The crystalloid endoplasmic reticulum (ER) of UT-1 cells is a specialized smooth ER that houses 3-hydroxy-3-methylglutaryl-CoA reductase, a membrane protein that regulates endogenous cholesterol synthesis. The biogenesis of this ER is coupled to the overproduction of 3-hydroxy-3-methylglutaryl-CoA reductase. To understand better this membrane system and the relationship between the synthesis of a membrane protein and the formation of membrane, we have purified the crystalloid ER. Purified crystalloid ER did not contain significant amounts of membrane derived from the Golgi apparatus, mitochondria, or plasma membrane. Approximately 24% of the protein in this organelle corresponded to 3-hydroxy-3-methylglutaryl-CoA reductase; however, at least eight other proteins were detected by gel electrophoresis. One of these proteins (Mr 73,000) was as abundant as reductase. These results suggest that the biogenesis of this ER involves the coordinate synthesis of multiple membrane and content proteins.

Progress in the understanding of cell function often is achieved through the study of cells that overexpress an activity that ordinarily takes place at low levels within the cell. For the study of membrane biogenesis and control of intracellular protein traffic, the UT-1 cell (1) is unique. This cell contains a proliferated smooth endoplasmic reticulum, called the crystalloid endoplasmic reticulum (ER), that arises in response to the synthesis of large amounts of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), a membrane protein of the endoplasmic reticulum that regulates the endogenous synthesis of cholesterol (2).

The UT-1 cell is a Chinese hamster ovary cell line that has been adapted to grow in the presence of compactin, a competitive inhibitor of HMG-CoA reductase. Biochemical (1) and morphological (3) studies have shown that these cells contain 100–500 times more reductase than parental Chinese hamster ovary cells and numerous membrane tubules that are packed together into a hexagonal array that occupies 15% of the volume of the cell. HMG-CoA reductase appears, by immunocytochemical measurements, to be confined to this membrane.

An important feature of the UT-1 cell is that the expression of the crystalloid ER is regulated by cholesterol (1). UT-1 cells grown in the absence of compactin do not have a crystalloid ER. Within several hours after the addition of the inhibitor, however, reductase-containing smooth ER emerges from the nuclear envelope (4); and this membrane subsequently transforms into the tubular elements of the crystalloid ER. If compactin is withdrawn from the differentiated cells or if cholesterol is added to the medium, within 24 h most of the HMG-CoA reductase is degraded and the ER membrane begins to disappear from the cell (5).

The crystalloid ER membrane appears to arise in response to the synthesis of HMG-CoA reductase, a 97-kDa membrane protein with seven membrane-spanning regions (6). Unlike many membrane proteins that are synthesized in the rough ER but travel to the Golgi apparatus and beyond, HMG-CoA reductase is retained in the smooth ER membrane. Therefore, a biochemical analysis of this membrane may yield clues about the mechanism of membrane biogenesis and lead to the discovery of the signals that prevent HMG-CoA reductase, as well as other resident ER proteins, from moving to the Golgi apparatus. For these reasons, we have purified this interesting intracellular membrane organelle and begun to characterize its molecular composition.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following chemicals were from Sigma: HEPES-HCl (H3375), high molecular weight standards for sodium dodecyl sulfate gels (MW SDS3000), low molecular weight standards for sodium dodecyl sulfate gels (MW SDS70L), bovine serum albumin (A7906), and chemicals for both the 5'-nucleotidase assay and cytochrome oxidase assay. All reagents for polycyramide gel electrophoresis were obtained from Bio-Rad. Dulbeco's phosphate-buffered saline, trypsin/EDTA, penicillin/streptomycin, and Ham's F-12 medium were obtained from Gibco. Peroxidase-conjugated goat antimouse IgG (3611-3151) antibody was obtained from Cappel (Malvern, PA). Glutaraldehde and paraformaldehyde were from Electron Microscopy Sciences (Port Washington, PA). Araldite was from Ladd Research Industries, Inc. (Burlington, VT), and Lowicryl K4M was from Polysciences, Inc. (Warrington, PA). Deoxyribonuclease I (6330) was from CooperBiomedical, Inc. (Malvern, PA). Polyclonal anti-HMG-CoA reductase IgG was prepared as previously described (1). Monoclonal anti-HMG-CoA reductase IgG (clone A9) was prepared as previously described (7). Protein A-gold was prepared as previously described (5) using protein A (17-0770-01) obtained from Pharmacia (P-L, Biochemicals). New birth calf lypoprotein-deficient serum (d > 1.215 g/ml) was prepared by ultracentrifugation (1).

**Cell Culture**—The UT-1 cell is a cell line derived from Chinese hamster ovary K1 cells that was adapted to compactin resistance as previously described (1) and grown continuously in 40 µM compactin. Cells were grown in a monolayer in a 5% CO₂ incubator at 37 °C in...
medium A (Ham's F-12 medium containing 10% (v/v) lipoprotein-deficient serum, 40 μM compactin, 2 mM glutamate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM Hepes, pH 7.4). On day 0, 5 × 10^6 cells were seeded into each roller bottle, and 79 ml of medium A were added. Fresh medium was added every 2 days, and cells were harvested on day 6. To harvest the cells, each roller bottle was washed twice with 4°C with 20 ml of Dulbecco's phosphate-buffered saline. 5 ml of medium A containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA were added to each roller bottle and incubated while rotating the bottle for 5 min at 37°C. Then, 25 ml of cold medium B (Ham's F-12 medium containing 25 mM Hepes, pH 7.4). The cells from eight roller bottles were combined, and the total volume was brought to 30 ml with medium C. A 0.7-ml sample of the suspension was removed (designated whole cell), and the remaining material was centrifuged at 600 × g for 10 min at 25°C. Isolation of Crystalloid ER—The steps in the purification of the crystalloid ER are shown in Fig. 1. The cell pellet from each roller bottle was resuspended in 3 ml of medium C (Ham's F-12 medium containing 25 mM Hepes, pH 7.4). The cells from eight roller bottles were combined, and the total volume was brought to 30 ml with medium C. A 0.7-ml sample of the suspension was removed (designated whole cell), and the remaining material was centrifuged at 6000 × g for 10 min at 25°C to collect the cells. This cell pellet was resuspended in 3 ml of ice-cold buffer A (40 mM Tris maleate, pH 6.8, 5 mM MgCl₂, 5 mM EGTA, 10 mM dithiothreitol, 0.1 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride) and homogenized in 1-ml batches by passage through a 27-gauge needle 35 times at 4°C. Each 1-ml batch of homogenate was centrifuged at 1000 × g for 10 min at 4°C, and the supernatant fractions were combined and saved (designated low speed supernatant 1 (LSS-1)). The pellets were resuspended in 3 ml of ice-cold buffer A containing 600 units of DNase I, gently mixed by inversion, and incubated for 10 min at 37°C. Following this incubation, each sample was passed through a 27-gauge needle 10 times at 4°C and centrifuged at 1000 × g for 10 min at 4°C. The supernatant fractions were combined and saved (designated low speed supernatant 2 (LSS-2)). The pellets were resuspended in ice-cold buffer A containing 0.5 M sucrose to a final volume of 600 μl.

To separate the crystalloid ER from other cellular contaminants, the pellet from the second low speed spin was centrifuged on a sucrose gradient. Gradients were then centrifuged for 20 h at 40,000 × g at 4°C using a Sorvall AH627 swinging bucket rotor. Three distinct bands, designated B1, B2, and B3, were visible at the end of the centrifugation and had refractive indices of 1.390, 1.403, and 1.413, respectively. Each band from the gradient was stored at −70°C.

Electrophoresis and Immunoblotting—Each sample was solubilized in buffer B (15% sodium dodecyl sulfate, 10% sucrose, 62.5 mM Tris-HCl, pH 6.8, 8 M urea, 100 mM dithiothreitol) and electrophoresed in an 8% polyacrylamide slab gel according to the method of Laemmli (8). Either the gels were stained with 0.04% Coomassie Blue or the proteins were transferred to nitrocellulose paper (9). All gels that were transferred contained prestained molecular weight standards. Following transfer, the nitrocellulose paper was incubated for 2 h at 25°C in buffer C (50 mM Tris- HCl, pH 7.4, 80 mM NaCl, 5% (v/v) Carnation nonfat milk, 0.02% (v/v) Nonidet P-40). To localize HMG-CoA reductase, nitrocellulose strips were incubated with monoclonal anti-HMG-CoA reductase IgG (25 μg/ml) in buffer C for 2 h at 25°C. After two brief washes in buffer C at 25°C, the nitrocellulose strips were incubated for 15 min at 25°C in buffer C. Anti-HMG-CoA reductase IgG-binding sites were visualized by incubating the nitrocellulose strips with affinity-purified anti-mouse IgG coupled to horseradish peroxidase (0.3 μg/ml in buffer C) for 2 h at 25°C. The strips were then washed five times with buffer C in the absence of Carnation nonfat dry milk and Nonidet P-40. Following the wash, each strip was incubated in buffer D (0.6 mg/ml 4-chloro-1-naphthol, 50 mM Tris-HCl, pH 7.4, 6.5 mM NaCl, and 0.01% (v/v) freshly added H₂O₂ for 10-30 min at 25°C. The reaction was stopped by washing with distilled water, and the nitrocellulose samples were air-dried and stored in the dark in an air-tight wrapping at 4°C.

Quantification of Immunoblots—For each fraction, three different concentrations of protein were electrophoresed, transferred to nitrocellulose, and processed for detection of HMG-CoA reductase by indirect immunoperoxidase staining as described. Each of the immunoblots was then scanned with an LKB densitometer (Model 2202), and the area under the peak curve was cut out and weighed. Following electrophoresis, the replica of the HMG-CoA reductase peak was plotted as a function of the amount of protein added to the gel. From this curve, the specific activity of HMG-CoA reductase in each fraction was calculated as the area/μg of protein.

Immunoelectron Microscopy—Immediately after isolation, fractions were fixed for 1 h at 4°C by pelleting through fixative containing 1% glutaraldehyde, 0.1 M sodium cacodylate, pH 7.4, 0.3 M sucrose, and 4 mM CaCl₂. Samples were washed five times in 0.1 M sodium cacodylate and incubated for 30 min at 4°C in 0.1% sodium borohydride. After washing five times in distilled water, samples were stained en bloc with 0.1% uranyl acetate in distilled water for 30 min at 25°C. After washing three times in distilled water, the samples were embedded in Ezecryl K4M as previously described (5). All tissues were sectioned on an M2B Sorvall ultramicrotome, and the sections were collected on Formvar-coated nickel grids. Grids with sections were floated on phosphate-buffered saline (PBS), pH 7.4, containing 1% (w/v) ovamblin for 1 h at 25°C. Grids were then floated on 70 μl of PBS containing either 0.5 mg/ml polyclonal anti-HMG-CoA reductase IgG or 0.5 mg/ml nonimmune rabbit IgG and 0.5% ovamblin for 16-24 h at 4°C. IgG-binding sites were localized with protein A-gold (15-nm diameter) prepared as previously described (5). Grids were jet-washed three times with PBS and then floated on 300 μl of protein A-gold (1:30 dilution in PBS, pH 6.8) for 1 h at 25°C. Grids were jet-washed thoroughly with PBS and then soaked in PBS for 5 min. This sequence was repeated three times before the grids were rinsed with distilled water and dried.

Grids were stained with 4% uranyl acetate for 15 min and lead citrate for 5 min at 25°C.

Electron Microscopy—Immediately after isolation, B1 was fixed by pelleting through 3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4. The pellets were post-fixed with 2% OsO₄ in 0.1 M sodium cacodylate, pH 7.4, and stained en bloc with 0.5% uranyl acetate. Samples were dehydrated and embedded in Araldite.

Other Assays—HMG-CoA reductase activity was measured as previously described (10). Protein was determined by the procedure of Lowry et al. (11) after precipitation with 18% trichloroacetic acid in the presence of 0.1% sodium dodecylsulfate, 5'-Nucleotidase activity was measured according to a modification of the method of Dixon and Purdom (12). Cytochrome oxidase was measured according to the method of Wharton and Tzagoloff (13).
RESULTS

Strategy for Purification of Crystalloid ER—Our goal during the development of the purification procedure was to obtain large quantities of intact crystalloid ER. This required gentle homogenization to keep the tubular elements together and low speed centrifugation to separate the larger intact structures from other organelles and membranes. A major contaminant in the low speed pellet, however, was the nuclei. These were separated from the intact crystalloid ER by treating the pellets with DNase, followed by flotation from 2.2 M sucrose

FIG. 2. Quantitative analysis of HMG-CoA reductase in B1 fraction. UT-1 cells were grown in roller bottles and harvested on day 6. The cells were then processed to isolate the crystalloid ER as described (see Fig. 1). Three different amounts of B1 protein (10, 5, and 1 µg) were electrophoresed and transferred to nitrocellulose. Each sample was then processed to localize HMG-CoA reductase as described under "Experimental Procedures." The peroxidase-positive band was scanned for each sample (inset), and the area under the curve was plotted as a function of the amount of protein loaded onto the gel. The slope of the line corresponds to the specific activity of HMG-CoA reductase in the fraction. This method was used to determine the amount of reductase in each fraction shown in Table I.

TABLE I

Quantification of HMG-CoA reductase in cell fractions

UT-1 cells were harvested and processed to isolate the crystalloid ER as described. Three different concentrations for each fraction as well as the whole cell starting material were electrophoresed, transferred to nitrocellulose, and immunoblotted with anti-HMG-CoA reductase IgG using an indirect immunoperoxidase procedure. Each lane was scanned by densitometry, and the area under the peak was calculated. As described for Fig. 2, a standard curve was generated, and the specific activity for each fraction was derived from the slope of this curve.

| Fraction | Specific activity | Total protein | Total activity | Activity |
|----------|------------------|---------------|----------------|----------|
|          | area/µg | mg | µg | µg |
| Whole cell | 0.70 | 62.13 | 43.50 | 100 |
| LSS-1 | 0.34 | 42.00 | 14.28 | 32.8 |
| LSS-2 | 0.77 | 16.50 | 12.70 | 29.2 |
| B1 | 6.90 | 1.46 | 10.07 | 23.2 |
| B2 | 2.25 | 0.47 | 1.05 | 2.4 |
| B3 | 0.21 | 1.66 | 0.34 | 0.8 |

Fig. 3. Ultrastructural analysis of B1 fraction. UT-1 cells were grown in roller bottles and harvested on day 6. The cells were then processed according to the procedure shown in Fig. 1. B1 fraction was pelleted into glutaraldehyde and embedded in Araldite. A, magnification × 5,500; B, magnification × 15,000.

into a linear 1.5 to 0.5 M sucrose gradient. Due to the long centrifugation times and the susceptibility of HMG-CoA reductase to proteolytic cleavage (11), all buffers used in this procedure, as well as the sucrose gradients, contained protease inhibitors that are known to prevent the hydrolysis of this protein (14). At the end of the centrifugation, there were three distinct bands in the sucrose gradient with refractive indices of 1.390, 1.403, and 1.413. These bands were designated B1, B2, and B3, respectively.

To monitor the purification of the crystalloid ER, we used quantitative immunoblotting. Three different protein concentrations for each fraction were electrophoresed into polyacrylamide gels and transferred to nitrocellulose. HMG-CoA reductase was detected on the nitrocellulose by immunoperoxidase staining using a monoclonal anti-HMG-CoA reductase IgG. Peroxidase bands were then scanned by a densitometer, and the area under the curve was plotted as a function of the protein concentration loaded onto the gel (Fig. 2). Protein concentrations were chosen to ensure that the densitometry tracings were in the linear range, and the specific activity of HMG-CoA reductase in the fraction. This method was used to determine the amount of reductase in each fraction shown in Table I.
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FIG. 4. Indirect immunogold localization of HMG-CoA reductase in B1 fraction. B1 fraction was isolated as described (see Fig. 1). After fixation, the pellet was embedded in Lowicryl K4M. Thin sections were prepared and incubated with either polyclonal anti-HMG-CoA reductase IgG (A) or nonimmune IgG (B) before incubation with protein A-gold. Magnification X 27,000.

As seen in Table I, B1 contained the highest specific activity of HMG-CoA reductase. Compared to the whole cell, this band was enriched 10-fold in HMG-CoA reductase and contained 23% of the enzyme. B2 also was enriched in HMG-CoA reductase but contained only 2.4% of the total enzyme. The majority of the remaining HMG-CoA reductase was in the supernatant fraction from the low speed centrifugation step; this may correspond to smooth ER fragments created during the homogenization procedure. Typically, in this fractionation scheme, we recovered nearly 90% of the HMG-CoA reductase. By this analysis, B1 should contain the most purified crystalloid ER.

Characterization of B1—When each of the three bands was fixed and embedded for electron microscopy, B1 contained structures that corresponded in appearance to the crystalloid ER. As seen in Fig. 3, this fraction contained numerous clusters of membrane-bound vesicles that were joined together by amorphous matrix material. The diameter of these vesicles (120-330 nm) was two to three times larger than the diameter of the crystalloid tubules within the UT-1 cell (3). Longitudinal tubular profiles were not evident in these membrane aggregates, which indicates that the tubules formed vesicles during the isolation procedures. As judged from these electron micrographs, there were very few contaminating organelles in the B1 fraction. B2, on the other hand, contained fewer bundles of vesicles and larger numbers of mitochondria, whereas B3 was largely composed of amorphous material and unidentified membranes (data not shown).

To determine whether the aggregates of membrane-bound vesicles in B1 contained HMG-CoA reductase, this fraction was fixed, embedded in Lowicryl K4M, and analyzed for the distribution of anti-HMG-CoA reductase IgG-binding sites using indirect immunogold labeling (5). Fig. 4A shows that although the membrane morphology of the vesicle aggregates was difficult to discern, owing to the absence of a post-
The gel was then scanned using a laser densitometer (B), and the area under each peak was determined. The area under each peak was used to calculate the percent protein in that peak compared to the total protein loaded on the gel (the sum of all peaks).

**Fig. 6. Quantitative analysis of distribution of proteins in crystalloid ER.** Purified crystalloid ER was electrophoresed into polyacrylamide gels and stained with Coomassie Blue (A). The gel was then scanned using a laser densitometer (B), and the area under each peak was determined. The area under each peak was used to calculate the percent protein in that peak compared to the total protein loaded on the gel (the sum of all peaks).

**DISCUSSION**

To proceed further with the study of the UT-1 cell and to take advantage of the potential of the crystalloid ER for studying membrane biogenesis and protein sorting, this membrane system needed to be isolated. The method that we have developed specifically selects for the crystalloid ER, leaving behind other smooth ER elements that may be free in the cytoplasm of the cell or loosely attached to the tubules of the crystalloid ER. Although the tubular structure is disrupted during the isolation procedure, these membranes withstand the stress of isolation and remain attached to one another. The isolation procedure routinely yielded 1–2 mg of crystalloid ER protein from 6 × 10⁶ cells.

The criteria for purity of the crystalloid ER were based on quantitative immunoblotting, electron microscopy, and measurement of known marker enzyme activity. Numerous attempts were made to monitor purification by measuring HMG-CoA reductase enzyme activity. These measurements, however, proved to be unreliable because the enzyme appeared to be inactivated during purification. The reasons for this inactivation are not clear. The enzyme was not degraded during the purification process because the monoclonal anti-reductase IgG, which recognizes the 58- and 50-kDa proteolytic fragments of reductase (7), bound to only the 97-kDa intact form of the enzyme on immunoblots. A major difference between our purification protocol and other methods for purifying HMG-CoA reductase (15) is that the enzyme remained intact and embedded in the phospholipid bilayer. Conceivably, this form of the enzyme is susceptible to inactivation. Alternatively, the activity of the membrane-bound form of the enzyme may require a cytosolic factor.

Within the UT-1 cell, reductase represents 2% of the total protein (1). In the purified crystalloid ER, this enzyme is enriched 10–12-fold compared to the whole cell; therefore, 20–24% of the crystalloid ER protein should be reductase. This value is in agreement with the contribution of reductase to the total amount of crystalloid ER protein determined by densitometric scanning of Coomassie Blue-stained polyacrylamide gels (Fig. 6). On the other hand, if we use stereological techniques to calculate the quantity of reductase in this organelle, we obtain a far lower value. We have calculated previously that there are 700 molecules of reductase/μm² of the crystalloid ER membrane (9), yet this type of membrane

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2 R. Kornfeld, personal communication.
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should contain about 30,000 molecules/μm² (16). This means that only 2.3% (700/30,000) of the membrane protein should be reductase. The discrepancy between the stereological measurements and the direct measurements is difficult to explain. One possibility, however, is that the density of membrane proteins is lower in the crystalloid ER than it is in other ER membranes.

Regardless of the method used to calculate the quantity of reductase in these membranes, there is little doubt that many of the proteins in the purified crystalloid ER are also membrane proteins. Particularly intriguing is the 73-kDa protein, which is as abundant as reductase. Therefore, even though the crystalloid ER is synthesized in response to the production of large amounts of reductase, this ER contains other membrane proteins. This is unlike rod outer segment membranes that contain nearly pure rhodopsin (20,000 mol/μm² (17)), another transmembrane protein with multiple membrane-spanning regions (18).

The purification of the crystalloid ER represents a major advance in the development of techniques to study the structure and function of this organelle. Further studies will focus on determining whether the other proteins in the crystalloid ER are regulated by cholesterol and whether they correspond to ER proteins that are found in all cells. Purified crystalloid ER may also be useful for identifying molecules that pass through smooth ER elements during transport from their site of synthesis in the rough ER to the cell surface. Conceivably, the crystalloid ER is a staging area for movement of proteins to the Golgi apparatus; if this is the case, then this organelle could be used to study ER to Golgi transport.

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