T-System Membranes in Microsomal Fractions of Crayfish Muscles: Identification and Differences in Protein Composition

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Abstract. Specific binding of ^{3}H-ouabain and ruthenium red (RR) to membranes of T-tubules in crayfish muscles was used to identify the subfraction containing vesicles originating from the T-system. The microsomal fraction was prepared by differential centrifugation, and subfractions were separated in continuous or discontinuous sucrose density gradients. ^{3}H-ouabain binding was estimated by scintillation counting; RR binding was examined by electron microscopy. The light subfraction was identified using both methods as that containing vesicles of T-tubules. Protein separation by SDS-electrophoresis revealed marked differences between the subfraction containing vesicles of T-tubules and other subfractions, the most distinctive feature being the presence of a protein of M_r 46,000 predominantly in the light subfraction.

Key words: Crayfish sarcotubular fractions — T-tubule membranes — ^{3}H-ouabain — Ruthenium red — SDS electrophoresis

Introduction

In the past 20 years, the study of sarcotubular fractions has greatly expanded the knowledge on mechanisms underlying the regulation of intracellular calcium concentration in muscle cells (for a review, see Tada et al. 1978). The possibility of separating a population of vesicles originating from T-tubules would be important for the elucidation of molecular events involved in excitation-contraction coupling. This was attempted for the first time on rabbit muscle using ^{3}H-ouabain as a marker of T-tubule membranes (Caswell et al. 1976; Lau et al. 1977).

The presence of calcium electrogenesis in T-system membranes of the crayfish muscle (Zachar 1971; 1981), and distinctive morphological features, with the non-junctional T-tubules comprising about 65 % of the total T-system membrane area (Uhrík et al. 1980), make this preparation interesting from the comparative point of view. Moreover, selective staining of crayfish T-tubules with ruthenium red without concomitant binding of the dye to the peripheral sarcolemma (Uhrík and Zacharová 1982) allowed the use of this marker in addition to ^{3}H-ouabain as

an independent tool for the differentiation of subfraction obtained by gradient centrifugation.

Data obtained by SDS electrophoresis suggested significant differences in composition of this subfraction as compared with microsomes from other layers of the sucrose gradient.

A preliminary account of the results was given elsewhere (Kontšeková et al. 1982).

Materials and Methods

Muscle fractionation

Sarcotubular membrane fraction was prepared by a slightly modified method of Caswell et al. (1976). All operations were carried out in cold. Approximately 30 g of the crayfish (*Astacus fluviatilis*) claw muscles were needed to prepare the sarcotubular fraction containing 30 mg of protein. The muscles were excised, minced by scissors, transfered into a solution containing 250 mmol/l sucrose and 0.5 mmol/l EDTA, pH 7.4 and homogenized in 30 s intervals for 3 min in 3.3 vol sucrose and subsequently 1 min in 10 vol sucrose using a Polytron homogenizer (PT 1020 Kinematika Luzern). The homogenate was then centrifuged at 2000*g* for 15 min in order to separate myofibrils and nuclei, and subsequently at 15,000*g* for 20 min to eliminate mitochondria and their fragments.

The supernatant containing sarcoplasmic reticulum and other membrane material was passed through eight layers of cheese-cloth and centrifuged at 95,000*g* for 70 min. The pellet was resuspended in 150 ml of 250 mmol/l sucrose and centrifuged at 95,000*g* for 70 min. The precipitate, referred to as „sarcotubullar fraction", was rehomogenized in 250 mmol/l sucrose using a glass-Teflon homogenizer.

The sarcotubular fraction was placed on continuous and discontinuous sucrose density gradients, respectively. Continuous density gradients were prepared in centrifuge tubes (SW 27 Ti swinging bucket rotor, Beckmann ultracentrifuge) by passing 12.5 ml of 1.9 mol/l (65 %) sucrose into 12.5 ml of 0.36 mol/l (12 %) sucrose. Discontinuous gradients were prepared from 0.43; 0.58; 0.73; 0.87; 0.95; 1.02; and 1.16 mol/l sucrose concentrations. Three mg of protein in 2 ml of the sarcotubular fraction (suspended in 250 mmol/l sucrose) was layered on the top the gradient and centrifuged for 5 hours at 77,000*g*.

Ouabain binding

37 MBq of ^{3}H-ouabain per 100 g muscle in 2 ml of crayfish saline (van Harreveld 1936) was injected into the hemolymph of living animals. After one hour, the muscles were excised and fractionated. ^{3}H-ouabain binding in each subfraction of the sarcotubular fraction obtained from density gradient was estimated by scintillation counting.

Gel electrophoresis

Protein components and their molecular weights were evaluated by sodium dodecyl sulfate gradient gel electrophoresis according to King and Laemmli (1971) using 2-mercaptoethanol as the sulfur bridge reducing agent. The continuous density polyacrylamide gel gradients were prepared by passing 26 ml of 20 % into 26 ml of 10 % gels. Protein (150 μg) was loaded on each gel. Molecular weight standards were run simultaneously with the samples. Molecular weights were estimated by plotting the relative mobilities of the respective fractions againts the logarithm of molecular weights.

Purity control

No visible contamination of sarcotubular subfractions by any other cellular organelles could be found in electron microscope.

The activity of succinatedehydrogenase (succinate: oxidoreductase E. C. 1.3.99.1), as a marker enzyme of mitochondrial contamination, was measured in sarcotubular fraction by the method of Tisdale (1967); it was found to make up 5.4 % of the activity present in the mitochondrial fraction obtained at the beginning of the separation procedure.

Proteins

Protein concentration in the sarcotubular fraction was roughly estimated by the method of Campbell and Sargent (1967); the preparation was then diluted, if necessary. Final evaluation of protein concentration in each sample was performed by the method of Schacterle and Pollack (1973).

Ruthenium red staining

Ruthenium red (Luft 1971) was dissolved in crayfish saline and injected into the hemolymph of living animals in a dose of 500 mg per 1 kg body weight. The direct contact of the hemolymph with muscle fibres facilitated the diffusion of the dye into the T-system. After 60 min (the animals were still alive), the claw muscles were excised and fractionated.

Electron microscopy

Separate fractions were homogenized in 0.15 mol/l Na-cacodylate buffer supplemented with 2 mmol/l $CaCl_2$ (pH 7.4). The concentration of each fraction was set to about 1000 μg protein/ml. Six ml of the suspension was mixed with 0.52 ml of 25 % glutaraldehyde to give 2 % glutaraldehyde concentration, and centrifuged for 1—1.5 h at 40,000g. Fixed sediments were washed overnight with cacodylate buffer and postfixed with 1 % OsO_4 in the same buffer for 1.5 h. The samples were then shortly washed with H_2O and contrasted with 2 % uranyl acetate in H_2O for 24 h. This step was omitted in the case of ruthenium red staining. The next step included dehydration in ethanol series and transfer through propylene oxide into Epon 812 or Durcupan. Sections of silver interference colour (ultramicrotome Sorvall Porter-Blum MT2) were studied in an electron microscope Tesla BS 613 at 80 kV.

Results

Separation of the sarcotubular fraction on the sucrose density gradient

Fig. 1. illustrates the differentiation of the crayfish sarcotubular fraction obtained after separation on continuous or discontinuous sucrose density gradients.

On the continuous gradients, the pellets were separated to light (A), intermediate (B, C) and heavy (D) fractions. After separation on the discontinuous gradient the number of subfractions increased (A_1, A_2 — light; B, C — intermediate; and D_1, D_2 — heavy subfractions).

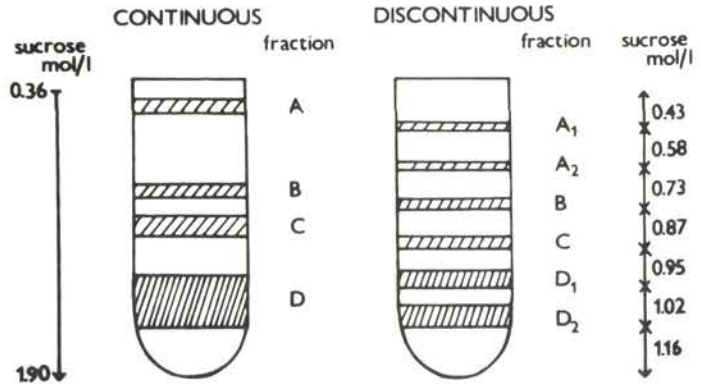

Fig. 1. Separation of the sarcotubular fraction on sucrose density gradients. A (A_1, A_2) — light fraction; B, C — intermediate fraction; D (D_1, D_2) — heavy fraction.

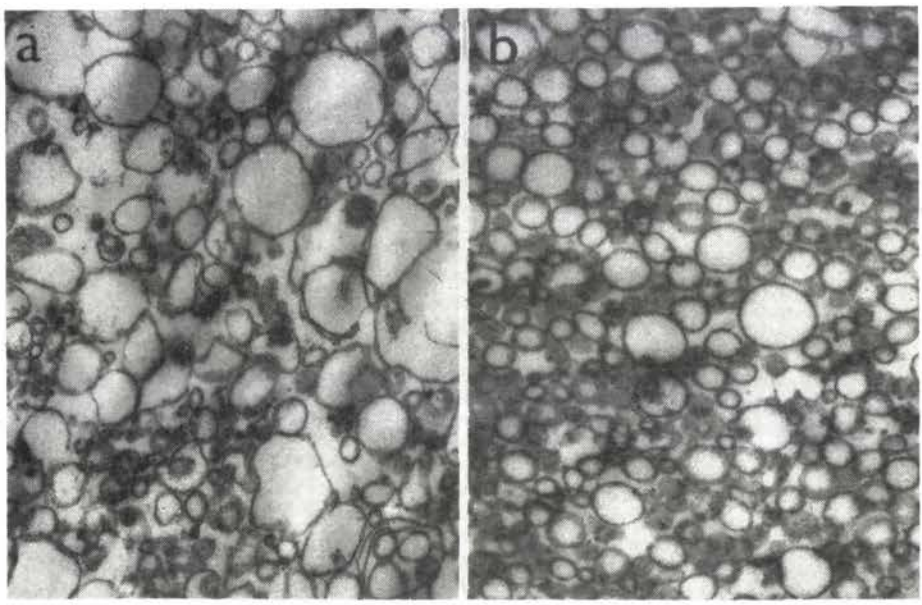

Fig. 2. Ultrathin sections of the light (*a*) and heavy fraction (*b*). (×21,800)

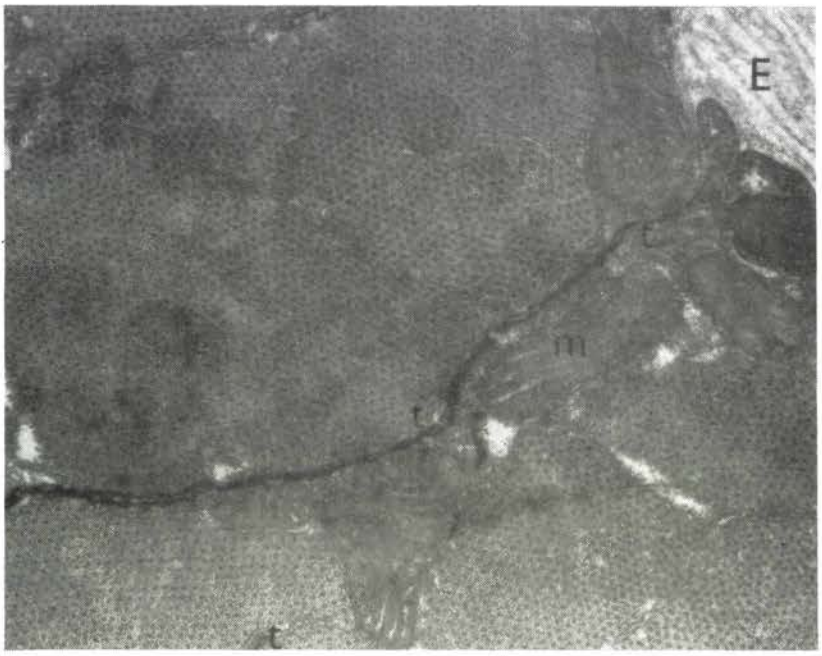

Fig. 3. Transverse section of the peripheral segment of a single crayfish muscle fibre pretreated for 30 min with 1 mmol/l ruthenium red dissolved in crayfish saline. Only T-system is stained, both the external lamina and the plasma membrane remained unstained. t — tubule of the T-system, E — external lamina, m — mitochondrion. (×29,000)

Electron microscopic identification of the T-tubule fraction

The differences in ultrastructural appearance between the individual subfractions processed conventionally were not specific enough to relate them to particular morphological features of the SR and T-system in intact fibres respectively (Fig. 2).

The selective staining of T-tubules in isolated crayfish muscle fibres with ruthenium red (Uhrík and Zacharová 1982) (Fig. 3) was the main impetus to develop the procedure of staining the whole musculature of the crayfish via the hemolymph system (see Materials and Methods). It should be noted, however, that even under most favourable conditioins for staining (perfusion of an isolated single muscle fibre with a ruthenium red solution, Fig. 3) contrasted T-tubules were encountered only in peripheral segments of the fibre. In fibres pretreated with the stain via the hemolymph system, the staining was rare and could be seen only exceptionally. It was not practical, however, to increase the amount of the stain injected into the hemolymph, due to the adverse effects of the stain on the viability of the animals.

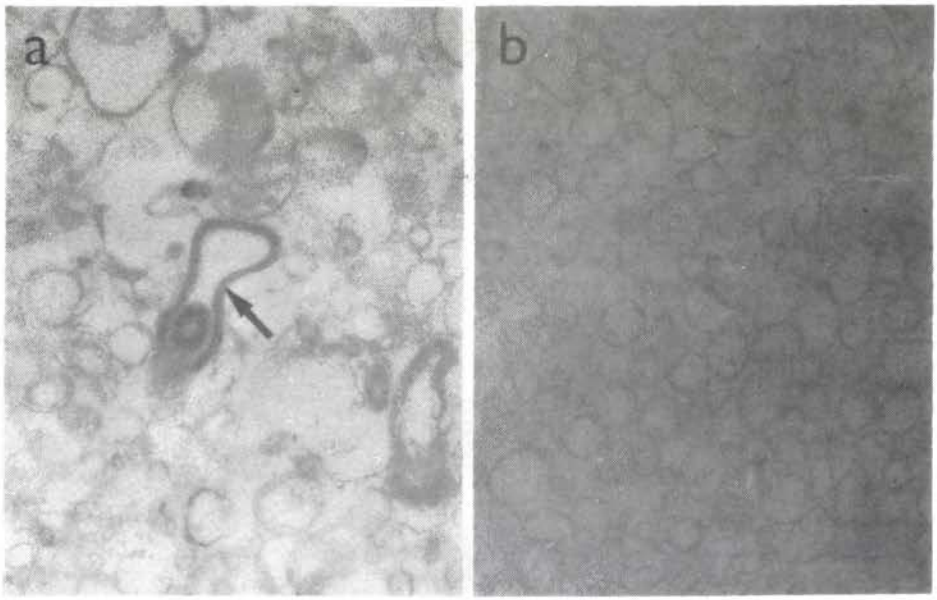

Fig. 4. Ultrathin sections (unstained) of the light (*a*) and heavy fraction (*b*) from ruthenium red pretreated muscles. Electron dense vesicles (arrow in *a*) could be detected only in the light fraction. (×52,000)

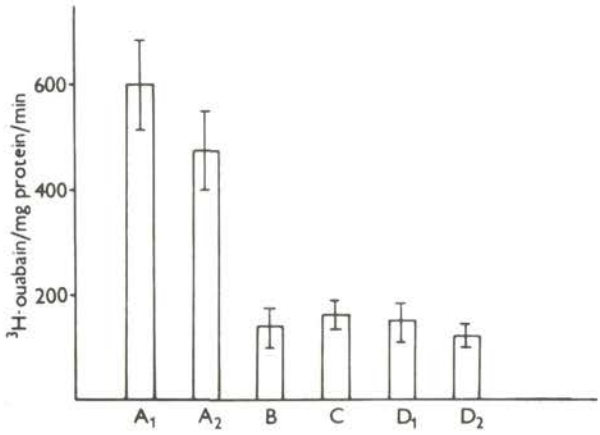

Fig. 5. The activity of ^{3}H-ouabain in vesicles of the crayfish sarcotubular fraction after the separation on the discontinuous gradient. A_1, A_2 — light fraction; B, C — intermediate fraction; D_1, D_2 — heavy fraction.

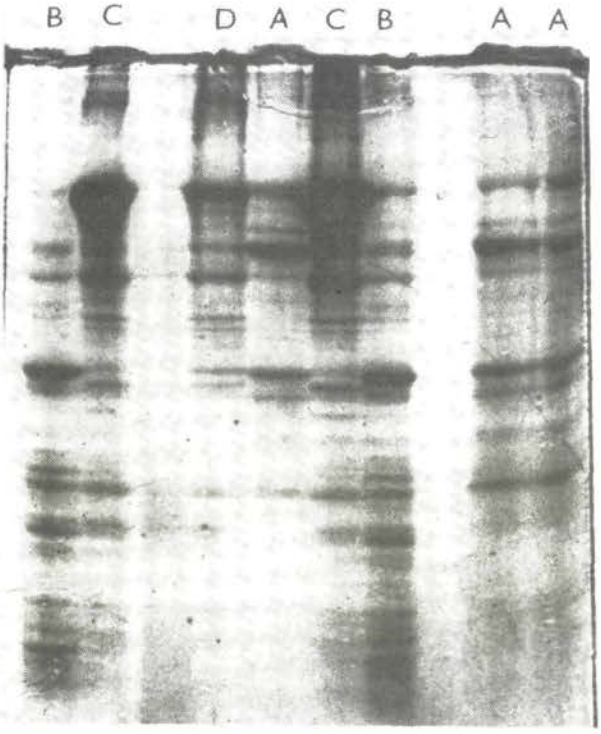

Fig. 6. SDS polyacrylamide gel electrophoresis of crayfish sarcotubular fractions after the separation on the continuous sucrose density gradient. Differences in protein composition between the individual fractions are evident. A — light fraction; B, C — intermediate fraction; D — heavy fraction.

Electron microscopic examination of fractions obtained from animals pretreated with ruthenium red showed the presence of electron-dense vesicles in the light sarcotubular fraction only (Fig. 4*a*). For comparison, an unstained section of the heavy fraction is shown in Fig. 4*b*.

The presence of many unstained vesicles in the light fraction (Fig. 4*a*) may have resulted both from only partial staining of the T-system with ruthenium red and from a contamination with vesicles of sarcoplasmic reticulum.

Identification of the T-tubule fraction with ^{3}H-ouabain

The marking of T-tubules with ^{3}H-ouabain resulted in a distribution pattern similar to that seen after ruthenium red staining. The activity of ^{3}H-ouabain in the light subfractions was about five times higher than that in the middle and heavy subfractions (Fig. 5), respectively.

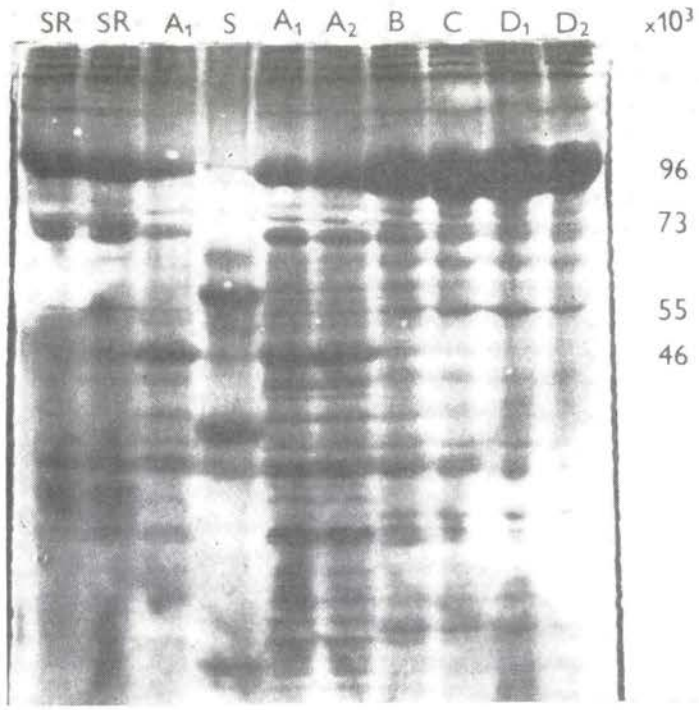

Fig. 7. SDS polyacrylamide gel electrophoresis of the crayfish sarcotubular fractions after the separation on the discontinuous sucrose density gradient. A_1, A_2 — light fraction; B, C — intermediate fraction; D_1, D_2 — heavy fraction; S — molecular weight standards; SR — sarcotubular vesicles prior to the separation on the sucrose density gradient.

The light fraction may therefore be considered as that containing elements originating from the T-system.

Differences in protein composition

Using SDS electrophoresis, differences in protein composition between individual subfractions isolated on the continuous (Fig. 6) or discontinuous (Fig. 7) sucrose density gradients, respectively, could be demonstrated. In all fractions, a conspicuous band was formed by a protein of M_r 96,000, the intermediate and heavy fraction being the major site of its localization.

Proteins of M_r 68,000 and 55,000, respectively, predominantly occurred in the heavy subfractions, their presence in the intermediate subfractions was less pronounced.

Protein of M_r 73,000 was contained both in the intermediate and light fractions.

The most pronounced band in the light fraction was formed by a protein of M_r 46,000. Only traces of this band could be detected in other fractions.

Discussion

The possibility of relating individual fractions of sarcotubular microsomes to ultrastructural components in intact muscle fibres may be considered a prerequisite for studies concerned with molecular mechanisms operating during the contraction-relaxation cycle.

The analysis presented herein represents the first attempt to elucidate this problem in an arthropod muscle. Previous investigations on rabbit muscle using ^{3}H-ouabain as a marker for T-tubule membranes (Caswell et al. 1976; Lau et al. 1977; Caswell et al. 1979) disclosed the presence of T-system elements in the heavy fraction of the sucrose density gradient (40 % sucrose). Ouabain-binding vesicles were there mechanically linked to terminal cisternae of the sarcoplasmic reticulum. Only after breaking this linkage with a French press, the ouabain-binding vesicles migrated to the lighter part of the density gradient (22 % sucrose).

With the crayfish muscle, no special measures had to be taken to detach T-tubules from the sarcoplasmic reticulum (SR). Following muscle homogenization and centrifugation, the ouabain-binding or ruthenium-red stained vesicles appeared in the light fraction of the sucrose density gradient. The failure to find a single ruthenium red stained vesicle in other than the light fraction suggests a complete detachment of SR from the T-system. This different behaviour may be explained by ultrastructural differences between the different types of muscle fibres. In contrast to rabbit muscle (Caswell et al. 1976) where T-tubules, the central element of triads, are firmly attached to both sides of the terminal cisternae of SR, in crayfish mostly diads are encountered and the non-junctional (non-attached) T-tubules make up about 65 % of the total T-system membrane area of long-sarcomere fibres (Uhrík et al. 1980). The proportion of the short-sarcomere (fast contracting) fibres in crayfish claw muscles is not exactly known; our preliminary observations suggest that they may form only a minority of the whole fibre population.

SDS polyacrylamide gel electrophoresis revealed marked differences in protein composition between the fraction containing T-system elements and other fractions.

The protein of M_r 96,000, contained mainly in the heavy fraction with the lowest ouabain binding may be assumed to correspond to Ca^{2+}-ATPase the molecular weight of which is known to be about 100,000 (Meissner et al. 1973;

Tada et al. 1978). Hasselbach and Migala (1975) reported a value of 102,000 ± 14,000.

The protein of M_r 55,000 could hypothetically be brought in connection with the high-affinity calcium binding protein termed M_{55} (Meissner and Fleischer 1973; Warren et al. 1974).

In view of the results of ouabain binding (Fig. 5), the major component of the light fraction (containing T-system vesicles), a protein of M_r 46,000, could tentatively be related to β-subunit of $(Na^+ + K^+)$-ATPase (M_r about 40,000; see Jørgensen 1982).

Any discussion on the possible physiological implications of these differences should, however, be reserved until more experimental data concerning protein identification are accumulated.

In comparing our results with those on protein composition of the rabbit T-tubule fraction both interspecies differences and divergent findings on rabbit sarcotubular fractions are to be taken into account. According to Brandt et al. (1980) the protein of M_r 102,000 constitutes only a minor part of the rabbit T-system vesicles, the main bulk being formed by proteins of M_r 112,000; 67,000; 77,000; and 68,000. On the other hand, Rosemblatt et al. (1981) found Ca^{2+}--ATPase (M_r 100,000) to be the major protein component (40 %) of rabbit T-tubules; proteins of M_r 75,000; 36,000; and 28,000 make up only 10 to 15 % of the total protein.

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