# Isolation and Characterization of the Major Protein Component of Sarcotubular Membranes*

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ABSTRACT: Treatment of highly purified sarcotubular membranes from rat skeletal muscle with 10 mM sodium dodecyl sulfate dissolved in a low ionic strength solution at pH 7.4 renders soluble more than 95% of the membrane protein. By Sepharose 4B column chromatography more than 90% of the soluble protein is recovered in the eluate comprising a single peak called fraction-2. The protein molecules in fraction-2 are large molecular weight aggregates of low molecular weight subunits. On the basis of gel electrophoresis and Sephadex gel filtration, all subunits were found to be either identical or similar molecular entities with a molecular weight of approximately 6500. These subunits contain N-terminal glycine residues only and C-terminal alanine residues. Calculation of molecular weight on the basis of alanine being the sole C-terminal amino acid yields a value of approximately 10,000. The probability that the protein isolated is the major protein unit responsible for the molecular architecture of these membranes is discussed.

Green *et al.* (1961) isolated a protein component of mitochondrial membranes which they called "structural protein." It was found to account for at least 30% of the protein in these membranes and on the basis of its several characteristics, these investigators suggested that structural protein plays a skeletal role in the molecular organization of mitochondrial membranes. Subsequently, Criddle *et al.* (1966) presented evidence which indicated that this structural protein is a single species of protein. Moreover, Richardson *et al.* (1963) found that structural protein similar to that in mitochondria can be isolated from a wide variety of biological membranes, and they hypothesized that structural proteins are necessary for the morphologic integrity of all biological membranes. On the basis of recent studies, Green and coworkers (1968) have concluded that structural protein is not a homogeneous protein entity but rather a family of closely related protein species. They also found that there is another family of proteins, called "core proteins," present in mitochondrial membranes and that core proteins also play a skeletal role in the morphology of these membranes. Even more recently Ward and Pollak (1969) reported that the structural protein of rat liver microsomes is heterogeneous and differs in several ways from mitochondrial structural protein.

The report of Schatz and Saltzgaber (1969), however, casts some doubt on the entire concept of structural protein for it indicates that a significant fraction of what has been called structural protein in mitochondria represents a denatured form of mitochondrial ATPase and therefore must be considered an artifact. They further point out that although specific mitochondrial structural protein may exist, such protein has not yet been isolated in a pure form. Moreover, Schnaitman (1969) has found that inner mitochondrial membrane contains 23 different protein species, the outer membrane 12, and smooth and rough microsomal membranes 15 different proteins and that no individual protein species accounts for more than 10 to 15% of the total protein of the given membrane fraction.

Clearly the protein structure of the membranes studied is so complex as to present a formidable barrier to the investigation of their molecular structure and its relationship to their biochemical activity. It seems apparent that a membrane with a much simpler protein composition could play an important role as an experimental prototype for the molecular investigation of membrane structure and function. That the sarcotubular membranes isolated from skeletal muscle might provide such a prototype is suggested by Mommaerts' concept (Mommaerts, 1967) that sarcotubular membranes are highly specialized for the task of ejecting and resorbing massive amounts of $Ca^{2+}$ exceedingly rapidly (in milliseconds). On the basis of currently available data, he further theorizes that there is little room in the sarcotubular membrane for protein that is not actively involved in the $Ca^{2+}$ transport process.

If Mommaerts' hypothesis is correct, it seems quite possible that most of the protein of the sarcotubular membrane is composed of a very limited number of molecular species; if so, this membrane would be the prototypic one to seek. We therefore initiated a study in this area. The first step was the development of a method for preparing a population of isolated membranes that are primarily, if not solely, derived from the sarcoplasmic reticulum. This was achieved with rat skeletal muscle by combining differential centrifugation with the sequential use of two density gradient centrifugation steps (Yu *et al.*, 1968a); purification of the membranes was monitored by measuring calcium transport activity and the $(Ca^{2+} + Mg^{2+})$-ATPase activity which is associated with the $Ca^{2+}$ transport process (Hasselbach and Makinose, 1963). The membrane fraction with the highest specific

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activity for these processes is called fraction $SF_1$[1] and is now routinely isolated. That fraction $SF_1$ is derived primarily, if not exclusively, from the sarcoplasmic reticulum is supported by the recent morphological analysis of it by Deamer and Baskin (1969). The present communication is a report of studies on the protein composition of fraction $SF_1$. A preliminary report of some of the data has been published (Masoro and Yu, 1969).

## Materials and Methods

Membrane fraction $SF_1$ was prepared from rat skeletal muscle by the method of Yu *et al.* (1968a). The proteins soluble at high ionic strength, which Martonosi (1968) reported to be contaminants of muscle microsomes, were removed from the $SF_1$ by the following modification of Martonosi's method: $SF_1$ is extracted with a 0.6 M KCl, 80 mM Tris-maleate, 0.3 M sucrose solution (pH 7.2) for 1 hr at 2°. The extracted membranes,[2] which have full ($Ca^{2+}$ + $Mg^{2+}$)-ATPase and $Ca^{2+}$ uptake activity, are harvested by centrifugation at 105,000*g* for 60 min, washed with 0.3 M sucrose, and again collected in a pellet by ultracentrifugation. This pellet is homogenized in 1 ml of the following medium per 3 mg of sarcotubular protein: 10 mM sodium dodecyl sulfate, 8 mM NaCl, 2.5 mM Tris, pH 7.4 at 25°. The homogenate rapidly becomes totally transparent. This "sarcotubular solution" is dialyzed at 2° for 36 hr against frequent changes of a solution A of the following composition: 8 mM NaCl, 2.5 mM Tris, pH 7.4 at 25°; in the initial experiments, both the homogenization solution and the dialysis solution (*i.e.*, solution A) also contained 0.5 mM 2-mercaptoethanol. Little loss of lipid or protein occurs during dialysis, but experiments with [$^{35}$S]sodium dodecyl sulfate showed that 97–98% of the detergent is removed from the dialysate during the dialysis. To remove readily sedimentable material from the dialysate, it is centrifuged for 30 min at 48,000*g*. The supernatant contains about 95% of the $SF_1$ protein and a precipitate which contains about 5% of the protein is discarded. This supernatant was the source of solubilized membrane protein for all the studies reported in this communication.

The protein components in this supernatant are fractionated by Sepharose 4B (Sigma Chemical Co.) gel filtration as follows. Sepharose 4B is equilibrated with solution A and packed in a column. Solution A is the eluent. A 1-ml aliquot of the supernatant, which contains 2.12 mg of sarcotubular protein and 5% Ficoll, is added to the column; solution flow rate is 6.35 ml/cm$^2$ per hr. Each collection tube contains 1.9 ml of eluate. The protein concentration profile is roughed out by ultraviolet monitoring of the eluate. The protein concentration of the contents of each tube is measured by the method of Lowry *et al.* (1951); the interference of Tris (and 2-mercaptoethanol when it was used) was assessed and found not of significant magnitude to interfere with interpretation of the data. The per cent recovery of applied protein is assessed in separate experiments in which the elution peaks are again monitored by ultraviolet; the contents of the tubes that comprise a peak are pooled and the protein content of this pool assayed by the method of Lowry *et al.* (1951).

The protein components of the supernatant are also fractionated by Sephadex G-200 gel filtration by procedures similar to those described for the Sepharose 4B gel filtration studies.

To determine the amino acid content of a given protein fraction, it is hydrolyzed in evacuated tubes in 6 N HCl at 110° for 24 hr. After hydrolysis, the HCl is evaporated and the dried sample is then dissolved in 0.2 M citrate buffer (pH 2.2). Amino acid analysis is performed with a Phoenix (Model M-7800) amino acid analyzer. The columns were packed with Phoenix-Spherix type XX8-10-0 for short columns and XX8-60-0 for long columns. Cysteic acid analysis is carried out by the method of Moore (1963): aliquots of protein are first oxidized with performic acid, acid hydrolysis and amino acid analysis followed as described above.

Analytical polyacrylamide gel electrophoresis of protein fractions is carried out in the vertical gel electrophoresis cells of the EC-Apparatus Corp. Gels containing 4 or more per cent polyacrylamide (cyanogum-41) are used as is a 3% cyanogum-41–0.5% agarose gel. The gels were prepared in a buffer of the following composition: 85 mM Tris, 80 mM boric acid, 2.5 mM $Na_2EDTA$, pH 8.4. The electrode chamber contains the same buffer and migration is toward the anode. Electrophoresis is carried out for 2 hr at 300 V before the gel is fixed and stained with Amido Black 10B in 10% acetic acid, 50% methanol solution.

Protein fractions are also analyzed by cellulose acetate strip electrophoresis. The Millipore Phoroslide system (Millipore Corp.) is used with buffers varying in pH from 6.5 to 9.5. Details of the procedures are delineated in the legend of Figure 2.

Analytical ultracentrifugation of protein fractions is carried out with a Model E Beckman analytical ultracentrifuge at a speed of 56,000 rpm. After full speed is reached, photographs are taken at 16-min intervals. The bar angle is 50°. Protein concentration is either 6 mg/ml or 9 mg/ml of solution A. In some cases, the system also contains 0.5 mM 2-mercaptoethanol. During the time required to reach 56,000 rpm the system was inspected visually for any evidence of rapidly sedimenting material.

To dissociate protein aggregates into polypeptide subunits, protein fractions are treated according to the method of Shapiro *et al.* (1967). Specifically 50 μg of protein is dissolved in 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, 0.01 M phosphate buffer, pH 7.1, and incubated for 3 hr at 37°. The dissociated proteins are dialyzed for 16 hr against 0.1% sodium dodecyl sulfate, 0.1% 2-mercaptoethanol, and 0.01 M phosphate buffer, pH 7.1, at 25°. These dissociated protein fractions are analyzed by electrophoresis in an EC vertical gel apparatus; 5%, 12%, and 15% cyanogum-41 gels are prepared in the following solution: 0.1% sodium dodecyl sulfate, 0.1 M phosphate buffer, pH 7.1. Electrophoresis is carried out in a buffer system of 0.1%

[1] The abbreviations used are: highly purified sarcotubular membrane fraction, $SF_1$; elution volume, $V_e$; void volume, $V_0$.

[2] The proteins made soluble by treating erythrocyte membranes with a high ionic strength salt solution have been considered to be components of these membranes by Rosenberg and Guidotti (1969); since the prime function of sarcotubular membranes is $Ca^{2+}$ transport associated with an ATPase and neither of these activities is damaged by 0.6 M KCl extraction we have proceeded on the basis of the veracity of the conclusion of Martonosi (1968) that the proteins extracted by the KCl treatment are contaminants (possibly actomyosin).

sodium dodecyl sulfate, 0.1 M phosphate, pH 7.1, with a voltage of 8 V/cm for 3 to 26 hr depending on the gel concentration. Migration is toward the anode. The gel is fixed in 20% sulfosalicylic acid for 16 hr and then stained in 0.25% Coomassie Blue for 2–5 hr depending on the gel concentration. The gel is destained in 7% acetic acid.

Protein fractions are also dissociated into subunits by a modification of the method of Pagé and Godin (1969) and then analyzed by Sephadex G-150 in the presence of detergent as they described. Details of the procedure are delineated in the legend of Figure 8.

The N-terminal amino acid residues in protein fractions are analyzed by preparing dansyl or DNP derivatives. DNP derivatives are prepared by the method of Fraenkel-Conrat *et al.* (1955). Identification of the DNP derivatives is made by thin-layer chromatographic methods of Rauderath (1963). Specifically the solution containing the DNP derivatives is acidified and extracted with ether. The DNP derivatives that enter the ether phase are separated and identified by a two-dimensional silica gel G thin-layer chromatographic system. The first solvent system used in development is toluene–pyridine–ethylene chlorohydrin–0.8 N $NH_4OH$ (100:30:60:60, v/v). The second solvent system is used in continuous flow and is composed of $CHCl_3$–$CH_3OH$–glacial acetic acid (95:5:1, v/v). The DNP derivatives that are not extracted by the ether are separated and identified by a one-dimensional silica gel G thin-layer chromatographic system. The solvent used for development is 1-propanol–34% $NH_3$ (7:3, v/v). The thin-layer plates are scanned for quantitative estimation of DNP derivatives by a densitometric method utilizing a 420-m$\mu$ filter. Dansyl derivatives are prepared by the method of Gray (1967). Identification of dansyl derivatives is made by electrophoresis on paper at pH 1.9 (formic acid buffer) and 4.4 [pyridine–acetic acid–$H_2O$ (10:20:2500)] at 2500 V for 1 hr (Gilson high voltage electrophoresis apparatus).

The C-terminal amino acid residues of protein fractions are analyzed by a carboxypeptidase A method (Cahill and Li, 1968). The proteins are dissolved in 0.05 M Veronal buffer (pH 7.5). The incubation is started by the addition of carboxypeptidase A or B. At various time intervals aliquots of the reaction mixture are removed and mixed with 0.2 M sodium citrate buffer pH 2.2. The precipitate is removed by centrifugation and 0.5-ml samples of the supernatant are analyzed for their amino acid content by the automatic amino acid analyzer technique described above. DFP-treated carboxypeptidase A and carboxypeptidase B were purchased from the Worthington Biochemicals Corp.

Peptide mapping of the protein in fraction-2 is carried out as follows. A protein–trypsin (Mann), mass ratio of 16:1, is dissolved in $H_2O$ and the pH is maintained at 8.0 by delivering NaOH titrant from a pH-Stat automatic titrator (Radiometer). The reaction is terminated when no further addition of NaOH titrant is required. The peptides formed were mapped by a modification of the two-dimensional chromatography and electrophoresis procedure of Katz *et al.* (1959). The pH of the electrophoretic system was 4.4. All peptides were found to migrate toward the cathode.

Phospholipid is analyzed by a modification of the method of Masoro *et al.* (1964) as described previously (Yu *et al.*, 1968b). The $^{35}S$ content of sodium dodecyl sulfate is assayed by a liquid scintillation method previously described (Yu *et al.*, 1968b).

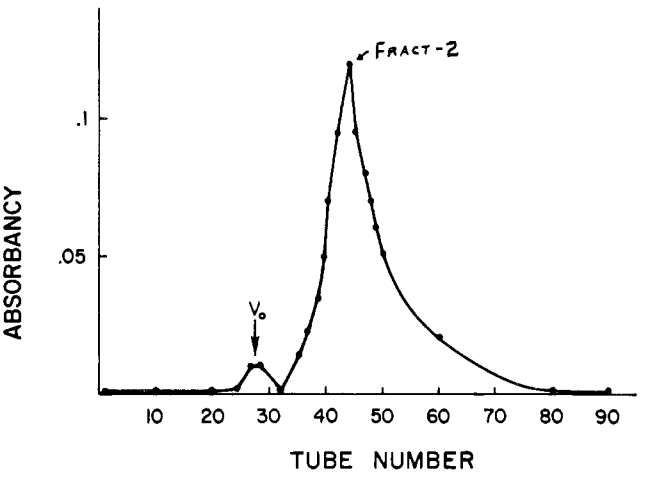


FIGURE 1: Sepharose 4B gel filtration analysis of solubilized proteins from $SF_1$. (For procedure see Materials and Methods.)

## Results

Before developing the above method for the solubilization of the sarcotubular membrane proteins, several other approaches were attempted without success. Since these approaches were carefully explored, it is probably of value to briefly mention some of them. Sonic treatment of the $SF_1$ membranes was the first method tried because of the report of Barclay *et al.* (1967) that mild sonic treatment of isolated rat liver plasma membranes solubilized the membranes and yielded three classes of high density lipoprotein and one protein class. Although sonic treatment of $SF_1$ did cause the membrane suspension to become transparent, analysis by electron microscopy showed this "solution" to be composed of finely divided fragments of membrane structure. Another approach involved delipidation of the membranes by $CHCl_3$–$CH_3OH$ (2:1) extraction. The resulting protein powder was suspended in 8.0 M urea and succinylated by the method of MacLennan (1965). This treatment did not yield an appreciable amount of solubilized protein, nor did attempts to dissolve the delipidated protein in formic acid (0.9%) as described by Rosenberg and Guidotti (1968). Several other approaches were also tried, but only the method utilizing sodium dodecyl sulfate described in the Materials and Methods section yielded almost all of the sarcotubular membrane protein in a soluble form.

Analysis of the solubilized $SF_1$ protein by Sephadex G-200 column chromatography yielded 2 protein fractions, one with a $V_e$ equal to the $V_0$ and another with a $V_e > V_0$; the latter contained most of the protein and exhibited a $V_e:V_0$ ratio of 1.27. Since Sephadex G-200 column chromatography did not permit good resolution of these two protein peaks, Sepharose 4B gel filtration was used. More than 90% of the $SF_1$ protein solution applied to the Sepharose 4B column was eluted in two separate peaks. The first peak (called fraction-1) emerged at the $V_0$ and accounted for about 0.5% of the applied protein; the second peak (called fraction-2) accounted for 90% or more of the applied protein

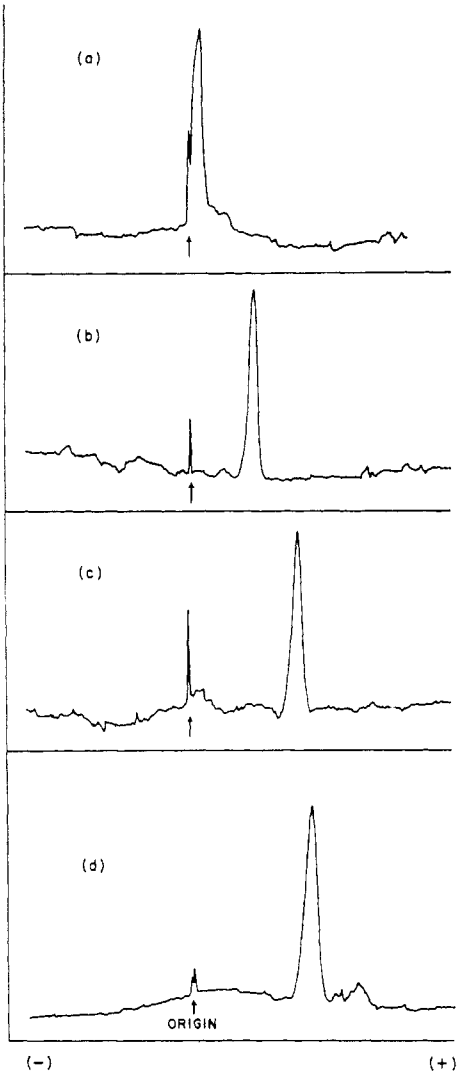


FIGURE 2: Cellulose acetate strip electrophoretic analysis of fraction-2. The Millipore Phoroslide system was used; the buffer was sodium barbital–HCl (0.05 M). The electrophoresis was carried out under the following conditions: temperature, 23°; time, 0.5 hr; voltage, 100 V. The strip was fixed and stained by treating for 10 min with a solution containing 1.8% ponceau S, 26.8% trichloroacetic acid, and 26.8% sulfosalicylic acid. The records above are densitometric scans of the fixed and stained cellulose acetate strips. Scan a is a densitometric recording of electrophoresis carried out at pH 7.0; scan b at pH 7.6; scan c at pH 8.5, and scan d at pH 9.3.

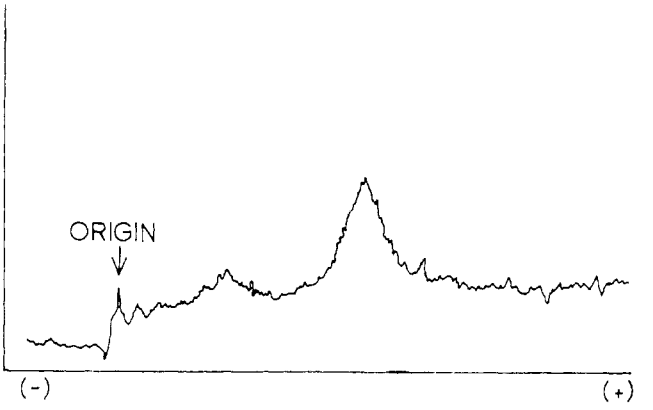


FIGURE 3: Polyacrylamide–agarose gel electrophoretic analysis of fraction-2. The record above is a densitometric scan.

TABLE I: Amino Acid Composition of Subfractions of Fraction-2.[a]

| | Subfraction[a] | | |
|---|---|---|---|
| | 1st | 2nd | 3rd |
| Lysine | 110.8 | 123.3 | 118.4 |
| Histidine | 37.9 | 42.6 | 41.1 |
| Arginine | 123.1 | 125.4 | 115.8 |
| Aspartic acid | 303.2 | 301.1 | 292.4 |
| Threonine | 143.0 | 153.1 | 123.7 |
| Serine | 191.2 | 184.0 | 214.5 |
| Glutamic acid | 296.1 | 355.1 | 319.4 |
| Proline | 139.9 | 152.4 | 121.7 |
| Glycine | 205.8 | 205.1 | 178.9 |
| Alanine | 207.1 | 222.9 | 195.7 |
| Valine | 129.7 | 142.6 | 126.3 |
| Isoleucine | 89.2 | 97.9 | 90.1 |
| Leucine | 264.6 | 269.0 | 271.2 |
| Tyrosine | 49.4 | 49.0 | 44.7 |
| Phenylalanine | 82.7 | 105.2 | 102.3 |
| Cysteic acid | 10.1 | 9.8 | 9.9 |

[a] Expressed in μmoles/100 mg of protein. Tryptophan was not measured.

(Figure 1). The sodium dodecyl sulfate:protein weight ratio of fraction-2 was <0.001; *i.e.*, fraction-2 was either free of or contained only traces of sodium dodecyl sulfate. The phospholipid:protein weight ratio of fraction-2 was 0.05 compared to a ratio of 0.49 found for intact sarcotubular membrane (Yu *et al.*, 1968b). A possible reason for the lack of symmetry in peak 2 is considered in the Discussion section.

Amino acid composition studies were carried out on three subfractions obtained from fraction-2 during its elution, a first, second, and third subfraction in respect to the order of elution from the Sepharose 4B column. Data for one of the five preparations studied are reported in Table I. The amino acid composition was similar for each of the subfractions; indeed the differences between subfractions are within the range possible with this analysis as is indicated by the fact that the differences between subfractions noted in Table I were not consistently observed in the other four preparations so studied. If the average of all five preparations were presented, there would be almost no differences noted between the amino acid composition of the three subfractions.

Fraction-2 was analyzed by cellulose acetate strip electrophoresis at pH 6.5 to 9.5 and the results at pH 7.0, 7.6, 8.5, and 9.3 are shown in Figure 2. At pH 6.5 the protein did not migrate but at all other pH values the protein migrated in a single band towards the anode. Attempts to analyze the protein of fraction-2 by polyacrylamide vertical gel electrophoresis were unsuccessful with gels containing 4% or more polyacrylamide because most protein did not enter the gel.

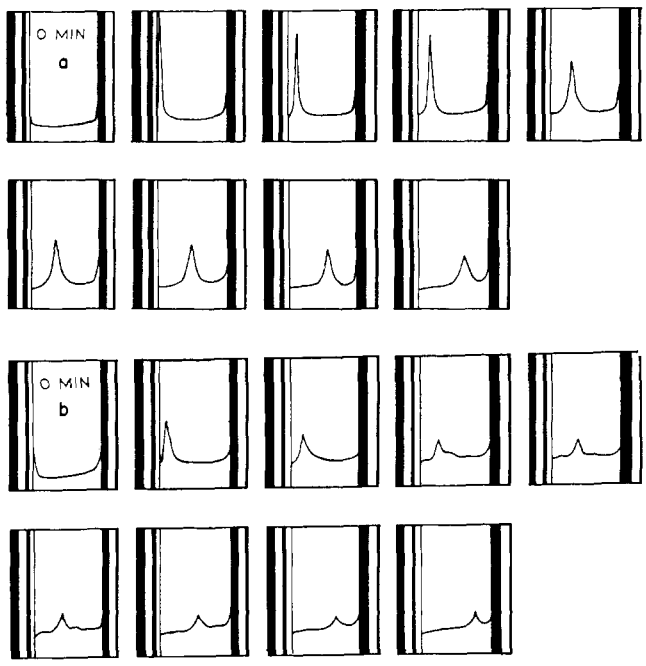


FIGURE 4: Analytical ultracentrifugation of fraction-2: (a) in presence of 2-mercaptoethanol; (b) in absence of 2-mercaptoethanol. Pictures were taken at 16-min intervals after full speed was reached. (For procedures see Materials and Methods.)

A 3% polyacrylamide gel proved too difficult to handle with the vertical system but became manageable when 0.5% agarose was also present. With this polyacrylamide–agarose system, all protein entered the gel and migrated in two bands (Figure 3). Both bands are broad which may relate either to the properties of this complex gel system or to the nature of fraction-2 (see Discussion section) or both.

Fraction-2 was further studied by analytical ultracentrifugation. When fraction-2 was prepared in the presence of 2-mercaptoethanol, at least three components were observed (Figure 4a); the major component has an $s_{20}$ value of 6.5. However, when the analysis was made in the absence of 2-mercaptoethanol the fast-moving minor component was no longer observable (Figure 4b).

The results of the foregoing experiments make it clear that the protein in fraction-2 is composed of molecules of high molecular weight. Since it seemed possible that these molecules are aggregates of smaller molecular weight protein species, an attempt was made to dissociate the protein of fraction-2 into possible subunits by treating it with sodium dodecyl sulfate and 2-mercaptoethanol according to the method of Shapiro *et al.* (1967). The resulting solution was then analyzed electrophoretically in a 5% polyacrylamide vertical gel containing 0.1% sodium dodecyl sulfate (see Experimental Section for details). All protein entered the gel and most migrated in a single band (Figure 5); a comparison of the extent of migration of this band relative to that of standard proteins indicated that the band is composed of polypeptides of molecular weight of about 17,000, a value reported in a preliminary communication (Masoro and Yu, 1969). However, Shapiro and Maizel (1969) recently published further data in regard to their method which

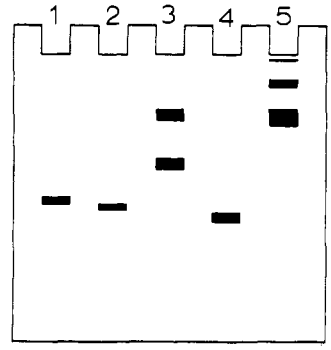


FIGURE 5: Analysis of dissociated protein of fraction-2 by electrophoresis in 5% polyacrylamide gel containing dissociating agents. The slots are numbered 1 to 5 consecutively from left to right; slot 1 contains apoferritin; slot 2, fraction-2; slot 3, pepsin; slot 4, hemoglobin; and slot 5, bovine serum albumin. All proteins were treated with dissociating agents as described in Materials and Methods as is the electrophoretic procedure. Electrophoresis was carried out for 3 hr.

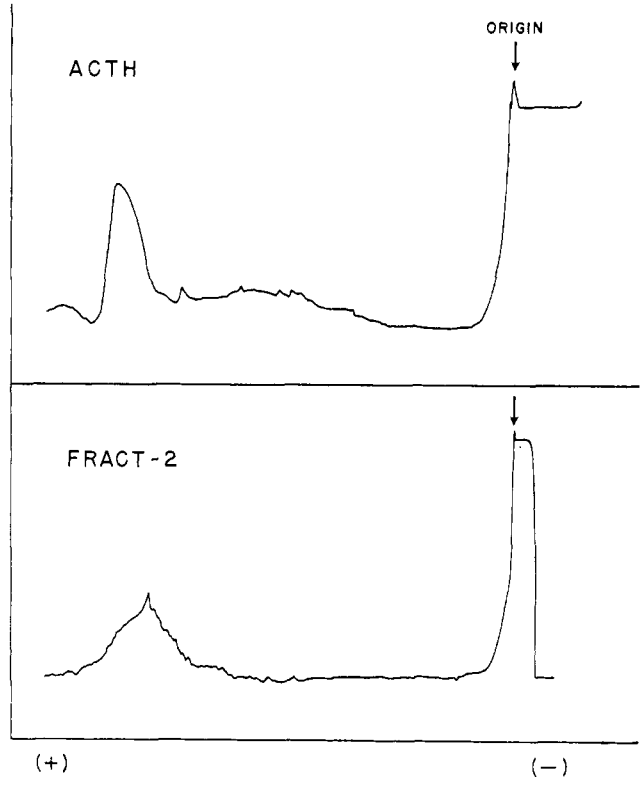


FIGURE 6: Analysis of dissociated protein of fraction-2 by electrophoresis in a 12% polyacrylamide gel containing dissociating agents. Upper densitometric scan is for dissociated ACTH and lower scan for dissociated fraction-2. Both proteins as well as the other standards reported in Figure 7 were treated with dissociating agents as described in Materials and Methods, as is the electrophoretic procedure. Electrophoresis was carried out for 24 hr.

indicate that analysis by 5% polyacrylamide gel electrophoresis may lead to a false estimation of molecular weight for proteins of low molecular weights. Indeed with gels containing a higher percentage of polyacrylamide than 5%,

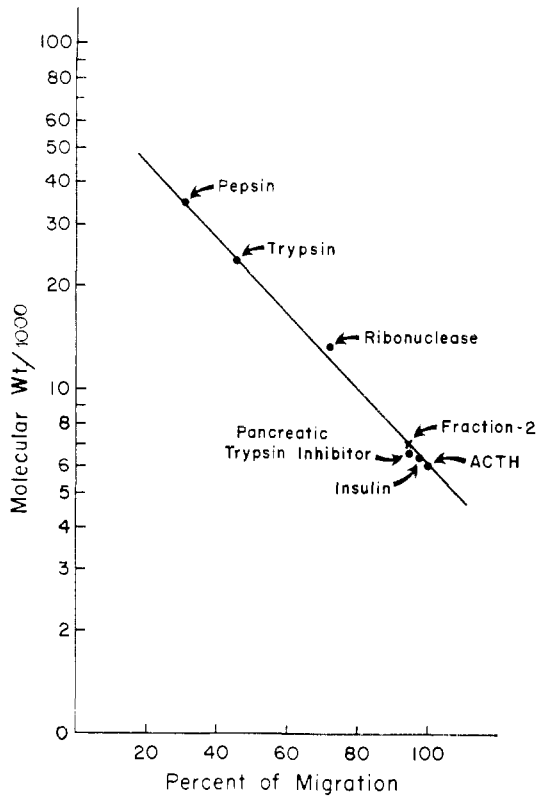


FIGURE 7: Relationship between molecular weight of a dissociated protein and its migration distance in the 12% polyacrylamide gel containing dissociating agents. All proteins were treated and assayed as described in Figure 6.

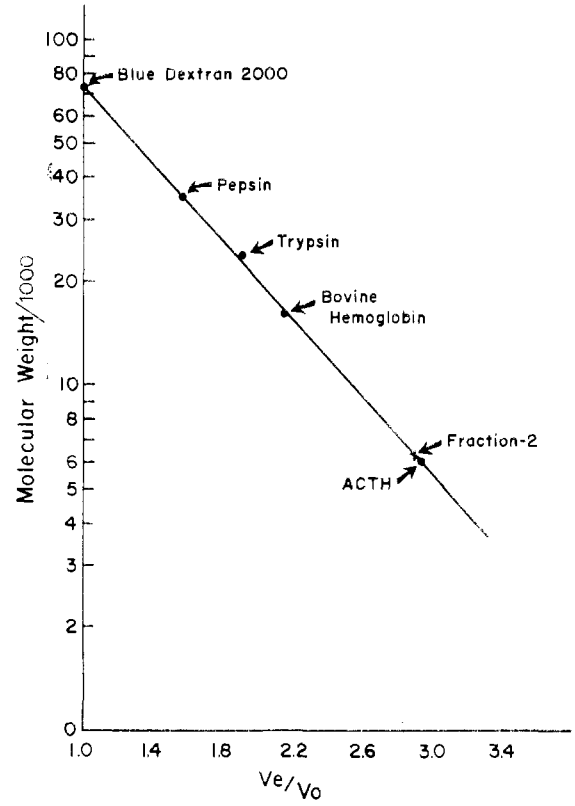


FIGURE 8: Relationship between molecular weight of a dissociated protein and its elution behavior on a sodium dodecyl sulfate containing Sephadex G-150 column. The protein of fraction-2 and the standard proteins at a concentration of 6 mg/ml were preincubated with 0.075 M sodium dodecyl sulfate containing $1.5 \times 10^{-4}$ M *p*-hydroxymercuribenzoate at pH 8.0 at 37° for 24 hr prior to the Sephadex gel filtration. Sephadex G-150 was equilibrated in a 0.05 M sodium dodecyl sulfate solution containing $1.5 \times 10^{-4}$ M *p*-hydroxymercuribenzoate at pH 8.0. The eluent had the same composition as the equilibrating solution. Chromatography was carried out at room temperature at an elution rate of 3–4 ml/cm² per hr.

electrophoretic analysis of the dissociated protein indicated our original estimate of molecular weight to be in error. The results of electrophoresis in a 12% gel are shown in Figure 6; all protein entered the gel and migrated as a single band. On the basis of migration distance, the dissociated protein components of fraction-2 have an estimated molecular weight of approximately 6500 (Figure 7). Analysis of dissociated fraction-2 in a 15% gel also indicated homogeneity of protein species, since only a single migrating band was observed. The validity of the method of Shapiro *et al.* for the determination of molecular weight of proteins has recently been confirmed by Weber and Osborn (1969) and Dunker and Rueckert (1969).

Analysis of dissociated protein of fraction-2 by Sephadex G-150 column chromatography also provided evidence of a single protein species since all protein eluted in a single sharp peak. A comparison of the $V_e:V_0$ ratio of the dissociated fraction-2 with that of other known proteins treated in a similar fashion led to an estimated molecular weight of 6500 (Figure 8).

Analysis of the N-terminal amino acid residues of the protein in fraction-2 as DNP and dansyl derivatives showed only N-terminal glycine present. A typical densitometric scan of the thin-layer chromatographic analysis of DNP derivatives is presented in Figure 9. On the basis that glycine is the sole N-terminal amino acid residue of these 6500 molecular weight protein subunits, the yield of DNP-glycine from these proteins is 33% of theoretical. Since N-terminal analysis of glycylglycine by the same method results in a DNP-glycine yield of 46% of theoretical, this quantitative analysis of N-terminal glycine in protein fraction-2 is consistent with the qualitative evidence indicating glycine to be the sole N-terminal amino acid residue present. High voltage electrophoretic analysis of dansyl derivatives also indicated that the only N-terminal residue present is glycine.

Analysis of the C-terminal amino acid residues of the protein in fraction-2 by carboxypeptidase A or B indicates that alanine is a C-terminal residue. The results with carboxypeptidase A are presented in Figure 10. Calculation of molecular weight on the basis of alanine being the sole C-terminal amino acid of identical protein subunits results in an estimated value of 9880 from carboxypeptidase A analysis and 10,300 from carboxypeptidase B analysis. These results agree rather well with the estimated molecular weight of 6500 from gel electrophoresis and gel filtration analysis when the uncertainties involved in each of these methods of molecular weight estimation are taken into account.

Peptide mapping of the protein in fraction-2 was carried out after tryptic digestion. The data are reported in Figure 11. Fifteen different peptides were detected.

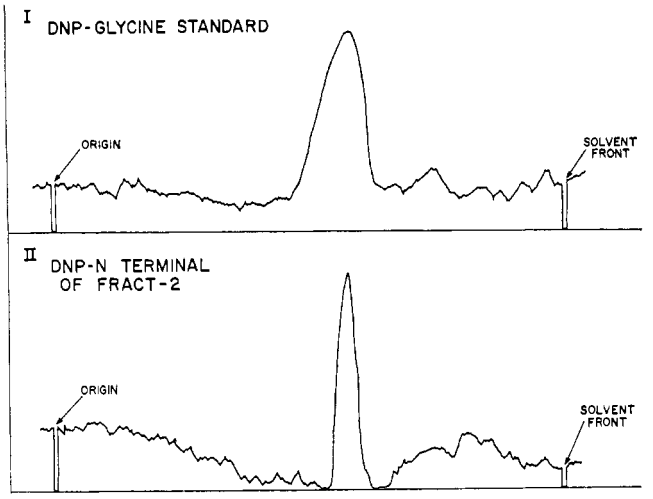


FIGURE 9: Thin-layer analysis of DNP derivatives prepared from fraction-2. The above is a scan of ether-extractable acidified DNP derivatives. The nonether-extractable fraction was similarly studied and no evidence for the presence of an N-terminal derivative was found. Procedures are described in Materials and Methods.

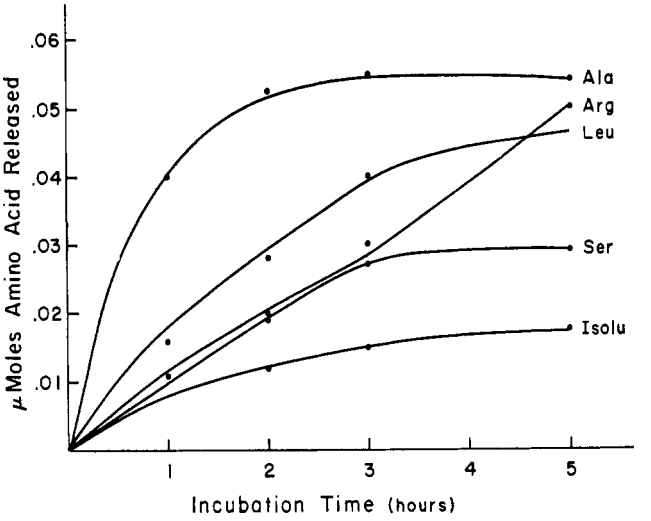


FIGURE 10: Rates of release of amino acids by carboxypeptidase A treatment of fraction-2. (For procedure see Materials and Methods.)

## Discussion

More than 95% of the protein of sarcotubular membranes can be solubilized by the sodium dodecyl sulfate treatment described in this paper. Of this solubilized protein, more than 90% can be isolated by Sepharose 4B column chromatography in a single fraction, called fraction-2. Fraction-2 is comprised of large aggregates of identical or very similar protein subunits of low molecular weight with N-terminal glycine residues and probably only C-terminal alanine residues.

The shape of the Sepharose 4B elution peak for the protein in fraction-2 (shown in Figure 1) implies that this fraction is not a totally homogeneous population of protein molecules. Also the data from ultracentrifugation analysis of fraction-2 (reported in Figure 4a) in the presence of 2-mercaptoethanol shows the presence of more than one component; as discussed below this heterogeneity may merely reflect different states of aggregation. When the ultracentrifugation was carried out in the absence of 2-mercaptoethanol (Figure 4b), the protein appeared less heterogeneous; yet Sepharose 4B gel filtration analysis of fraction-2 in the absence of 2-mercaptoethanol still results in an elution peak similar to that shown in Figure 1. The polyacrylamide–agarose gel electrophoresis studies (reported in Figure 3) also imply that fraction-2 is not homogeneous. Therefore although fraction-2 is composed of remarkably similar components there appears to be some heterogeneity.

From the data presented in Figures 5 through 9 it seems evident that the proteins of fraction-2 are aggregates, and that the heterogeneity noted above relates to different states of aggregation. The dissociation system changed the protein in fraction-2 from molecular structures that would not enter a 4% polyacrylamide gel to one that enters a 15% gel. Moreover, upon electrophoresis in varying gel concentrations, the migration pattern of dissociated protein of fraction-2 involved a single migrating band. The electrophoretic analysis by the Shapiro and Maizel system (1969) relates migration distance to molecular size alone since charge differences are masked in this system. The gel filtration on Sephadex G-150 in the presence of dissociating agents also provides evidence for a single protein component from fraction-2, but again this system screens molecules almost exclusively on the basis of molecular size. It is noteworthy, however, that both the gel filtration and the gel electrophoretic systems indicate the same estimated molecular weight for the subunits of the protein in fraction-2, *i.e.*, a molecular weight of approximately 6500.

In addition to the evidence that the protein subunits of fraction-2 are all of nearly identical size, the following data suggest that these subunits are of either identical or very similar polypeptide species. First, glycine is the only N-terminal amino acid residue found in this protein fraction. Second, estimation of the molecular weight of this hypothetical polypeptide species on the basis of alanine being the only C-terminal amino acid present yields a value of approximately 10,000, remarkably close to the value estimated from gel filtration and gel electrophoresis analysis when the uncertainties inherent in each of these estimations is considered.

The finding of 15 peptides on peptide mapping of fraction-2 requires some comment. On the basis of a molecular weight of 6500 and the amino acid composition reported in Table I, it is to be expected that a homogeneous protein sample would yield less than 15 peptides. However if the molecular weight is 10,000 or so as indicated by C-terminal amino acid analysis, 15 peptides might well be expected. Therefore the finding of 15 peptides, a relatively small number, indicates either the presence of a single polypeptide species with a molecular weight of approximately 10,000 or at most two polypeptide species which on the basis of other data must be of similar size and composition.

In a recent preliminary report, Martonosi (1969) stated that he was unable to substantiate the claim made in our preliminary report that a single polypeptide species accounts for most of the protein of sarcotubular membranes. He based this statement on his finding that proteins obtained from sarcotubular membranes yield a large number of distinct

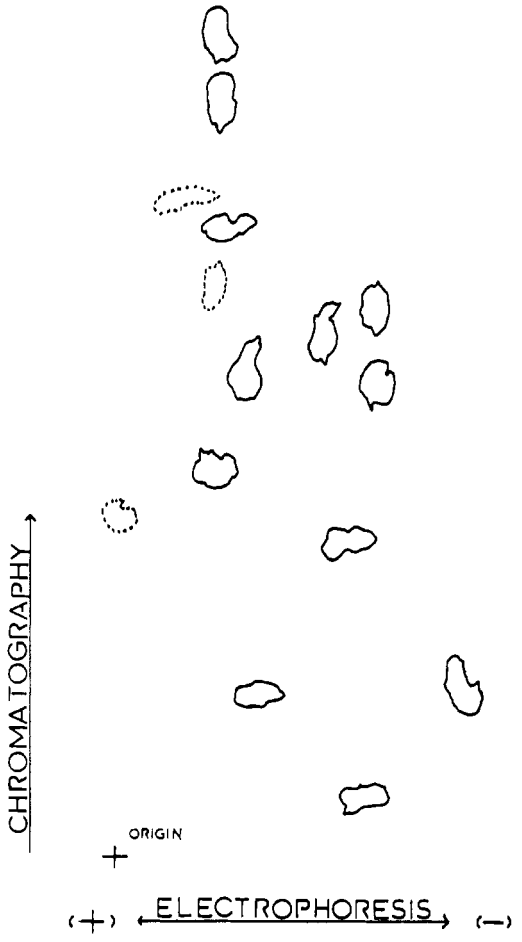


FIGURE 11: Peptide mapping of fraction-2. The procedures are described in the Materials and Method section. The peptides are detected by spraying with 0.2% ninhydrin reagent in 1-butanol saturated with water. Color is developed by heating. Spots outlined by dashed line exhibited only faint color.

bands in various polyacrylamide gel electrophoretic systems. It is difficult to ascertain the reason for the discrepancy between our work and Martonosi's, particularly since his communication does not include his procedure for preparing the soluble sarcotubular membrane material used in his electrophoretic studies. From our results reported above only complete dissociation of lipid-poor sarcotubular protein made the homogeneity of the polypeptide structure obvious on gel electrophoresis.

The finding that fraction-2 contains approximately 90% of the protein of sarcotubular membranes and that this is made up of identical or similar polypeptide subunits of molecular weight in the range of 6500–10,500 means that the protein subunit which is part of the molecular architecture of these membranes has been isolated. The isolation of such a protein may be an excellent starting point for a stepwise and chemically defined reconstitution of the membranous structure which should provide insight regarding the molecular nature of this membrane system. Also if Mommaerts' theory is correct and most of the protein of sarcotubular membrane is involved in the $Ca^{2+}$ transport function, the isolation of this predominant molecular species of protein would provide an important tool for the biochemical investigation of this transport system. Moreover, our belief that sarcotubular membranes might offer an experimental prototype for the study of molecular aspects of membrane function and structure in general by virtue of a relatively simple protein composition seems well borne out by our findings. However it must be recognized that in the process of isolating this protein from the sarcotubular membranes the naturally occurring lipoprotein structure of biological membranes (Morgan and Hanahan, 1966; Barclay *et al.*, 1967) has been destroyed and the protein component denatured. Although the isolation of this protein component is clearly an important step in the analysis of the protein structure of these membranes, successful use of such an isolated component for reconstitution of membrane structure remains to be established and probably depends on the development of procedures which will "renature" the protein, *e.g.*, the reintroduction of lipid into the protein structure.

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# 21,25-Dihydroxycholecalciferol. A Metabolite of Vitamin $D_3$ Preferentially Active on Bone*

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ABSTRACT: A metabolite of vitamin $D_3$ has been isolated in pure form from the plasma of pigs given large doses of vitamin $D_3$. It has been identified as 21,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectra, and behavior on gas–liquid chromatography.

This metabolite is 0.5 as active as vitamin $D_3$ in the rat in the cure of rickets and in intestinal calcium transport, but is more active than vitamin $D_3$ in the mobilization of bone mineral.

The successful identification of vitamin $D_3$ (cholecalciferol) by Windaus and collaborators essentially brought to a close the search for dietary antirachitic factors (Windaus *et al.*, 1936; Schenk, 1937). A new search in the vitamin D field made its appearance with the successful demonstration in this laboratory of biologically active metabolites of vitamin $D_3$ (Lund and DeLuca, 1966). One group was identified almost immediately as esters of vitamin D and long chain fatty acids (Lund *et al.*, 1967; Fraser and Kodicek, 1966) but these appeared to be of minor functional importance (DeLuca, 1967). However another metabolite fraction (peak IV) more polar than vitamin D contained at least one metabolite more effective than vitamin $D_3$ in the cure of rickets, in the stimulation of intestinal calcium transport, and in the mobilization of bone mineral (Lund and DeLuca, 1966; Morii *et al.*, 1967). In addition it acted more rapidly than vitamin $D_3$ itself. The peak IV fraction was subsequently subfractionated into seven radioactive components, one of which retained the ability to cure rickets (Ponchon and DeLuca, 1969; Blunt *et al.*, 1968b) (peak IV). This substance was subsequently isolated in pure form and identified as 25-hydroxycholecalciferol (Blunt *et al.*, 1968a,b). It was successfully synthesized chemically and shown to be the circulating active form of the vitamin (DeLuca, 1969).

Synthesis of [$^3$H]-25-hydroxycholecalciferol made possible the demonstration that this metabolite is further metabolized to more polar metabolites in intestine, bone, and kidney (Cousins *et al.*, 1970; R. J. Cousins and H. F. DeLuca, 1970, unpublished data). Haussler *et al.* (1968) and Lawson *et al.* (1969) have also shown a metabolite of vitamin $D_3$ in intestinal nuclei more polar than 25-hydroxycholecalciferol. Thus the identification of these metabolites appeared important to a thorough understanding of the mechanism of vitamin D action. One of these polar metabolites has now been isolated in pure form, identified as 21,25-dihydroxycholecalciferol, and shown to have a marked action on mobilization of bone mineral while having a small but significant effect on intestinal calcium transport. In addition it is one-half as active as vitamin $D_3$ in curing rickets in rats. It is the purpose of this communication to report these results.

## Methods and Results

*General Procedures.* All radioactive determinations were carried out by means of a Packard Tri-Carb Model 3003 liquid scintillation counter equipped with an automatic

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