16 mM glucose and 3.0 mM Hepes at pH 7.1). The cells were incubated for 1 h at 37°C, rinsed 5—10 times with normal Hepes buffer, the cells scraped off and added to some minced neonatal rat hearts. The normal preparative procedure was then followed. Aliquots of gradient fractions were counted in a gamma counter (Nuclear Chicago, Los Angeles, CA) and/or filtered (using Millipore 0.22 μm membrane filters) and counted in a scintillation counter (Beckman LS 200, Beckman Instruments, Fullerton, CA) with a scintillation cocktail (0.5% PPO and 0.01% POPOP in toluene).

Measurements of the densities of sucrose solutions and gradient fractions were made using a Fisher refractometer (Fisher Scientific Co., Pittsburgh, PA) at room temperature. Chemicals were either reagent or analytical grade and obtained primarily from Mallinkrodt (St. Louis, MO) or J.T. Baker (Phillipsburg, NJ). Biochemicals were obtained from Sigma (St. Louis, MO).

## Results

$K^+$-phosphatase, 5'-AMPase and succinate dehydrogenase activities and protein were measured for all fractions in all experiments. $Ca^{2+}$-ATPase and glucose-6-phosphatase assays were performed frequently and these enzymes were used as putative sarcoplasmic reticular markers. $(Na^+ + K^+)$-ATPase, sialic acid content and the amount of radioiodine incorporated into tissue culture cell membranes were used as additional sarcolemmal markers. Monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase were used occasionally as outer mitochondrial membrane markers.

An early, somewhat successful technique, not using KCl, involved pelleting low speed (100 × *g*) supernatants at high speed (177 000 × *g*) and layering this material on top of a gradient. Typically the purification of $K^+$-phosphatase was 5—7-fold (with respect to homogenate) and the yield of purified membranes accounted for only 3—5% of the starting $K^+$-phosphatase (see Fig. 1A). When KCl/$PP_i$ was used to extract myofilaments the first high speed pellet had to be washed free of KCl to prevent subsequent aggregation upon density gradient centrifugation. The second high speed spin served this purpose. The use of either the 30 min (122 000 × *g*) 36% sucrose density shelf centrifugation or a 10 min (510 × *g*) centrifugation between the two 177 000 × *g* centrifugations (discarding the pellets) allowed exclusion of much aggregated material which

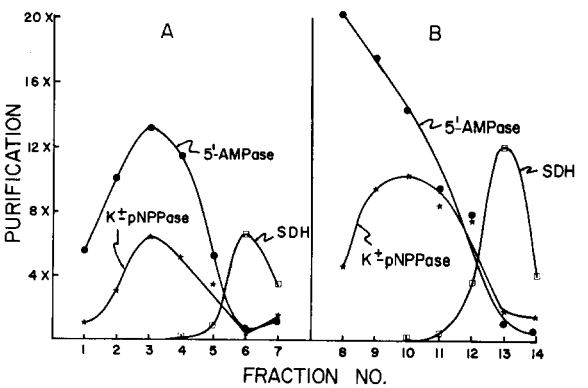


Fig. 1. Marker distribution on gradients with or without prior KCl/$PP_i$ treatment. This figure shows the distribution of $K^+$-phosphatase ($K^+$-pNPPase), 5'-AMPase and succinate dehydrogenase (SDH) specific activities on parallel sucrose density gradients without (A) or with (B) KCl/$PP_i$ treatment. In the experiment shown in (A) pooled supernatants from 100 × *g*, 10 min centrifugations of homogenate were pelleted (177 000 × *g*, 40 min) and layered under a 27—36% sucrose gradient. The experiment in (B) was done in the manner described in the text without the centrifugation over 36% sucrose. Fractions (1—7 and 8—14) were from the top to the bottom of the gradients in order. Purifications are specific activities with respect to homogenate specific activities.

would otherwise remain in the pellet of the density gradients. The density shelf spin also decreased greatly the amount of mitochondria subsequently applied to the density gradient. These procedures allowed more prospective sarcolemma to be purified on one gradient. This general procedure was found to increase the amount and purity of sarcolemma (as judged by $K^+$-phosphatase) that could be isolated from the homogenate by density gradient centrifugation. The purification factor was higher (approx. 10) and more reproducible and the yield was substantially higher (8—15%).

Fig. 1 shows an interesting result with regard to the use of $KCl/PP_i$. Fig. 1A shows the distribution of specific enzyme activities of a gradient on which material never exposed to $KCl/PP_i$ was purified. Fig. 1B shows a parallel gradient on which material exposed to $KCl/PP_i$ was purified. It can be seen that the addition of $KCl/PP_i$ produced a higher purification of $K^+$-phosphatase, but also changed the distribution of 5′-AMPase. Without $KCl/PP_i$, the 5′-AMPase activity paralleled the $K^+$-phosphatase activity consistently. With $KCl/PP_i$, the 5′-AMPase seemed to have a lower equilibrium density, while that for $K^+$-phosphatase remained the same. Several possible explanations will be discussed later. Alkaline phosphatase activity had a distribution similar to that of 5′-AMPase on gradients. However, the 5′-AMPase activity was about 50 times that of alkaline phosphatase with *p*-nitrophenylphosphate as the substrate and about 20 times that of alkaline phosphatase when 5′-AMP was the substrate. This and the relatively low activity of nonspecific phosphatase (as measured with phenylphosphate as substrate) provided evidence that the 5′-AMPase activity was not due to alkaline phosphatase or nonspecific phosphatase activity.

Application of the material to the bottom of the density gradient by resuspending the second high speed pellet in 42% sucrose increased the purification and yield of the sarcolemmal markers and decreased contamination by succinate dehydrogenase. Electron micrographs of sarcolemmal fractions from bottom-loaded gradients also showed less extraneous particulate matter than did top-loaded gradients. Overnight centrifugation (14—20 h) of the gradients also increased the specific activity and the yield of sarcolemmal marker enzymes. Fig. 2 shows an experiment in which two parallel identical gradients were centrifuged for 3 h. One gradient was then fractionated and the other one was centrifuged 18 more hours before fractionation. It should be pointed out that these gradients were bottom loaded and that the sarcolemmal vesicles were moving up toward their equilibrium density. It can be seen that the peak of specific activity has shifted to a much lower density fraction in the longer isopycnic run. In addition, while fraction 5 (from the shorter spin) contains only 3% of the initial $K^+$-phosphatase and has considerable contamination, three fractions of greater purity in the longer spin (8—10) contain 9% of the initial $K^+$-phosphatase and have minimal contamination by succinate dehydrogenase and $Ca^{2+}$-ATPase (see Fig. 2).

Polytron homogenization produced a higher purification and yield of $K^+$-phosphatase than did homogenization with a motor-driven Teflon pestle in a fitted glass tube. Decreasing the pH of the homogenizing medium or changing its composition generally decreased sarcolemmal yield and purity. Homogenization in 10 mM bicarbonate gave comparable results if sucrose was added to the homogenate immediately to bring the sucrose concentration up to 250 mM.

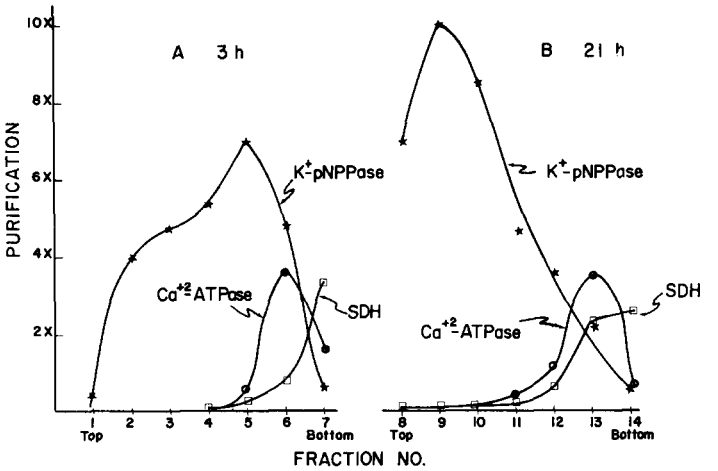


Fig. 2. Marker distribution gradients centrifuged 3 or 21 h. This figure shows the distribution of $K^+$-phosphatase, $Ca^{2+}$-ATPase and succinate dehydrogenase specific activities on sucrose density gradients centrifuged for 3 h (A) or 21 h (B). The experiments were performed as described in the text. Purifications are specific activities with respect to homogenate specific activities.

Higher KCl concentration (e.g. 0.9 M) increased yield slightly, but not purification of $K^+$-phosphatase.

Fig. 3 and Table I show the distributions of several markers used in an experiment. In this experiment neither the 36% sucrose density shelf nor the 510 $\times g$ spins were used. It can be seen that a large proportion of the $K^+$-phosphatase applied to the gradient was in the bottom fraction (fraction 6, Table I) and nearly 70% of the succinate dehydrogenase activity was recovered on the gradient. This is probably due to the inclusion of aggregated material (which the intermediate steps remove) on the gradient (mostly in fraction 6 or 40% pellet). Fig. 3 shows that $K^+$-phosphatase, $(Na^+ + K^+)$-ATPase and sialic acid were purified in a parallel fashion and were purified 10—20-fold over the homogenate activity in the second and third fractions (30—32% sucrose; 1.12—1.13 g/ml). It can be seen that the percent yield in these fractions is 13%, 18% and 23% of the homogenate activities for $K^+$-phosphatase $(Na^+ + K^+)$-ATPase and sialic acid content, respectively. The yield of $K^+$-phosphatase (which was purified roughly 10-fold) was usually 8—15%. It can be seen that there is an increase in yield and specific activity of $K^+$-phosphatase, and sialic acid $(Na^+ + K^+)$-ATPase at the bottom of the gradient. This is, presumably the, aggregated material previously discussed. The general distribution of 5′-AMPase is essentially the same as in Fig. 1B and was discussed above. The distribution of succinate dehydrogenase activity is much like that in Fig. 1. Most of the activity is found in a brown mitochondrial band at about 36% sucrose. Most of the rest of the succinate dehydrogenase is found in the bottom fraction, presumably as part of the afore-mentioned aggregate. In Fig. 2 the peak of succinate dehydrogenase is in the bottom fraction. In the experiment shown there, the second to last fractions were in 34% sucrose so the peak of succinate dehydrogenase would be expected to be in the pellet.

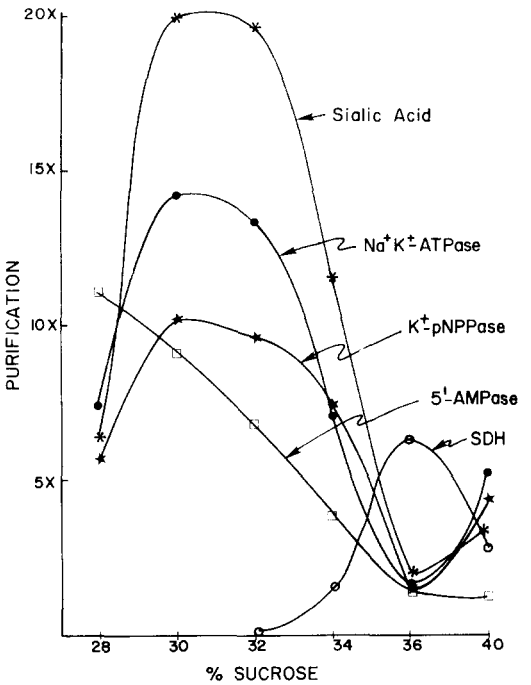


Fig. 3. Marker distributions on a gradient. This figure shows the distribution of $K^+$-phosphatase, ($Na^+$ + $K^+$)-ATPase, succinate dehydrogenase, 5'-AMPase activities and sialic acid on a sucrose density gradient. This is the same experiment as in Table I and 28, 30, 32, 34, 36 and 40% sucrose fractions correspond to fractions 1—6 in that table. Purifications are as in Figs. 1 and 2.

TABLE I

DISTRIBUTION OF SEVERAL MARKERS ON A GRADIENT

This experiment is the same as in Fig. 3. There was no intermediate low speed centrifugation or centrifugation over a 36% sucrose cushion. H (homogenate) was treated with KCl/$PP_i$ spun down hard yielding $P_1$ which was resuspended in homogenizing medium and spun down hard again yielding $P_2$. $P_2$ was layered under a continuous 27—36% sucrose gradient and centrifuged 14 h at 122 000 × *g*. Succinate dehydrogenase activity as arbitrary units and was not detectable (n.d.) in fractions 1 and 2. Numbers in parentheses are % yields.

| | Protein (%) | $K^+$-phosphatase (μmol/mg per h) | ($Na^+$ + $K^+$)-ATPase (μmol/mg per h) | Sialic acid (nmol/mg) | AMPase (μmol/mg per h) | Succinate dehydrogenase |
|---|---|---|---|---|---|---|
| H | 100.0 | 0.59 (100.0) | 1.32 (100.0) | 23 (100.0) | 2.67 (100.0) | 1.15 (100.0) |
| $P_1$ | 44.0 | 1.29 (95.9) | 2.85 (95.0) | 41 (78.4) | 5.36 (88.3) | 2.74 (104.8) |
| $P_2$ | 24.5 | 1.88 (78.0) | 4.31 (80.2) | — (—) | 7.03 (64.5) | 4.49 (95.8) |
| 1 | 1.1 | 3.39 (6.0) | 9.64 (8.0) | 146 (6.1) | 29.3 (11.9) | n.d. (—) |
| 2 | 0.7 | 5.98 (6.6) | 18.72 (9.3) | 451 (11.4) | 24.0 (5.9) | n.d. (—) |
| 3 | 0.4 | 5.70 (6.4) | 17.51 (9.0) | 448 (11.6) | 17.9 (4.5) | 0.10 (0.1) |
| 4 | 0.7 | 4.42 (5.0) | 9.18 (5.0) | 260 (7.2) | 10.2 (2.7) | 1.86 (1.2) |
| 5 | 7.0 | 0.87 (10.2) | 2.23 (11.8) | 43 (13.0) | 4.1 (10.8) | 7.16 (43.1) |
| 6 | 9.1 | 2.52 (38.6) | 6.71 (42.3) | 73 (28.7) | 4.0 (13.5) | 3.17 (24.9) |
| 1—6 | 18.9 | 2.30 (72.8) | 5.95 (85.4) | 93 (78.0) | 6.9 (49.3) | 4.19 (69.3) |

Fig. 2 shows a demonstration of the distribution of $Ca^{2+}$-ATPase on a gradient. This enzyme is known to be associated with the sarcoplasmic reticulum in cardiac muscle, but it is also a notoriously labile enzyme. When $Ca^{2+}$-ATPase was measured, the pattern was consistently similar to that in Fig. 2. The peak in specific activity of this enzyme was just above the mitochondrial band and could sometimes be seen as a thin white band in that region. It should be emphasized that there was virtually no $Ca^{2+}$-ATPase activity in the high $K^+$-phosphatase fractions (see Fig. 2B). Glucose-6-phosphatase activity was very low and has not been clearly demonstrated as a sarcoplasmic reticular marker in this tissue, although several investigators have used it as such [10,13,14]. The distribution of glucose-6-phosphatase activity on a gradient showed a small sharp peak in specific activity in the fraction just above the mitochondrial band (at about 34% sucrose) similar to the $Ca^{2+}$-ATPase. The relative purification of glucose-6-phosphatase in this fraction was about 3—4-fold but the activity in homogenate was very low to begin with. Results with skeletal muscle gave a clear indication of the location of the sarcoplasmic reticulum on the gradient due to its powerful $Ca^{2+}$-ATPase. The skeletal sarcoplasmic reticulum was visibly (and enzymatically) apparent in a band just above the mitochondria in the same location as the putative sarcoplasmic reticulum from cardiac muscle. The patterns for $Ca^{2+}$-ATPase and glucose-6-phosphatase suggests that separation of the sarcolemma from the sarcoplasmic reticulum has been achieved to a fairly good extent.

Monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase activities were measured in a limited number of experiments, but the distribution of these outer mitochondrial membane marker enzymes were very similar. Fig. 4 shows the distribution of monoamine oxidase in a typical experiment. The highest peak in specific activity of monoamine oxidase is at the very top of the gradient, but contained a relatively small amount (11%) of the total activity on the gradient. The second peak in specific activity was not as high as the first and coincided with the peak of succinate dehydrogenase activity in the mitochondrial band. This peak (fractions 6 and 7) accounted for approximately 40% of the monoamine oxidase on the gradient and showed a distribution much like the succinate dehydrogenase in the lower part of the gradient. The logical conclusion is that while most of the outer mitochondrial membranes stay in the mitochondrial fraction, some are free and are partially purified at the top of the gradient. This is in agreement with electron microscope observations of the mitochondrial band, which show little evidence of intact mitochondria. The peaks of specific activity of these enzymes do not coincide with that of $K^+$-phosphatase, nevertheless outer mitochondrial membranes may be a contaminant of the sarcolemmal preparation. It should be pointed out that the amount of $K^+$-phosphatase activity in the pellet is very low with the 36% sucrose cushion centrifugation used here. This is in contrast to the results shown in Table I and Fig. 3 where no intermediate step was performed.

Table II shows resultant purifications for some of the markers used (averages from several experiments). It should be noted that the $K^+$-phosphatase purification factor is the same as that for $(Na^+ + K^+)$-ATPase as would be expected since they are believed to be functions of the same protein [25]. The activity of these enzymes were always parallel on gradients. The purification of sialic

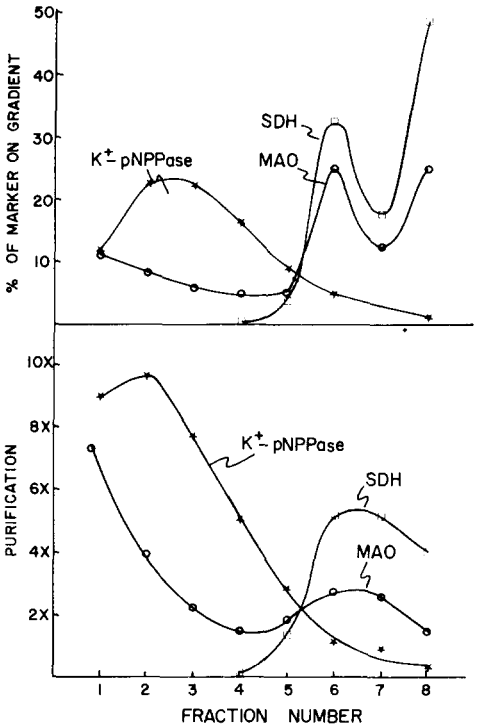


Fig. 4. Distribution of monoamine oxidase (MAO) on a gradient. The lower graph shows the relative purifications of monoamine oxidase, $K^+$-phosphatase and succinate dehydrogenase on a sucrose density gradient. The upper graph shows the percents of total marker activities applied to the gradients for the same enzymes and gradient. This experiment was performed in the normal manner, including the 36% sucrose density shelf centrifugation. The amount of $K^+$-phosphatase activity in the pellet is very low. 32% of the homogenate $K^+$-phosphatase was applied to the gradient and 45% of that is in fractions 2 and 3.

TABLE II

PURIFICATION OF SOME MARKERS

Enzyme activities are in μmol/mg protein per h except succinate dehydrogenase which is in arbitrary units. Sialic acid is in nmol/mg protein. The number of independent experiments is in parentheses, and values are ±S.E.

| | $K^+$-phosphatase (22) | $(Na^+ + K^+)$-ATPase (6) | Sialic acid [a] (6) | 5′-AMPase (20) | Succinate dehydrogenase (22) |
|---|---|---|---|---|---|
| Homogenate activity | 0.617 ± 0.043 | 1.30 ± 0.19 | 15.5 ± 3.9 | 2.68 ± 0.26 | 1.08 ± 0.08 |
| Sarcolemmal fraction activity | 6.17 ± 0.42 | 13.4 ± 1.6 | 165 ± 35 | 22.5 ± 2.5 | 0.12 ± 0.04 |
| Purification factor (-fold) | 10 | 10.3 | 10.6 | 8.6 | 0.11 |

[a] It is believed that in two of these experiments (including the one in Fig. 3 and Table I) artifactually high values for sialic acid contents were obtained. The relative purification and yields are likely to be valid. Without these two experiments the homogenate and sarcolemmal specific contents are 9.2 ± 1.7 and 86.2 ± 8.2 giving a purification factor of 9.4.

acid is similar to both $K^+$-phosphatase and ($Na^+ + K^+$)-ATPase. 5'-AMPase is somewhat less purified. If the values of the peak 5'-AMPase from these experiments are used an activity of 31.5 ± 2.7 μmol/mg protein per h and a purification of 11.8-fold are obtained. Succinate dehydrogenase activity on the other hand was reduced to approximately one-tenth of its activity in the homogenate and was often not detectable, especially in later experiments where the centrifugation over a 36% sucrose density shelf was used.

Fig. 5 shows the distribution of $^{131}I$ bound to the sarcolemma of tissue culture cells which were enzymatically radioiodinated, on a sucrose gradient. The distribution of $^{131}I$ clearly parallels the $K^+$-phosphatase activity. In conjunction with results from Heller and Harary [19], showing copurification of $^{131}I$ and $K^+$-phosphatase in tissue culture cells, $K^+$-phosphatase from neonatal rat hearts copurifies with $K^+$-phosphatase of tissue culture cells from the neonatal rat heart. This supports the use of $K^+$-phosphatase as a sarcolemmal marker.

Fig. 6 is an electron micrograph of a sarcolemmal fraction. It appears to contain primarily rounded vesicles of various diameters (0.1—0.8 μm) with little evidence of extraneous particulate or fibrillar material or recognizable portions of mitochondria.

The above results were obtained from neonatal rat hearts. Additional experiments were performed on adult rat heart and adult rabbit heart. The adult rat and rabbit heart results were qualitatively the same as those for the neonate rat hearts. The only significant differences are that the rabbit sarcolemmal markers are isolated at a lower density and thus the overlap of $K^+$-phosphatase with sarcoplasmic reticular and mitochondrial markers is decreased and the activity of 5'-AMPase in the rabbit is much lower.

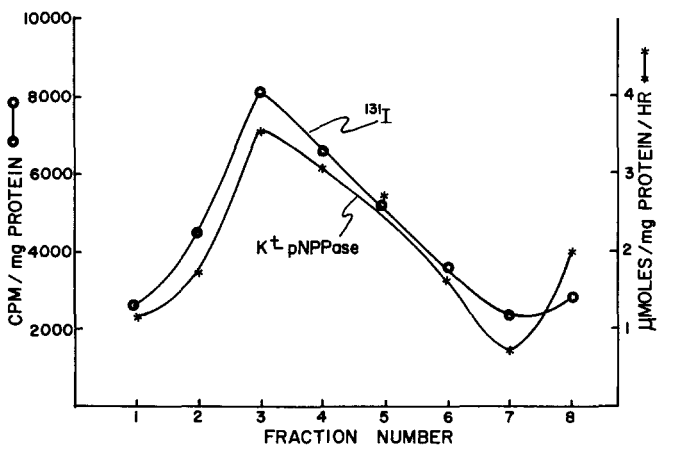


Fig. 5. Distribution of $^{131}I$ from radioiodinated membranes on a gradient. Sarcolemma of cardiac tissue culture cells were radioiodinated, the cells were added to some minced hearts and the normal isolation procedure was then followed. Specific activities of $^{131}I$ and $K^+$-phosphatase are indicated on the ordinate axes.

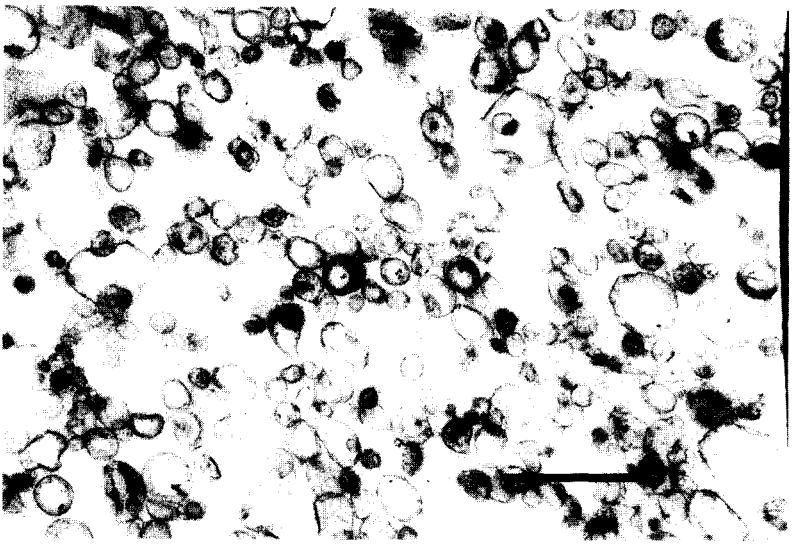

Fig. 6. Electron micrograph of sarcolemmal vesicles. This figure shows a typical sarcolemmal fraction from the normal procedure. Magnification 18 200× (calibration bar = 1 μm).

## Discussion

A potentially useful preparation of cardiac sarcolemma has been described here. This method uses a brief mild KCl/$PP_i$ treatment and various centrifugation procedures to produce a reasonable yield of cardiac sarcolemmal vesicles. The sarcolemmal vesicles produced have a low intrinsic density ($\rho \approx 1.13$ g/ml), are enriched in several sarcolemmal markers and lack measureable $Ca^{2+}$-ATPase activity.

Plasma membranes isolated from most tissues which have been studied are of relatively low density ($\rho < 1.17$ g/ml) [23,24]. St. Louis and Sulakhe [17] and Hui et al. [16] have developed procedures which isolate 'heavy' membranes ($\rho \approx 1.21$ g/ml). Heavy membrane preparations [15—17] have yielded fractions 4—7-fold enriched in ($Na^+ + K^+$)-ATPase activity, but with mitochondrial contamination as evidenced by 0.25—1.0-fold purification of mitochondrial markers. Heller and Harary [19] have suggested that cardiac sarcolemma may have a high density because of attachment to dense particles or because of changes in their intrinsic density. It is possible that these heavy sarcolemma are aggregated with some more dense cellular components such as myofibrils or DNA from disrupted nuclei.

High concentrations of KCl have frequently been used to extract myofibrillar proteins from cardiac muscle homogenates. However, high salt solutions are known to extract membrane proteins and lipids and could markedly alter membrane structure and function [23]. Longer and stronger KCl/$PP_i$ treatments than described produced some increase in yield, but the relatively mild extraction used here was chosen as a compromise between increasing purification and a possible detriment to membrane integrity. The lack of parallelism of 5′-AMPase and $K^+$-phosphatase on gradients when KCl was used

illustrates this point. The KCl/$PP_i$ treatment may change the density of a subclass of vesicles making them separable. It may allow a different type of vesicles to reach the gradient or cause a differential loss of membrane protein. It is possible that KCl/$PP_i$ extracts $K^+$-phosphatase (and perhaps other proteins) from the vesicles, yielding a subpopulation of vesicles enriched in 5'-AMPase and of lower density. Many other explanations are possible, but at this point there is no evidence to suggest that any one interpretation is correct. Further study may elucidate a mechanism for this effect.

Most sarcolemmal preparations have employed a brief (1—2 h) density gradient centrifugation with material loaded from the top. Substantial improvements were made here by both longer centrifugation (14—16 h) and by loading material onto the bottom of the gradient. The longer spins allow more of the vesicles to reach their equilibrium density (see Fig. 2). The bottom loading decreases the amount of contaminant and aggregated material which travels through the part of the gradient of most interest.

Recent cytochemical experiments by Wollenberger and Schulze [41] and Perissel et al. [42] show that ($Na^+ + K^+$)-ATPase, adenylate cyclase and 5'-AMPase are located exclusively at the sarcolemma in cardiac muscle. $K^+$-phosphatase activity has been attributed to, and is normally found to copurify with the ($Na^+ + K^+$)-ATPase [25]. The enzymatic radioiodination of intact tissue culture cells provides an independent method for labelling only the sarcolemma of these cells. Also, at least 61% of cellular sialic acid has been found to be exposed on the surface of tissue culture cardiac cells [43]. All of these sarcolemmal markers were purified in a parallel fashion (except for 5'-AMPase as discussed above) and had comparable purification factors (see Table II). This provides evidence that sarcolemmal markers are well purified (approx. 10-fold), but does not give information concerning contamination of this fraction.

Inner mitochondrial membrane contamination of the sarcolemmal fraction was small and often non-detectable as measured by succinate dehydrogenase activity. The results with outer mitochondrial membrane markers suggest some contamination of the sarcolemmal fraction by these membranes. Further examination of these outer mitochondrial membrane markers and their separation from the sarcolemmal fraction should be undertaken in further studies of this preparation. It should be noted that other cardiac sarcolemmal preparations have been done without consideration of outer mitochondrial contamination. Due to the apparent similarity of the equilibrium density of outer mitochondrial membranes and sarcolemmal (with the gradients used in this study) it should seem compelling to evaluate their distribution in cardiac cell fractionations.

Glucose-6-phosphatase and NADPH cytochrome *c* reductase have frequently been used as marker enzymes for endoplasmic reticulum [23]. There is no definitive evidence that these enzymes are localized in the sarcoplasmic reticulum of muscle cells. Nevertheless, several investigators have used them as markers for this organelle [10,13,14]. $Ca^{2+}$-ATPase is more widely accepted as a component of the sarcoplasmic reticulum of cardiac muscle, but the activity is rather labile and this may be why some investigators do not report the distribution of this enzyme in cardiac sarcolemmal isolation procedures. The results of $Ca^{2+}$-ATPase and glucose-6-phosphatase together with the parallel

results from skeletal muscle were taken as evidence for the lack of sarcoplasmic reticular contamination in sarcolemmal fractions. It should be noted that several authors find $Ca^{2+}$-ATPase activity concentrated in their sarcolemmal fractions [16,39,40]. It is reasonable from a physiological point of view that there be some $Ca^{2+}$-pumping mechanism in cardiac sarcolemma. Indeed, Sulakhe and coworkers have presented evidence that $Ca^{2+}$-ATPase in both skeletal [38] and cardiac sarcolemma [39] is not due to sarcoplasmic reticular contamination. Continuous density gradients which show a parallel purification of, for example, $(Na^+ + K^+)$-ATPase and $Ca^{2+}$-ATPase would provide more compelling evidence that these enzymes are on the same vesicles (especially if aggregation is not suspected). Thus, whether or not there is a prominent $Ca^{2+}$-ATPase in cardiac sarcolemma is an unresolved question which is complicated by there being a $Ca^{2+}$-ATPase in the sarcoplasmic reticulum.

Electron microscopic observations cannot provide quantitative information concerning the purity of a sarcolemmal fraction. It can be seen, however, that the sarcolemmal fraction is composed mostly of small smooth vesicles and

TABLE III

PURIFICATION OF SARCOLEMMAL AND MITOCHONDRIAL MARKERS IN CARDIAC SARCOLEMMA PREPARED BY VARIOUS METHODS

N.D., non-detectable.

| Reference | Species | Purification factor (% yield) | |
|---|---|---|---|
| | | Sarcolemmal marker | Mitochondrial marker |
| Bers (present study) | rat | 10.0 (12) [a,b] | 0.1 (0.1) [d] |
| Langer et al. [22] | rat | 15.2 (12) [a] | N.D. [d] |
| St. Louis and Sulakhe [17] | rat | 5.6 (60) [a,g] | — |
| Limas [14] | rat | 6.8 [a,g] | 0.02 [e,h] |
| Jarott and Picken [9] | rat | 3.0 (49) [c] | 0.4 (6) [d] |
| Dietze and Hepp [12] | rat | 2.7 [a,g] | — |
| Kidwai et al. [10] | rat | 2.3 (3) [b] | 0.13 [e,h] |
| Heller and Harary [19] | rat | 5.0 (38) [b] | 1.4 (26) [e] |
| Feldman and Weinhold [18] | rat | 1.5 (30) [c] | 0.5 (10) [d] |
| St. Louis and Sulakhe [17] | guinea pig | 7.0 (20) [a] | 0.3 (0.6) [d] |
| Hui et al. [16] | guinea pig | 4.1 (8) [a] | 1.0 (2.6) [e] |
| Scarpa and Williamson [15] | guinea pig | 3.7 (22) [a] | 1.0 (6.3) [e] |
| Tada et al. [11] | guinea pig | 2.5 (52) [a] | 0.04 (0.1) [f] |
| Bers (present study) | rabbit | 16.2 (14) [b] | N.D. [d] |
| St. Louis and Sulakhe [17] | rabbit | 3.7 (18) [a,g] | — |
| McNamara et al. [13] | dog | 7.0 [a] | 0.02 [e,h] |
| Stam et al. [8] | dog | 3.5 (3) [a,g] | 0.2 (1.0) [e] |
| Paris et al. [20] | chick | 10.0 (6) [b] | 0.3 (0.1) [e] |
| Pang and Weglicki [21] | hamster | 7.3 (7) [a] | 1.5 (1.3) [e] |

[a] $(Na^+ + K^+)$-ATPase.
[b] $K^+$-phosphate.
[c] 5'-AMPase.
[d] Succinate dehydrogenase.
[e] Cytochrome *c* oxidase.
[f] Azide sensitive ATPase.
[g] Assuming homogenate $(Na^+ + K^+)$-ATPase = 1.5 μmol/mg per h.
[h] Sarcolemmal fraction compared to mitochondrial fraction where homogenate values were unavailable.

there is no evidence of particulate or fibrillar material. The distribution of some other cellular membranes (e.g. lysosomal or those of Golgi apparatus) were not determined.

Table III shows purification factors and yields of sarcolemmal and mitochondrial markers of sarcolemmal fractions prepared by a variety of techniques. Either $(Na^+ + K^+)$-ATPase or $K^+$-phosphatase was chosen (when available) as the marker for comparing sarcolemmal preparations since they are probably the most widely used sarcolemmal markers. This may be somewhat unfair, since several groups have used adenylate cyclase as their primary sarcolemmal marker and have higher purification factors for this enzyme. Some investigators do not clearly indicate their mean or even typical specific activities of these enzymes. Kidwai et al. [10] reported $(Na^+ + K^+)$-ATPase activity of 18.0 μmol/mg per h, but did not state the homogenate activity for this enzyme. This result could not be reproduced here (or by others [13,19]) and the purification of $K^+$-phosphatase of only 2.3-fold makes a purification of $(Na^+ + K^+)$-ATPase of greater than 10-fold seem unlikely in the light of the preceding discussion. Some investigators did not report homogenate activities for mitochondrial markers [10,12,14] or $(Na^+ + K^+)$-ATPase [12,14]. This makes evaluation of these techniques difficult. The preparation developed in this study is quite good with respect to sarcolemmal purification and lack of mitochondrial contamination when compared to those in Table III. It should be pointed out that the results obtained with rabbit heart are even better than for the neonate rat (although the number of experiments was smaller ($n = 5$)). This procedure for the preparation of a relatively pure sarcolemmal fraction should be very useful in further studies of the cardiac sarcolemma and its physiology. This sarcolemmal preparation has been used by Bers and Langer [44] in a study which correlates cardiac sarcolemmal $Ca^{2+}$ binding and contractile function in intact cardiac muscle.

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## References

1 Ebashi, S. and Endo, M. (1968) Prog. Biophys. Mol. Biol. 18, 123—183
2 Shine, K.I., Serena, S.D. and Langer, G.A. (1971) Am. J. Physiol. 221, 1408—1417
3 Sanborn, W.G. and Langer, G.A. (1970) J. Gen. Physiol. 56, 192—217
4 Langer, G.A. and Frank, J.S. (1972) J. Cell Biol. 54, 441—455
5 Andersson, K.E. and Edman, K.A.P. (1974) Acta Physiol. Scand. 90, 113—123
6 Rich, T.L. and Langer, G.A. (1975) J. Mol. Cell. Cardiol. 7, 747—765
7 Langer, G.A., Frank, J.S. and Brady, A.J. (1976) Int. Rev. Physiol. 9, 191—237
8 Stam, A.C., Weglicki, W.B., Feldman, D., Shelburne, J.C. and Sonnenblick, E.H. (1970) J. Mol. Cell. Cardiol. 1, 117—130

9 Jarrot, B. and Picken, G.M. (1975) J. Mol. Cell. Cardiol. 7, 685—695
10 Kidwai, A.M., Radcliffe, M.A., Duchon, G. and Daniel, E.E. (1971) Biochem. Biophys. Res. Commun. 45, 901—910
11 Tada, M., Finney, J.O., Swartz, M.H. and Katz, A.M. (1972) J. Mol. Cell. Cardiol. 4, 417—426
12 Dietze, G. and Hepp, K.D. (1971) Biochem. Biophys. Res. Commun. 44, 1041—1049
13 McNamara, D.B., Sulakhe, P.V., Singh, J.N. and Sachs, G. (1974) J. Biochem. 75, 795—803
14 Limas, C.J. (1977) Arch. Biochem. Biophys. 179, 302—309
15 Scarpa, A. and Williamson, J.R. (1974) in Calcium Binding Proteins (Drabikowski, W., Strzelecka-Gobaszewska, H. and Carafoli, E., eds.), pp. 547—585, Elsevier, Amsterdam
16 Hui, C.W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415—427
17 St. Louis, P.J. and Sulakhe, P.V. (1976) Int. J. Biochem. 7, 547—558
18 Feldman, D.A. and Weinhold, P.A. (1977) Biochem. Pharmacol. 26, 2283—2289
19 Heller, M. and Harary, I. (1977) Biochim. Biophys. Acta 467, 29—43
20 Paris, S., Fosset, M., Samuel, D. and Ailhaud, G. (1977) J. Mol. Cell. Cardiol. 9, 161—174
21 Pang, D.C. and Weglicki, W.B. (1977) Biochim. Biophys. Acta 465, 411—414
22 Langer, G.A., Frank, J.S. and Philipson, K.D. (1978) Science 200, 1388—1391
23 Steck, T.L. (1972) in Membrane Molecular Biology (Fox, C.F. and Keith, A., eds.), pp. 76—114, Sinaver, Stamford, CT
24 Graham, J.M. (1975) in New Techniques in Biophysics and Cell Biology (Pain, R.H. and Smith, B.J., eds.), Vol. 2, pp. 1—42, Wiley, London
25 Schwartz, A., Lindenmayer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3—134
26 Fiske, C.R. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375—400
27 Edwards, M.J. and Maguire, M.H. (1970) Mol. Pharmacol. 6, 641—648
28 Mircheff, A.K. and Wright, E.M. (1976) J. Membrane Biol. 28, 309—333
29 Nordlie, R.C. and Arion, W.J. (1966) Methods Enzymol. 9, 619—625
30 Pennington, R.J. (1961) Biochem. J. 80, 649—654
31 Wurtman, R.J. and Axelrod, J. (1963) Biochem. Pharmacol. 12, 1439—1441
32 Sottocasa, G.L., Kuylenstierna, B., Ernstner, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415—437
33 Warren, L. (1959) J. Biol. Chem. 234, 1971—1975
34 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
35 Phillips, D.R. and Morrison, M. (1971) Biochemistry 10, 1766—1771
36 Hubbard, A.L. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390—405
37 Langer, G.A., Nudd, L.M. and Ricchiuti, N.V. (1976) J. Mol. Cell. Cardiol. 8, 321—328
38 Sulakhe, P.V., Drummond, G.I. and Ng, D.C. (1973) J. Biol. Chem. 248, 4150—4157
39 St. Louis, P.J. and Sulakhe, P.V. (1976) Can. J. Biochem. 54, 946—956
40 Dhalla, N.S., Anand, M.B. and Harrow, J.A.C. (1976) J. Biochem. 79, 1345—1350
41 Wollenberger, A. and Schulze, W. (1976) in Recent Advances in Studies on Cardiac Structure and Metabolism (Fleckstein, A. and Dhalla, A., eds.), Vol. 9, pp. 101—115, University Park Press, Baltimore
42 Perrisel, B., Charbonne, F., Moalic, J.M. and Maleb, P. (1977) Cell. Mol. Biol. 22, 341—346
43 Langer, G.A., Frank, J.S., Nudd, L.M. and Seraydarian, K. (1976) Science 193, 1013—1015
44 Bers, D. and Langer, G.A. (1979) Am. J. Physiol., in the press