Purification of Morphologically Intact Triad Structures from Skeletal Muscle

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ABSTRACT A procedure has been devised for isolation of triads (t-tubule/sarcoplasmic reticulum (SR) junctional complexes) from rabbit skeletal muscle. The procedure consists of preparation of a heavy microsomal fraction followed by two sequential 90-min sucrose gradient centrifugations to enrich the triads. A pyrophosphate/phosphate/magnesium buffer system was introduced to decrease aggregation in order to achieve effective separation. The preparation time is 12 h. Some differences between purified triads isolated by two variants of this method are noted. The purity of the triad fractions has been estimated by particle counting to be in the vicinity of 50%. There is good retention of morphology and Ca^{++}-loading activity and enrichment in Na^+,K^+-ATPase and adenylate cyclase. The triads are practically devoid of contractile elements, mitochondria, and free plasmalemma, and low in content of light SR. The method for obtaining enriched triads is reproducible, and sufficient yields are obtained for structural, biochemical, and functional characterization.

Muscle contraction and relaxation in skeletal muscle are regulated by the intracellular concentration of Ca^{++}. During excitation-contraction coupling, Ca^{++} is released from the sarcoplasmic reticulum (SR) compartment elevating the Ca^{++} concentration in the myoplasm, thereby triggering muscle contraction (1). Relaxation involves the reuptake and storage of Ca^{++} by sarcoplasmic reticulum, which can be simulated, in vitro, and therefore has been characterized in depth (2). The physiological Ca^{++}-release process is less readily simulated, in vitro, and is poorly understood in molecular or mechanistic terms. Of particular interest here is the intracellular triad junction, purported to be the link between the signal process of the surface membrane (excitation) and the release of Ca^{++} from the terminal cisternae of the SR resulting in contraction. The isolation of triads,[1] which are junctional associations of transverse tubular invaginations of the surface membrane with the terminal cisternae of the SR, could make possible in vitro simulation of the Ca^{++}-release process, especially Ca^{++} release induced by transverse tubule depolarization.

The only procedures in the literature that deal with the isolation of triads are by Caswell and co-workers (3, 4). The fraction described was somewhat enriched in triads and was used mainly for the subsequent isolation of transverse tubule (4–7). No estimate of purity was given. We now report the preparation of highly purified triad structures that retain architectural properties resembling that seen in situ (8–11). The purity of the fraction has been assessed by diagnostic marker enzyme assay and by particle counting. This paper describes the isolation procedures. A companion paper (11) emphasizes morphology and structural susceptibility of isolated triads to a variety of conditions.

MATERIALS AND METHODS

Materials

Solutions were prepared in sequentially distilled and deionized water and all stock chemicals were reagent grade from Fisher Scientific Co. (Pittsburgh, PA) unless otherwise stated. "Density gradient grade" sucrose was obtained from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, NY) and from EM laboratories, Inc. (Elmsford, NY). All sucrose concentrations are given as percent sucrose (wt/wt), determined using a Bausch and Lomb refractometer (Bausch

[1] The term triad derives from electron microscopy of muscle in which the transverse tubule is apposed between two terminal cisternae of sarcoplasmic reticulum, each in junctional association. Throughout the text, the term "triad" will be used to denote a structure composed of one or more junctional associations of transverse tubule with terminal cisternae. In this context, triad is not intended to suggest that it necessarily consists of three components but rather refers to a structure consisting of two or more components in junctional association.

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& Lomb Inc., Scientific Optical Products Div., (Rochester, NY). The pH of all solutions was adjusted at room temperature to the values indicated. Radioactive α-labeled ^{32}P-ATP for adenylate cyclase was prepared by the method of Walseth and Johnson (12) in Dr. R. Johnson's laboratory at Vanderbilt University or obtained from New England Nuclear (Boston, MA), as was [^{3}H]cAMP (30–50 Ci/mmole). Myokinase, adenosine deaminase, arsenazo III (~98%), Na_2ATP, and creatine phosphate were obtained from Sigma Chemical Co. (St. Louis, MO) while creatine phosphokinase was obtained from both Sigma and Boehringer Mannheim Biochemicals (Indianapolis, IN).

New Zealand White rabbits (2–3 kg) were obtained from Hilltop Rabbits, Inc. (Columbia, TN). All centrifugation steps were performed with Beckman medium speed centrifuges (J-21) and ultracentrifuges with appropriate rotors.

Assay Methods

Protein concentrations were determined by the procedure of Lowry et al. (13) using bovine serum albumin as standard. Total phosphorus was measured by a modification of the method of Chen et al. (14), as described by Rouser and Fleischer (15), and provided an estimate of lipid phosphorus. Radioactivity was measured with a Searle Mark III scintillation counter with appropriate programming.

Samples were prepared for thin-section electron microscopy as described in Mitchell et al. (11) and/or Palade et al. (16). The former uses a small amount of sample to form a thin pellet that can be sectioned and viewed from top to bottom; the latter is a filtration procedure that makes use of dextran to insure a representative distribution of sample.

ENZYME ASSAYS: Samples from gradient centrifugation were diluted slowly over approximately one-half hour to 10% sucrose, sedimented with a Type 35 rotor (30,000 rpm for 60 min), and resuspended in 10% sucrose, 5 mM HEPES, pH 7.2 before assaying. Adenylate cyclase activity was determined by measuring the production of radioactive cAMP produced from α-^{32}P-ATP according to a modification of the method of Jakobs et al. (17). In our experience this activity is stable and can be carried out on samples frozen and thawed one time. Generally, assays were run for 5 min at 37°C. The assay medium contained final concentrations of 25 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$; 1 mM 1-methyl-3-isobutyl xanthine (M.I.X.) was included to inhibit phosphodiesterase activity. An ATP-regenerating system, consisting of 25 U/ml myokinase, 12.4 U/ml creatine phosphokinase, 1 U/ml adenosine deaminase, and 20 mM creatine phosphate, was used to insure constant ATP concentration and to avoid buildup of competitive inhibitors.

Ouabain sensitive Na^+-K^+ ATPase activity was titrated with SDS according to the method of Seiler and Fleischer (18). The sample (1 mg/ml) was pretreated with varying concentrations of SDS ranging from 0 to 0.3 mg/ml in a medium containing 40 mM imidazole-HEPES, 2 mM Tris-EDTA for 30 min at room temperature before enzyme assay. Ouabain sensitivity was assayed in the presence and absence of 1 mM ouabain. Reactions were started by the addition of sample (10–20 μl) to a 37°C assay mix containing 120 mM NaCl, 5 mM NaN_3, 20 mM KCl, 3 mM $MgCl_2$, 0.5 mM EGTA, 3 mM Na_2ATP in 30 mM imidazole buffer, pH 7.5 in a final volume of 0.5 ml. Reactions were allowed to proceed for 5–20 min and were stopped by the addition of 0.5 ml of 5% SDS, 10 mM EDTA, and placing on ice. Phosphate production was measured by a modification of the method of Baginski et al. (19). Ouabain-sensitive Na^+-K^+ ATPase was calculated as the difference between the amount of phosphate released in the presence and absence of 1 mM ouabain at the optimal SDS concentration, usually between 0.17 and 0.23 mg SDS/ml.

Succinate-cytochrome *c* reductase activity was measured as described by Fleischer and Fleischer (20) by measuring the increase in optical density at 550.5 nm in the presence of cyanide at 32°C.

Ca^{++} "loading" rates in the presence of phosphate were assayed using the metallochromic indicator arsenazo III (21) with a Hewlett-Packard type 8450A spectrophotometer. Removal of calcium from the medium was measured by following A_{660}-A_{740}. Samples (20–50 μg protein) were assayed at room temperature in the presence of 125 mM potassium phosphate buffer, pH 7.0, 1 mM $MgCl_2$, 1 mM Na_2ATP, and 10 μM arsenazo III. Several loadings were made with 25 μM $CaCl_2$ additions per loading; further additions of calcium were made after all the calcium was taken up. Each addition served as its own internal calibration; in this regard the uptake of such additions required many tens of seconds, so that any uptake during this addition was negligible. The loading rates from such individual additions did not differ significantly and were averaged.

RESULTS

The procedure for triad isolation is described with two variants, *standard* and *pyrophosphate*. These will be dealt with sequentially in the text, although the figures and tables may include data from both variants for comparison.

The two variants were run side-by-side using portions of the same stock of rabbit muscle. For each preparation, the hind leg muscles of a single rabbit (~200–250 g) were utilized. Connective tissue fat, nerve, and red muscle were removed. The leg muscle was supplemented with back muscle (100–150 g). The mixture was passed through a General model H meat grinder fitted with a 2-mm hole mincing plate to obtain 300 g of ground muscle. In total, 180 g was used for the *standard* variant and 120 g for the *pyrophosphate* variant.

A. *STANDARD* VARIANT

Packets of 60 g of ground muscle were homogenized in a Waring Blender at top speed for 1 min in 300 ml of medium consisting of 10% sucrose, 0.5 mM EDTA, pH 7.2 (Fig. 1). The homogenate was centrifuged at 9,000 rpm for 15 min in a JA-10 rotor. The supernatant was filtered through cheesecloth and recentrifuged at 14,000 rpm in a JA-14 rotor for 30 min to produce the heavy microsome fraction. Microsomes derived from 180 g of ground muscle were resuspended in a Dounce homogenizer with a loose fitting pestle in 15 ml of 10% sucrose, 5 mM HEPES, pH 7.1.

Preparation of Stage I Gradient (Triad-enriched) Material

Triads were enriched from microsomes by velocity gradient centrifugation. A combination step and continuous gradient was prepared (Fig. 2). Centrifugation was carried out using a precooled Beckman SW 27 rotor at 27,000 rpm (96,260 g_{av}) at 2–5°C for 90 min (at speed). The distribution of material at completion of the run is shown in Fig. 2 (tube designated *S*). The 90-min centrifugation permitted only the most dense material to approach its isopycnic density, resulting in good separation of triad plasmalemmal activities from light SR and two even lighter density plasmalemmal fractions which we previously described (22). Two main bands were well resolved. The lower band was enriched in triads. It consisted of light brown turbid material containing visible small white aggregates. The fraction contained substantial levels of adenylate cyclase activity (20–25 pmol/mg-min) (Table I*A*). The Ca^{++}-phosphate "loading" was roughly half that of the light SR. Examination by electron microscopy (not shown) indicated that the band contained a heterogeneous population including (*a*) intact triads (or dyads), (*b*) free "heavy" SR (24), (*c*) some free transverse tubule, (*d*) intact mitochondria, and (*e*) contractile protein. The triad-enriched fraction accounted for 15–20% of the total microsomal protein (See Table I*A*).

The upper band designated "light" SR layered at the interface between the 25% sucrose step and the continuous gradient (see Fig. 2). It exhibited much lower adenylate cyclase activity but higher rates of calcium phosphate loading (Table I*A*). Electron microscopy of this fraction revealed mostly light SR vesicles with relatively little heavy SR and few triads (not shown).

Stage II Gradient Purification

The major impurities in Stage I triad-enriched material were mitochondria and aggregated contractile material. Separation of triads from mitochondria required dissociation of contractile elements, which was achieved using a pyrophosphate mixture (20 mM sodium pyrophosphate, 20 mM NaH_2PO_4, and 1 mM $MgCl_2$, pH 7.1) and a second velocity (90-min) centrifugation. The Stage I gradient triad-enriched material was diluted slowly

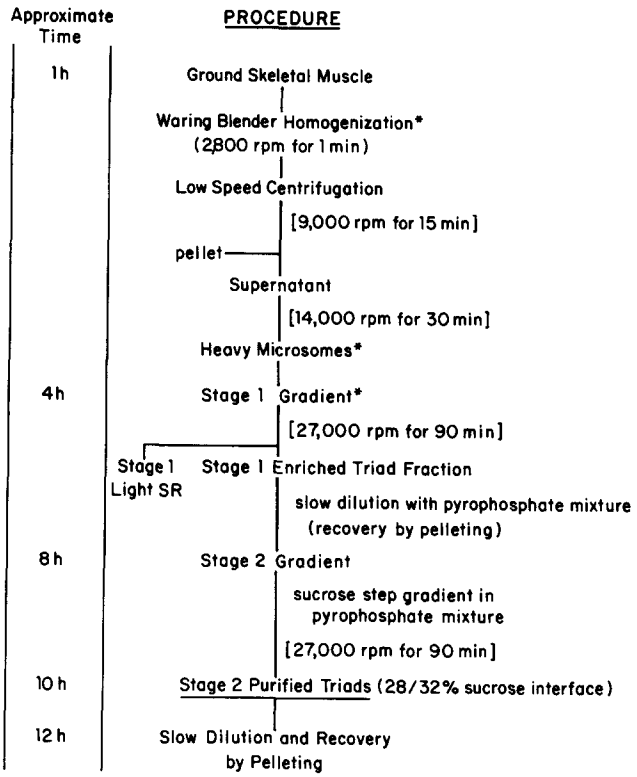

FIGURE 1 Flow diagram for isolation and purification of triads. Two variants are described. The *standard* variant does not introduce pyrophosphate mixture until the slow dilution of the Stage I gradient triads. The *pyrophosphate* variant contains pyrophosphate mixture in *all* solutions at all stages. The pyrophosphate mixture contains 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, and 1 mM $MgCl_2$, pH 7.1. The asterisk indicates the absence of pyrophosphate mixture in the solutions for the *standard* variant. The Stage I gradient and separation is illustrated in Fig. 2 and is summarized in Table I. The Stage II gradient separation is shown in Fig. 3 and is summarized in Table II. The time required to reach the stages of the purification is indicated on the left. The *standard* and *pyrophosphate* variants are discussed sequentially in the text.

with pyrophosphate mix to 10% sucrose, pelleted, and resuspended in pyrophosphate mix containing 10% sucrose. The sucrose step-gradient (Fig. 3) was fortified with the pyrophosphate mixture.

Protein and diagnostic enzyme distribution of the Stage II gradient purification is detailed in Table II*A*. Fractions F3, F4, and F5 contain the majority of the adenylate cyclase activity, and were enriched in triads as viewed by electron microscopy. Fraction F4, appearing at the 28/32% interface, displayed peak activity (50–65 pmol/mg·min). Identification of this material as purified triads is shown in Fig. 4*a* by electron microscopy; more extensive quantitative data are presented later in the text (see Table IV). Fraction F3 was contaminated with light SR, as indicated by its increased Ca^{++}-phosphate loading rates (Table II*A*) and by electron microscopy (not shown). Both fractions F4 and F5 consisted mainly of, and were considered, purified triads. Fraction F4 had only minor mitochondrial contamination (1.5%) while fraction F5 contained about 7% mitochondria as measured by particle counting. F4 appeared to be the more enriched of the two by adenylate cyclase and Ca^{++}-phosphate loading activity. The combined protein yield of fractions F4 and F5 is 5–6 mg/100 g starting muscle, accounting for ~60% of the adenylate cyclase activity of the Stage I triad-enriched starting material.

A preparation of purified nontriadic SR can also be obtained as a byproduct of the *standard* variant. The upper "light" SR band of the first stage enrichment is applied to a Stage II gradient; residual adenylate cyclase activity concentrates in the LF4 fraction (Table II *C*), leaving low levels of contaminating plasmalemmal adenylate cyclase activity in LF3 fraction.The highest levels of Ca^{++}-phosphate loading were found in Fraction LF3, which was generally 60% greater than that of Stage II purified triad and ~10% higher than that of Stage I light SR fractions. Examination by electron microscopy (not shown) indicated that this fraction was composed predominantly of vesicles without electron-opaque contents and was therefore considered to be light SR.

B. *PYROPHOSPHATE* VARIANT

This procedure is similar to the *standard* variant method, except that the pyrophosphate mixture is included throughout the purification. Because more protein is suspended during homogenization in the presence of the pyrophosphate mix, less ground muscle (40 g) was homogenized for every 300 ml of medium containing 0.5 mM EDTA, 10% sucrose (wt/wt) and pyrophosphate mixture. Otherwise, the preparation of microsomes employed the same centrifugation steps as outlined in Fig. 1 for the *standard* variant. The yield of microsomes was ~1.6-fold higher in the *pyrophosphate* variant compared with

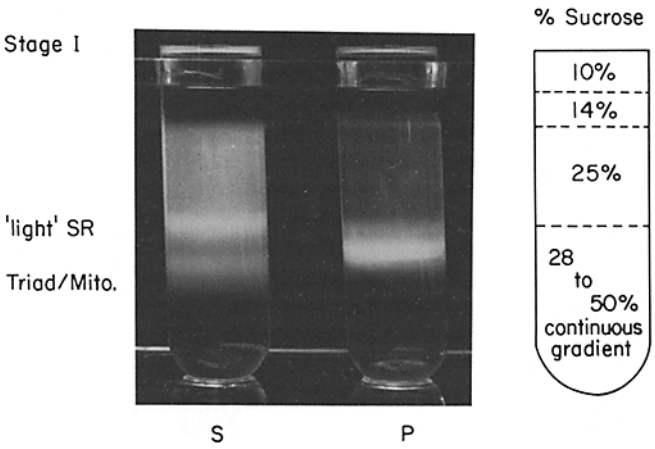

FIGURE 2 Preparation of Stage I enriched triads by fractionation of skeletal muscle microsomes. Purification of skeletal muscle microsomes is carried out by 90-min centrifugation in a combination step and continuous sucrose gradient. The right-hand diagram shows the concentrations of sucrose used in the gradient. Linear gradients from 28 to 50% sucrose were prepared 18.5 ml using 5 mM HEPES, pH 7.1 buffered solutions of 19% sucrose (wt/wt) and 57% sucrose (wt/wt) using a Beckman gradient former. The continuous gradients were formed at room temperature using chilled solutions and were equilibrated overnight at cold room temperature before application of the step gradient. A 12-ml step of 25% sucrose (wt/wt) was applied atop the continuous gradient followed by a second step (4 ml) of 14% sucrose (wt/wt) and 5 ml sample in 10% sucrose (wt/wt) were placed on top of the gradient in an SW 27 rotor tube. Results are shown for both variants. *S* refers to Stage I *standard* purification in 5 mM HEPES and sucrose, pH 7.2. *P* refers to Stage I *pyrophosphate* purification in 20 mM $Na_4P_2O_7$, 20 mM NaH_2PO_4, 1 mM $MgCl_2$ and sucrose. In each case, the lowest lying band in the tube is the Stage I enriched triad fraction. In the *standard* preparation the light SR and triad/mitochondrial fractions are clearly separated. For the *pyrophosphate* variant the two bands are less well resolved. The light SR fraction is located above and appears whiter. The triadic/mitochondrial fraction is immediately below and is whitish in appearance at the top and orange-brown at the bottom.

TABLE I

Summary of Stage I Gradient Fractionation

	Recovery			Enzymic activity		
	Tissue	% of microsomal protein	Ratio of protein triads/light SR	Adenylate cyclase	Ca^{++}-Phosphate Loading	Succinate-cytochrome *c* reductase
	mg/100 g			*pmol/mg·min*	*μmol/mg·min*	*nmol/mg·min*
A. *Standard* Variant						
Light SR	21.3 ± 3.7	21.7 ± 4.2	0.85 ± 0.21	5.6 ± 1.2	3.45 ± 0.56	7.5 ± 3.3
Enriched triadic material	17.9 ± 4.1	18.2 ± 4.9		21.4 ± 2.6	1.56 ± 0.05	79.3 ± 24.4
B. *Pyrophosphate* Variant						
Light SR	31.9 ± 5.9	21.1 ± 6.7	2.04 ± 0.23	Variable	1.94 ± 0.56	51.3 ± 15.3
Enriched triadic material	64.4 ± 10.7	40.7 ± 7.1		15.6 ± 2.2	1.04 ± 0.04	130.0 ± 13.0

A summary of Stage I enrichment of triads. Four successive preparations of Stage I light SR and triad-enriched material were isolated and assayed for protein and enzymic activities. The results are expressed as the mean with standard deviation. The gradient purification is shown in Fig. 2. *Standard* and *Pyrophosphate* variants are compared. Yields were normalized for 100 g of ground white muscle and are expressed in terms of mg obtained/100 g ground muscle, % of microsomal protein, and the ratio of protein in triad to light SR functions. Adenylate cyclase was assayed at 37°C as an index of transverse tubule. Rates of 375 pmol/mg min have been obtained in our laboratory for transverse tubule isolated using the French Pressure cell technique of Lau et al. (4) for disruption of triads and subsequent gradient centrifugation. Comparable rates (313 pmol/mg min) have been reported for purified surface sarcolemmal vesicles from rabbit skeletal muscle (18). Phosphate facilitated calcium "loading" was assayed as an index of sarcoplasmic reticulum. Purified light SR exhibits a rate approaching 4–5 μmol/mg·min when assayed promptly after preparation and without freezing. The determinations shown here were performed on material frozen and thawed once and are, therefore, less than optimal in activity. The mitochondrial marker enzyme, succinate-cytochrome *c* reductase activity was assayed at 32°C. In order to estimate mitochondrial contamination, a rate of 800–900 nmol/mg·min (20, 23) was used.

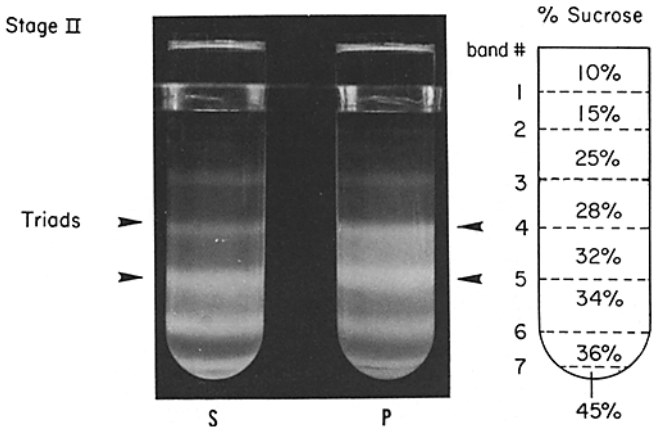

FIGURE 3 Stage II gradient purification of triads using *standard* (*S*) and *pyrophosphate* (*P*) variants. The sucrose step gradient employed is shown in the diagram at the right. Stage I triad-enriched material, in hypertonic sucrose from the gradient, was diluted slowly (30–45 min) with the pyrophosphate mixture to a sucrose concentration of 8–12%, using gentle stirring. The diluted material was sedimented (Type 35 rotor, 60 min at 30,000 rpm) and resuspended in the pyrophosphate mixture containing 10% sucrose (wt/wt) and applied to a discontinuous sucrose gradient. The gradient was constructed with steps of 0.5 ml 45%, 4.5 ml 36%, 6 ml 34%, 6 ml 32%, 6 ml 28%, 6 ml 25%, and 4 ml 15% sucrose. Each step was fortified with the pyrophosphate mixture. Sample, equivalent to one SW 27 tube of gradient I, was applied in 5 ml, and velocity centrifugation was carried out at 27,000 rpm for 90 min at 2–5°C using a Beckman SW 27 rotor. The distribution of material in the gradient after centrifugation is shown. Stage II gradients for both variants contain pyrophosphate mix. Band positions of fractions are indicated to the right of tube *P*. Purified triads are located in fractions 4 and 5. Enzymic characterization of the fractions in the gradient is given in Table II.

the *standard* variant (calculated from Table I). Microsomes from 120 g of ground muscle were resuspended in 15 ml of 10% sucrose containing pyrophosphate mixture.

The Stage I gradient separates microsomes into two partially resolvable bands (Fig. 2, tube labeled *P*). The separation of triads is incomplete and accounts for the variability in adenylate cyclase activity found in the upper band (Table I *B*). The lower band comprised ~40% of the total microsomal protein (Table I *B*) and was found by electron microscopy to primarily contain triads, mitochondria, and contractile aggregates (not shown). Compared with the *standard* variant, the yield of triad-enriched material was 3.5-fold greater, although adenylate cyclase specific activity was consistently ~30% lower (Table I *B*). The Stage I gradient triad-enriched material was diluted slowly with pyrophosphate mix to 10% sucrose, pelleted, and resuspended in pyrophosphate mix containing 10% sucrose.

The second stage gradient purification of the *pyrophosphate* variant was carried out as in the *standard* variant except for application of more sample. The separation pattern was similar (Fig. 3, tube labeled *P*). Table II *B* demonstrates that adenylate cyclase specific activity was again highest in the 28/32% interface fraction (F4), which consisted predominantly of morphologically well-preserved triads (Fig. 4 *b*). From the standpoint of yield, the final purified F4 triad fraction of the *pyrophosphate* variant was fourfold greater than that from the *standard* preparation, normalized per 100 g of starting ground muscle. Substantial adenylate cyclase levels were also found in F5, which, compared with F4, displayed higher succinate-cytochrome *c* reductase levels and lower Ca^{++}-phosphate loading rates. ~10 mg of F4 material, or 25 mg of F4 and F5 together, were isolated with the *pyrophosphate* variant from 100 g of starting ground muscle. Curiously, the adenylate cyclase specific activities were only about half as high as those of the *standard* variant.

COMPARISON OF TRIAD FRACTIONS

Diagnostic enzymic activities of triad fractions prepared by the two variants of the current purification procedure and by two other methods are compared in Table III. From the standpoint of adenylate cyclase activity, the Stage I gradient enriched triad material from both variants are in the same range, although that from the *standard* variant is significantly higher. Each Stage II gradient procedure results in enhanced cyclase activity, although purified triads prepared by the *standard* variant exhibit approximately twofold higher specific activity of adenylate cyclase than those prepared by the *pyrophosphate* variant.

Na,K-ATPase, another diagnostic for transverse tubule (4), demonstrated similar enhancement of levels through a second

TABLE II

Stage II Purifications of Triads and Light Sarcoplasmic Reticulum

Fraction	% sucrose at interface	Protein		Enzymic activity					
				Adenylate cyclase		Ca^{++}-Phosphate loading		Succinate-cytochrome c reductase	
		mg	% of Total	pmol/mg·min	% of Total	μmol/mg·min	% of Total	nmol/mg·min	% of Total
A. Summary of Stage II gradient purification of enriched triads prepared by the *Standard* variant									
Stage I	—	16.00	100.0	28.3	100.0	1.61	100.0	118.9	100.0
Stage II									
F1	10/15	0.43	2.7	ND	—	ND	—	34.3	0.0
F2	15/25	0.51	3.2	7.0	1.0	ND	—	40.0	1.3
F3	25/28	1.26	7.8	24.2	8.5	2.91	14.2	25.4	2.1
F4 (triads)	28/32	2.35	14.7	56.1	36.9	1.81	16.5	15.5	2.4
F5 (triads)	32/34	2.99	18.7	28.6	23.9	1.15	13.3	52.5	10.4
F6	34/36	3.49	21.8	8.6	8.4	0.92	12.5	163.8	38.1
F7	36/45	1.58	9.8	4.8	2.1	1.12	6.8	349.9	36.8
Recovery			78.7		80.8				91.1
B. Summary of Stage II gradient purification of enriched triads prepared by the *Pyrophosphate* variant									
Stage I	—	68.40	100	15.7	100.0	1.0	100.0	137.5	100.0
Stage II									
F1	10/15	2.09	3.0	33.0	6.5	ND	—	39.0	0.8
F2	15/25	2.85	4.2	10.1	2.7	ND	—	33.0	1.0
F3	25/28	6.52	9.5	16.8	10.2	ND	—	27.5	1.9
F4 (triads)	28/32	10.72	15.7	27.1	27.2	1.41	15.1	35.5	4.0
F5 (triads)	32/34	16.00	23.4	23.5	35.0	0.56	9.0	82.5	14.0
F6	34/36	12.56	18.4	ND	—	0.63	7.9	302.5	40.4
F7	36/45	8.85	12.9	ND	—	0.70	9.2	468.5	44.0
Recovery			87.1		>81.6				106.1
C. Summary of Stage II gradient purification of light SR prepared from the *Standard* variant									
Stage I	—	28.90	100.0	4.8	100.0	4.9	100.0	6.4	100.0
Stage II									
L F3	25/28	9.54	33.0	2.2	15.1	5.2	34.9	8.6	44.0
L F4	28/32	9.07	31.3	12.1	79.1	3.7	23.6	7.6	37.2

Stage II gradient purification of triads. Stage I triad material from *Standard* and *Pyrophosphate* variants was further purified using Stage II gradient purification (Fig. 3). The amount of Stage I triad material recovered from 100 g of ground muscle is given as Stage I (protein). A total of six SW 27 tubes (applying approximately 20 mg and 60 mg protein per tube for *Standard* and *Pyrophosphate* variants, respectively) is convenient for a single preparation involving one or two rabbits and one SW 27 rotor for both the *Standard* and *Pyrophosphate* variants. The fractions were assayed for protein, adenylate cyclase, phosphate facilitated calcium loading, and succinate-cytochrome c reductase. Protein and Ca^{++}-phosphate loading are values obtained from a single preparative run while succinate-cytochrome c reductase and adenylate cyclase are values averaged from several preparations. All assays were carried out on samples that were quick-frozen and singly thawed, except for the phosphate-facilitated loading in Table II *C*, which was performed on fresh material after the second stage of purification of light SR by the *Standard* variant. Purified triads, Stage II *Standard* (F4), assayed fresh without freezing and thawing, demonstrate Ca^{++}-loading rates of 2.5–3 μmol/min·mg protein. The reduction in activity revealed in Tables II *A* and II *B* is the result of assaying material that was frozen in the absence of 0.1 M KCl; the latter is required for stabilizing activity (25). *ND*, not determined.

stage of purification. However, for this enzymic activity, the purified triads from the *pyrophosphate* variant have more than twofold higher specific activity than the purified product from the *standard* variant. The calcium-phosphate loading rates of the purified triads (F4 fractions) from both variants are similar.

ELECTRON MICROSCOPIC ASSESSMENT OF TRIAD PURITY

Attempts have also been made to quantitate the triad content of fractions from both the *standard* and *pyrophosphate* variants. Triad fractions were prepared from four consecutive runs of both *standard* and *pyrophosphate* variants (Stage I gradient triad fraction, and Stage II gradient, Fractions F4 and F5). Quantitation of vesicle types and number were made by thin-section electron microscopy. To facilitate quantitation, a filtration procedure was developed that makes use of high molecular weight dextran as a nonosmiophilic spacer substance (16). This method insures uniform sample distribution permitting quantitation without necessitating sectioning entirely through the pellet, as is required for pellets obtained by centrifugation. In one instance, in which both methods of sample preparation were compared, essentially similar results were obtained.

Only vesicular structures were counted,[2] and these were segregated into the categories shown in Table IV. The identification of junctional structures involved subdivision into "probable" and "possible" categories (cf. Legend, Table IV) in order to reduce observer bias. Half or more of the vesicles counted in both *standard* and *pyrophosphate* Stage II, F4 fractions fell into the "probable" junctional structure category, which averaged 1.5 terminal cisternae per transverse tubule.

Particle counting (Table IV) confirms enzymic data (Tables II *A* and III) indicating a further enrichment of *standard* triads by the second stage of purification, but it does not reflect a similar purification for the second stage of the *pyrophosphate* variant. Particle count comparison of *standard*, F4 and F5 fractions suggests greater similarity than do the enzymic data

[2] Contractile protein was observed as a minor contaminant in Stage I preparations of both variants but was not evident in purified Stage II triads.

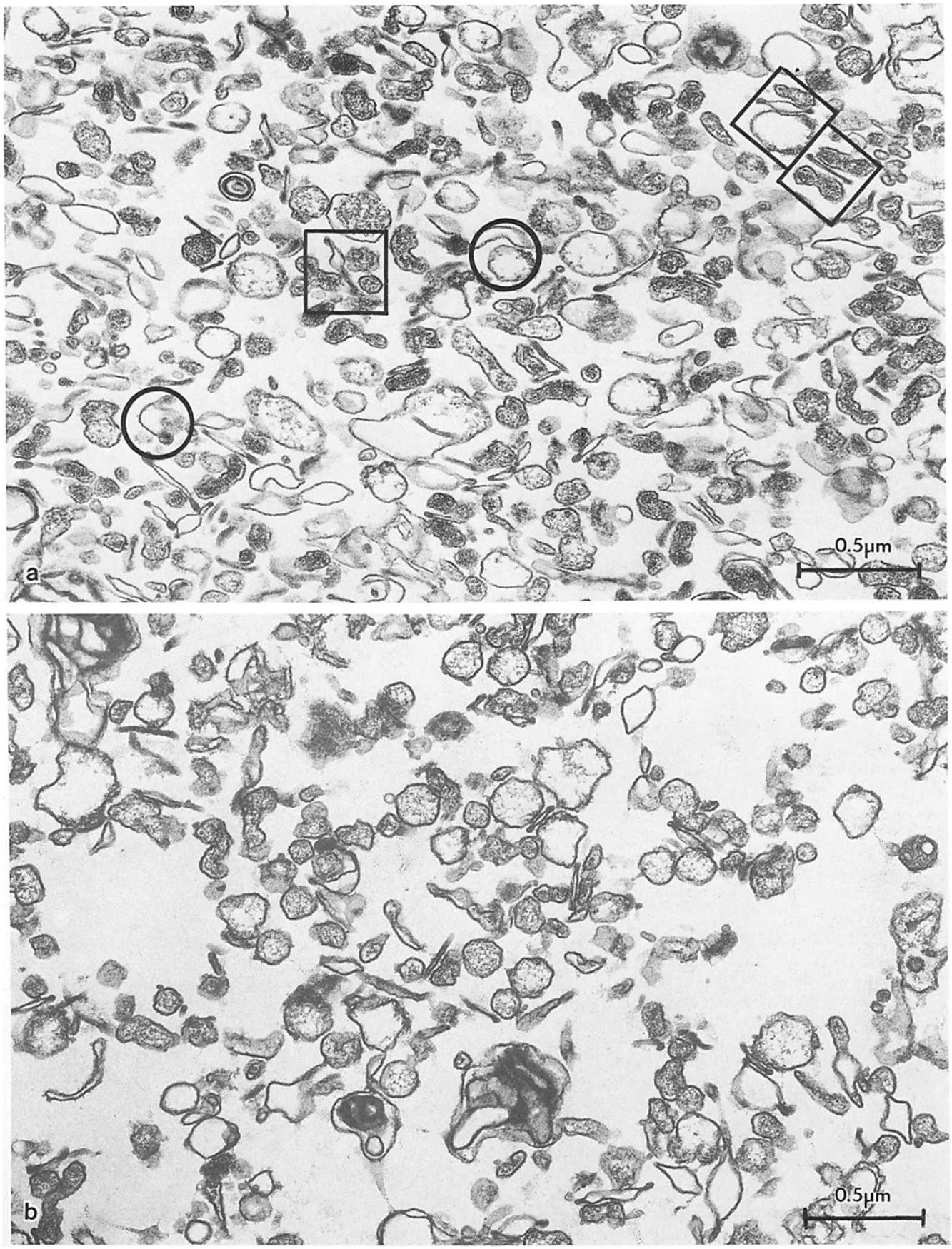

FIGURE 4 (*a*) Electron microscopy of a representative section Stage II gradient fraction 4 triads prepared according to the *standard* variant. The triads were fixed with glutaraldehyde in suspension and filtered according to (16) to ensure representative sampling. A representative field is shown. × 45,000. Material enclosed by square is considered a "probable" junctional structure (i.e. terminal cisternae and transverse tubule are juxtaposed appropriately to suggest junctional association and are in the field of focus). Material enclosed by circle is considered a "possible" junctional structure (see legend to Table IV). (*b*) Electron microscopy of Stage II gradient, fraction 5 triads prepared according to the *pyrophosphate* variant. × 45,000.

TABLE III

Comparison of Enzymic Characteristics of Isolated Triad Fractions Obtained by Different Procedures

	Na,K-ATPase	Adenylate cyclase	Ca^{++} Phosphate "loading"
	μmol/mg·h	*pmol/mg·min*	*μmol/mg·min*
I. Isopycnic enrichment by Caswell et al. (3, 5)	ND	13.0 ± 7.0	ND
II. KCl preparation (30)			
Stage I enrichment (isopycnic)	ND	17.4 ± 3.2	ND
Purification (Stage II gradient)	ND	41.2 ± 4.4	ND
III *A. Standard* variant			
Stage I enrichment	0.96 ± 0.19	21.4 ± 2.6	1.56 ± 0.05
Purified triads (Stage II gradient)	2.16 ± 0.79	57.5 ± 8.5	1.35 ± 0.20
III *B. Pyrophosphate* variant			
Stage I enrichment	2.76 ± 0.03	15.6 ± 2.2	1.04 ± 0.04
Purified triads (Stage II gradient)	4.96 ± 0.63	27.1 ± 7.0	1.43 ± 0.20

Comparison of enzymic characteristics of isolated triad fractions obtained by several different procedures. Procedure III *A* and III *B* are described in the text. The Stage II triad fractions given in the table are Fraction 4 (Table II). The Na,K-ATPase and adenylate cyclase assays were carried out at 37°C while the Ca^{++}-phosphate loading was at 25°C. The only triad fractionation in the literature is the isopycnic purification of Caswell et al. (3, 4). The first stage of the KCl preparation (30) has some similarity to the Caswell preparation in that it involves an isopycnic sucrose gradient of microsomes in the absence of KCl. The second stage of purification employed 0.7 M KCl in the gradient to disaggregate contractile protein during a 2.5-h centrifugation (30). Limited enzymic data is available for the Caswell fraction, which has been used primarily as an intermediate for transverse tubule isolation. Transverse tubule isolated by Lau et al. (4) was reported to have a Na,K-ATPase activity of 6.18 μmol/mg·h when assayed in the presence of NaI at 37°C. Transverse tubule isolated in our laboratory in a manner similar to Caswell from purified triads prepared by the *Standard* variant gave a Na,K-ATPase rate of 39 μmol/mg·h and 375 pmol/mg·min for basal adenylate cyclase activity. Maximal Na,K-ATPase activity was measured using optimal SDS concentrations as described in the Materials and Methods. *ND*, not determined.

(Table II *A*), but a similar comparison of *pyrophosphate* Stage II F4 and F5 fractions corresponds with the similarity of the two fractions enzymically (Table II *B*).

Comparison of particle counts of *standard* and *pyrophosphate* Stage II gradient fractions (F4) suggests that the purified triad fractions were similar (Table IV). We cannot explain by electron microscopic quantitation either the higher cyclase activity of the *standard* preparation or the higher Na,K ATPase activity of the *pyrophosphate* preparation (Table III). Nevertheless, both the enzymic and morphological data suggest that the purified triads are highly enriched (~50%).

DISCUSSION

The isolation of a subcellular structure is an important step in its biochemical and functional characterization. Thus far, a purified preparation of triads has not been available. We now report the development of a procedure for the isolation of a fraction highly enriched in triads. The purity is estimated by particle counting to be in the vicinity of 50% (Table IV). The isolated triads retain considerable architectural detail (11) characteristic of that observed in intact tissue (8–10), including the junctional feet structures that join transverse tubule and terminal cisternae (11).

The only previous work concerned with the isolation of triads is by Caswell et al. (3) who achieved some enrichment of triads from a microsomal fraction of rabbit skeletal muscle. The fraction was used mainly for the isolation of transverse tubule (4). Their study served to direct attention to the preparation of triads but did not include any quantitation that would permit an evaluation of enrichment or purity. In the six years that followed, no report of the purification of triads has appeared in the literature, although more recently Caswell's laboratory reported studies involving junctional reassociation (6, 26, 27) between purified transverse tubule and terminal cisternae.

The isolation of triads from rabbit skeletal muscle described here is straightforward and reproducible. The procedure consists of the preparation of a "heavy microsome" fraction and two sequential 90-min gradient centrifugations. The entire procedure takes about 12 h. The method yields adequate amounts of sample for biochemical study. The muscle from one rabbit will yield ~15 or 75 mg of purified triads (combined F4 and F5) by the *standard* or *pyrophosphate* variants, respectively.

The most critical problem in triad isolation was to devise conditions to disaggregate membrane components in order to achieve purification by centrifugation. The aggregation appears to be referable to contractile proteins and is therefore a problem inherent in the purification of membrane fractions from muscle. Most preparations of sarcoplasmic reticulum or plasmalemma from skeletal muscle make use of a salt presoak containing high concentrations of KCl or LiBr (22, 24, 28, 29). Initially, we used 0.6–0.7 M KCl (30), which enabled us to achieve separation and good purification of triads based on enzymic criteria, but the morphology was severely altered (11, 30).

Early work by Hasselbach and Schneider (31) and Hanson and Huxley (32) recognized that pyrophosphate could be used to solubilize actomyosin. Mg^{++} was found to be essential for the dissociation process (33). More recently, pyrophosphate has been used in the isolation of sarcolemma and sarcoplasmic reticulum from heart muscle (34–38). The application of pyrophosphate to the purification of triads required fine-tuning. In an effort to effectively solubilize actomyosin aggregates, a variety of PP_i/Mg^{++} combinations were substituted for KCl. Low levels of PPi (20 mM) were sufficient to break up actomyosin complexes for purification of triads. At higher levels (50 mM PPi/1 mM $MgCl_2$), swelling of the terminal cisternae portion of the triad was observed with loss of contents, similar to that seen (30) with high concentrations of KCl. In our experience, pyrophosphate from different batches and sources (Fisher Scientific Co. and Alfa Div., Ventron Corp.) can behave somewhat differently so that minor adjustment in concentration with a new batch of reagent may be necessary for optimization. Some $MgCl_2$ was required for the procedure to be effective, although $MgCl_2$ concentrations in excess of 1 mM caused gross aggregation. The pyrophosphate mixture described was satisfactory for dissociating contractile elements, enabling separation of membrane components with retention of triad morphology.

Two variants have been described for preparation of triads that differ mainly with regard to when the pyrophosphate

TABLE IV

Morphological Quantitation of Stage I and Stage II Gradient Fractions Obtained by Standard and Pyrophosphate Variants of the Triad Isolation Procedure

	Junctional structures				Nonjunctional structures		Other structures		
	Probable		Possible						
	TC	t-Tubule	TC	t-Tubule	TC	t-Tubule	Small empty vesicles (LSR)	Mitochondria	Large empty vesicles (PM)
A. *Standard* variant									
Stage I	18.9 ± 5.8	15.5 ± 4.1	13.9 ± 2.5	13.8 ± 3.5	4.7 ± 1.6	2.83 ± 0.77	16.7 ± 1.07	8.04 ± 2.66	1.06 ± 0.89
Stage II									
Fraction 4	31.1 ± 3.3	21.4 ± 3.0	15.4 ± 8.0	7.7 ± 3.3	11.8 ± 3.8	2.28 ± 0.61	6.56 ± 2.61	1.67 ± 0.80	0.43 ± 0.28
Fraction 5	28.3 ± 3.9	19.0 ± 2.8	14.4 ± 2.2	12.0 ± 1.3	11.6 ± 2.7	2.72 ± 1.36	2.94 ± 1.60	6.78 ± 2.05	0.25 ± 0.20
B. *Pyrophosphate* variant									
Stage I	31.8 ± 5.0	22.1 ± 1.6	8.8 ± 0.3	7.9 ± 0.4	9.4 ± 2.0	1.30 ± 1.05	7.81 ± 1.55	5.70 ± 1.92	0.47 ± 0.22
Stage II									
Fraction 4	33.3 ± 3.1	21.8 ± 3.4	13.7 ± 3.3	10.7 ± 2.5	12.7 ± 3.6	2.57 ± 0.84	3.35 ± 0.69	0.83 ± 0.32	0.24 ± 0.18
Fraction 5	34.9 ± 3.2	25.1 ± 2.8	10.1 ± 1.8	7.9 ± 1.2	12.1 ± 4.5	2.19 ± 1.03	3.43 ± 1.94	3.02 ± 0.65	0.18 ± 0.35

Morphological quantitation of Stage I and Stage II triad fractions by particle counting. Four consecutive preparations of *Standard* and *Pyrophosphate* triadic material were filtered according to (16) to obtain representative samples. Electron micrographs of representative fields were independently tallied by two investigators (R. D. Mitchell and P. Palade). Between 1,000 and 3,000 vesicles were counted for each fraction. Terminal cisternae and transverse tubule can readily be resolved morphologically. The term junctional structures refers to t-tubule and terminal cisternae, oriented with respect to one another so as to suggest junctional association. Triad material is classified as junctional material and is distinguished from nonjunctional triad components (i.e., free transverse tubule and terminal cisternae [TC]). Apposed transverse tubule and TC that were clearly visible with all portions of the structure in the plane of section were tallied as *probable junctional structures*, even if bridging structures were not clearly evident. *Possible junctional structures* refers to less certain identification in which t-tubule or terminal cisternae are identifiable and positioned near material that was out of the plane of section. See text for further discussion of morphological criteria for contaminating structures. Values, which appear in each category, are given as the percentage of all the vesicles counted for each fraction. Such values do not reflect the greater protein mass of some structures compared with others.

mixture is introduced. The *pyrophosphate* variant contained pyrophosphate mixture in all solutions beginning with homogenization, whereas the *standard* variant contains solutions fortified with pyrophosphate mixture beginning with the slow dilution of Stage I gradient triads. The early introduction of pyrophosphate gives a higher recovery of microsomes, and a four- to fivefold greater yield of triads. The *pyrophosphate* as compared with the *standard* variant is characterized by a higher Na,K-ATPase and lower adenylate cyclase at both stages of gradient purification. The basis for such differences will be dealt with in a later communication (Mitchell, R. D., P. Volpe, P. Palade, and S. Fleischer, manuscript submitted for publication.).

A variety of contaminants must be separated from triads including contractile protein, mitochondria, and nontriadic sarcoplasmic reticulum and transverse tubule. A "heavy" microsome fraction was obtained in order to reduce light SR contamination, even though the content of mitochondria and contractile protein initially may have been higher. Of the two variants described, the *pyrophosphate* microsomes contained more triads and mitochondria and less light SR. Velocity gradient centrifugation was effective for further purification in the *standard* Stage I enrichment. The two bands in *pyrophosphate* Stage I separation were less well separated, but the procedure was still effective to remove most free light SR from the triad-enriched material.

The mitochondrial contamination in our Stage I fraction is aggregated together with the triads and requires disaggregation conditions for further purification. A simple discontinuous sucrose gradient containing pyrophosphate mixture was effective in achieving the Stage II purification. As long as conditions for disaggregation were used, plasmalemma and free transverse tubule remained at the top of the gradient. The purified triads, especially Fraction 4, have only minor contamination by mitochondria (<1.7%), light SR (<6.6%) and larger vesicles probably of plasma membrane origin (<0.5%) as judged by particle counting by electron microscopy (Table IV). The low mitochondrial contamination is confirmed by marker enzyme assay (Table II). Fraction 5, though slightly less pure, is a respectable triad preparation as well, particularly in the *pyrophosphate* variant (Fig. 4*b*).

At present, no enzymic property has been localized histochemically that is biochemically unique to the triad structure and not to either of its component membranes, the transverse tubule or sarcoplasmic reticulum. Since triads could potentially co-isolate in regions of sucrose gradients containing sarcoplasmic reticulum, we employed transverse tubule markers, i.e., Na,K-ATPase and adenylate cyclase, which have been validated as simple diagnostics for triads by the work of Caswell and his colleagues (4, 5, 7). However, transverse tubule enzymic activities are shared by other sources of muscle and nonmuscle plasma membrane. A second consideration is that optimizing for even valid marker enzymes for transverse tubule and SR does not necessarily insure that these structures are in junctional association or retain good morphology. Thus, quantitation at the level of the electron microscope was used to check the purity of our fractions. The proportion of junctionally associated vesicles in both *standard* and *pyrophosphate* Stage II gradient F4 fractions was estimated to be in the vicinity of 50%.

Enzymic and electron microscopy criteria lead to somewhat different estimates of purity of the triads in comparing some of the fractions. This is because both methods, although the best available, are inadequate to the task. Both reflect uncertainties that are limiting. Particle counting is somewhat subjective with regard to triad identification; further, it counts the number and ignores size. Differences in soluble adventitious or compartmental protein are not considered by particle counting but markedly alter the specific activity by enzymic criteria. An additional problem is that the two diagnostic enzymes for t-tubule, adenylate cyclase and Na,K-ATPase, may be subject to differential inactivation during purification (Mitchell, R. D., P. Volpe, P. Palade, and S. Fleischer, manuscript submitted for publication).

Our *standard* and *pyrophosphate* procedures differ only in the extent of pyrophosphate exposure during preparation. At this point in time we have insufficient data available to recommend one variant over the other, despite certain clear enzymic differences. In both cases, the morphology of the purified triads is similar and resembles that of the structure in vivo. A highly enriched triad preparation is now available that may open the door to studies attempting to simulate a t-tubule mediated calcium release process in vitro. We have also de-

scribed the preparation of a defined light SR fraction, isolated under similar conditions to the triads, which may provide an important control for such Ca^{++}-release studies.

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