Molecular Organization of Prolactin Granules

II. Characterization of Glycosaminoglycans and Glycoproteins of the Bovine Prolactin Matrix

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ABSTRACT Prolactin (PRL) granules can be isolated from the anterior pituitary gland of adult cows in nearly 50% yield by use of a procedure previously developed for the fractionation of the rat pituitary. Treatment of the isolated bovine granules with 0.2% Lubrol PX results in the solubilization of most membranes present in the fraction but has only a limited effect on the matrices, which remain aggregated and can be recovered and purified by gradient centrifugation. These membraneless PRL granules, studied in detail by morphological and biochemical techniques, were found to contain only small amounts of contaminants (primarily growth hormone granules and small membrane fragments). SDS polyacrylamide gel electrophoresis revealed that, in comparison with other fractions isolated from the bovine pituitary, the membraneless granules have a simpler polypeptide composition including PRL (~85%), growth hormone (~8%), as well as ~13 minor bands with apparent mol wt ranging from 80,000 to 45,000. Many of these minor bands are accounted for by glycoproteins, as revealed by their binding of ^{125}I-concanavalin A, and two of these are also stained blue by the stains-all procedure, a reaction specific for acidic glycoconjugates. Chemical analyses of the membraneless granule fraction revealed the presence of a heterogeneous mixture of complex carbohydrates. Among glycosaminoglycans, the major component is heparan sulfate, while hyaluronic acid and chondroitin sulfate are present in smaller amounts. Moreover, some of the glycoproteins are sulfated and account for over 50% of the nondialyzable ^{35}S radioactivity found in the fraction isolated from labeled slices. Although the concentration of glycosaminoglycans and glycoproteins is relatively low in membraneless granules, the possibility that their presence in the fraction is largely due to cross-contamination and/or artifactual adsorption could be excluded on two grounds. These are: (*a*) electron microscope radioautography of preparations obtained from [^{35}S]sulfate- and D-[6-^{3}H]glucosamine-labeled slices showed a significant labeling of PRL granules in both intact cells and membraneless granule pellets, and (*b*) a mixing experiment showed that membraneless granules contain very little macromolecular sulfate radioactivity adsorbed from the soluble glycoconjugates present in the pituitary homogenate.

Experimental evidence obtained during the last several years clearly indicates that macromolecular carbohydrates are components of secretory granules not only in cell systems which are specialized for their secretion, but also in many others whose main exported products are of a different nature (e.g., amines and nonglycosylated proteins and peptides) (10, 19). The function of these minor components is still poorly defined. Recently, however, there has been considerable interest in these macromolecules since it has been proposed that they might be involved in the packaging of secretion products (21). This process, which occurs in the Golgi complex, results in the organization of soluble products into large, macromolecular aggregates, with a concomitant decrease (which in some cases can be quite substantial) in their osmotic activity. Packaging is

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important for the economy of cells because the aggregates can be stored within secretion granules without a large expenditure of energy.

To clarify the function of complex carbohydrates present in relatively small amounts in secretory granules, it will be necessary to know more about their actual concentrations, chemical composition, and localization in the organelles, as well as about their biosynthesis, transfer into secretory granules, and final disposal. The available information in this area is, however, quite limited. Complex carbohydrates of only two granule types (the chromaffin granules of adrenal medulla [6] and the zymogen granules of exocrine pancreas [23]) have been investigated by quantitative biochemical techniques. In the case of zymogen granules it is not yet even clear whether these molecules are mainly located in the granule content or in the limiting membrane (23, 13).

Previous work from one of our laboratories indicated that prolactin (PRL)[1] granules isolated from mammotroph cells of the rat pituitary might provide a suitable system for studying the role of macromolecular carbohydrates in storage mechanisms. These organelles can be isolated by a relatively simple centrifugation procedure (35), they are characterized by an extremely stable structure of their contents, which remain aggregated even after solubilization of the limiting membrane (8), and they contain sulfated glycosaminoglycans and glycoproteins (7). The latter results have been recently confirmed and extended by Slaby and Farquhar (27). However, because of the small size of the rat pituitary, the amounts of PRL granules which could be isolated were not sufficient for an adequate study of the composition, biosynthesis, and release of their complex carbohydrates. Because of this limitation we have used the pituitary gland of adult cows for the present studies. This report concerns the characterization of glycosaminoglycans and glycoproteins present in the matrix of bovine PRL granules, while the accompanying paper deals with the intracellular transport and fate of these macromolecules.

MATERIALS AND METHODS

Animals

Pituitary glands of lactating Holstein Friesian cows, 2–3 yr old, were removed soon after slaughter and placed either in oxygenated Krebs-Ringer-bicarbonate (KRB) medium containing glucose and amino acids (20), or in 0.32 M sucrose, at 0°C. Processing of the glands was begun within 30 min. Anterior pituitaries were dissected from the neurohypophyses and stripped of their connective tissue capsule.

In Vitro Incubation

Glands were sliced with a Stadie-Riggs hand microtome (Arthur H. Thomas Co., Philadelphia, Pa.) into slices ~0.5 mm thick. In some experiments, slices obtained from two to three pituitaries were transferred to 100-ml Ehrlenmeyer flasks containing 15–20 ml of KRB medium in which $MgSO_4$ had been replaced by $MgCl_2$, together with [^{35}S]sulfate (380 μCi/ml; sp act: 160–640 mCi/mmol). Incubation was carried out for 10 min at 4°C and then for either 90 or 180 min at 37°C and under 95% O_2-5% CO_2. At the end of the labeling period the slices were rinsed with 0.32 M sucrose and homogenized. In pulse-chase experiments, pituitary slices were labeled for 30 min in KRB medium containing either [^{35}S]sulfate (380 μCi/ml) or D-[6-^{3}H]glucosamine (85 μCi/ml; sp act: 20 μCi/μmol) and then reincubated in nonradioactive KRB for 15 min.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; GH, growth hormone; H and L, pellets obtained by high speed centrifugation of the heavy and light bands of the flotation gradient, respectively; KRB, Krebs-Ringer-bicarbonate; Lubrol supernate, material solubilized from PRL-G by Lubrol treatment; MLG, membraneless PRL granule fraction; PAGE, polyacrylamide gel electrophoresis; PRL, prolactin; PRL-G, PRL granule fraction.

Cell Fractionation

The procedures used (Fig. 1) were similar to those developed previously to prepare subcellular fractions from the rat pituitary (35). The postnuclear supernate, obtained by low-speed centrifugation of the homogenate, was fractionated by differential centrifugation to yield postnuclear and total microsomal pellets, as well as a final supernate. The postnuclear pellet was resuspended in a medium containing KCl (1 M), $MgCl_2$ (5 mM), and puromycin (0.5 mM), pH 7, incubated first for 15 min at 4°C, then for 10 min at 37°C, and finally resolved by isopycnic flotation on a discontinuous sucrose density gradient (SW41 rotor, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.; 190,000 *g* for 135 min). The bands collected from the gradient were diluted to 0.32 M sucrose and recentrifuged (Spinco 50 rotor, 120,000 *g*, 60 min). The resulting pellets were labeled L (light), PRL-G (PRL granules), and H (heavy). These fractions correspond to the combined bands 1 + 2, 3 + 4, and 5, respectively, of reference 35.

To solubilize the limiting membrane of PRL granules, aliquots of the PRL-G fractions were resuspended in 0.32 M sucrose containing 0.2% Lubrol PX (detergent:protein, wt:wt, = 4). The higher detergent concentration (0.4%), which was optimal in our previous studies on rat PRL granules (8), could not be used because it produces extensive solubilization of the bovine granule core. After incubation at 4°C for 60 min, the preparations were layered onto a 3-ml cushion of 1.2 M sucrose in Spinco SW 41 tubes. Centrifugation at 30,000 *g* for 60 min yielded a pellet containing PRL membraneless granules (MLG) and a supernate containing the detergent-solubilized material (lubrol supernate).

In the mixing experiment carried out to evaluate the adsorption of soluble sulfated macromolecules onto particular organelles, pituitary slices were labeled in vitro for 3 h with [^{35}S]sulfate as described above, then homogenized and fractionated by centrifugation. An aliquot of the final supernate (25,250 dpm; 0.1 mg of protein) was dialyzed extensively against 0.32 M sucrose to remove free sulfate radioactivity and added to the fluid used to homogenize another set of fresh, nonradioactive pituitary slices (~50 mg protein). The homogenate was then fractionated by centrifugation as shown in Fig. 1. The degree of contamination by [^{35}S]sulfate macromolecules of the various fractions isolated from such a homogenate was assessed by radiochemical determinations.

Light and Electron Microscopy

Small pituitary tissue fragments were fixed for 2 h at 4°C with 2.5% glutaraldehyde in 0.12 M cacodylate buffer, pH 7.4, containing 1% sucrose, then washed for 4 h at 4°C with the buffer-sucrose solution, postfixed with 1% OsO_4 in 0.056 M veronal acetate buffer, pH 7.4, for 2 h at 4°C, and stained in block with uranyl acetate (28). Other tissue fragments were fixed with OsO_4 alone and then block-stained. The centrifugation pellets were fixed *in situ* for 2 h at 4°C with 1% OsO_4 in 0.12 M cacodylate buffer, pH 7.4. Block staining was carried out as reported previously (8). All samples were embedded in Epon 812 and cut with a Reichert

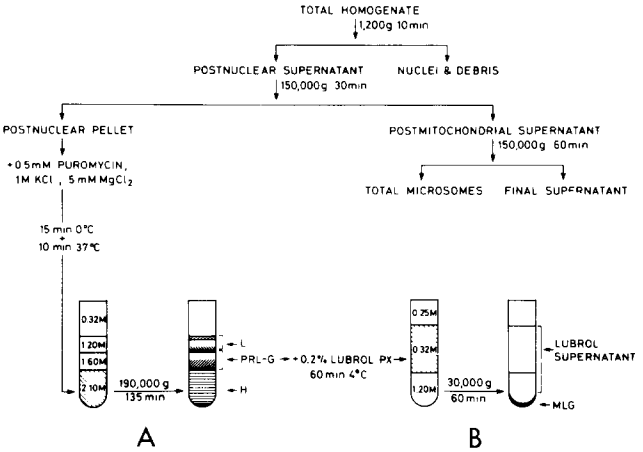

FIGURE 1 Fractionation procedures for bovine pituitary tissue. The postnuclear supernate, postnuclear pellet, total microsomes, and final supernate were isolated by differential centrifugation. The postnuclear pellet was resuspended in high salt-puromycin, incubated first at 4°C (15 min) and then at 37°C (10 min) (to lyse GH granules and detach bound polyribosomes), mixed with concentrated sucrose, and applied to the flotation gradient (*A*). The PRL granules, recovered from the PRL-G band by dilution and centrifugation, were treated for 60 min at 4°C with Lubrol PX (0.2%) in 0.32 M sucrose and sedimented through 1.2 M sucrose (*B*).

Om U_2 ultramicrotome (Reichert O. W. AG, Wien, Austria). Thick sections were stained with basic fuchsin and methylene blue and examined in the light microscope; thin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 or EM 400 electron microscope.

Electron Microscope Radioautography

60-80-nm-thick sections of both pituitary tissue slices labeled in vitro with [^{35}S]sulfate and MLG pellets prepared from slices labeled with either [^{35}S]-sulfate or D-[6-^{3}H]glucosamine (30 min of pulse followed by 15 min of chase incubation) were coated with Ilford L4 photographic emulsion (diluted 1:4) by the loop technique (3). After exposure at 4°C, the preparations were developed by Microdol X for 4 min. To determine quantitatively the density of the radioautographic grains in the components of the pellets, 14 grids labeled with ^{3}H and seven grids labeled with ^{35}S were analyzed. In each grid, five micrographs were taken at random at constant magnification × 7,000 and enlarged to × 22,750. Grains were then counted and assigned to MLG or to the extragranular space according to the procedure of Whur et al. (32). The percent of surface of the pictures occupied by MLG was evaluated by differential point counting (31).

Polyacrylamide gel electrophoresis (PAGE)

SDS PAGE was performed either in 1-mm-thick gel slabs (16) or in 11 × 0.6 cm gel tubes (disc gels), as previously described (36). The concentrations of acrylamide used were 7.5 and 10%. The gels were fixed with 10% TCA in 25% isopropanol, rinsed with several changes of 10% acetic acid in 25% isopropanol, and stained with Coomassie Brilliant Blue R (35).

Acidic macromolecular carbohydrates were identified by the stains all procedure of King and Morrison (12), in disc gels previously fixed in 25% isopropanol. Additional gels were stained by the periodic acid-Schiff procedure (34) for carbohydrates. However, because of the low sensitivity of this technique, the results obtained were useful only for the total microsomal fraction. To identify glycoproteins, we also used a modification of the ^{125}I-concanavalin A (Con A) labeling procedure described by Rostas et al. (25). ^{125}I-Con A (final concentration of 5×10^{6} cpm/ml in 0.5 M NaCl, 0.05 M sodium phosphate buffer, pH 6.5) was applied to 7.5% polyacrylamide gel slabs previously stained with Coomassie Blue. Attempts to use the same technique with 10% gels failed, on account of the poor penetration of the label. After overnight incubation at 37°C in a bath oscillating at 20 cycles/min, the gel slabs were rinsed with large volumes of buffer for 48 h (eight changes), then dehydrated in dimethyl sulfoxide, infiltrated with 2,5-diphenyloxazole, dried under vacuum, and exposed for 2–16 h to RP Royal X-Omat films at −70°C (2). To check the specificity of the ^{125}I-Con A binding, labeling of control slab gels was carried out in the presence of 0.2 M α-methyl-D-glucoside. Addition of this inhibitor completely prevented the appearance of the ^{125}I-Con A labeling.

Stained disc gels, slab prints, and autoradiographic films were analyzed quantitatively in an MK II microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England). The microdensitometric data were also used for the quantitative estimation of growth hormone (GH) and PRL according to techniques previously used for measuring the rat hormones (36).

Fractionation and Analysis of Glycosaminoglycans and Glycoproteins

Glycosaminoglycans and glycoproteins in PRL granules and other pituitary subcellular fractions were quantified as described previously (17, 18). Lipids were removed by extraction with chloroform/methanol, and the lipid-free protein residue was digested with pronase and desalted by gel filtration on Sephadex G-15. Glycosaminoglycans were precipitated with cetylpyridinium chloride, and excess cetylpyridinium chloride was removed from the supernatant solution containing the glycopeptides by extraction with *n*-amyl alcohol. Sulfated glycosaminoglycans were separated from hyaluronic acid by differential precipitation with cetylpyridinium chloride. Hexosamines were determined by use of the amino acid analyzer. The concentrations of glycosaminoglycans were calculated from the glucosamine content of the hyaluronate fraction and from the glucosamine and galactosamine contents of the sulfated glycosaminoglycans (for heparan sulfate and chondroitin sulfate, respectively).

Neutral sugars in the glycopeptides obtained by pronase digestion (see above) and hydrolyzed for 3 h in 2 N trifluoroacetic acid at 100°C, were quantitated by automated ion exchange chromatography of their borate complexes (14). A gradient containing 100 ml each of 0.27 M sodium borate buffer (pH 7.7) and 0.4 M borate buffer (pH 10) with a 0.3 × 70 cm column of Hamilton HAX6 resin was used.

In those cases where data were required concerning only the labeling of various components in subcellular fractions prepared from incubated pituitary slices, glycosaminoglycans and glycopeptides were isolated as described above after addition of unlabeled carrier glycosaminoglycans and glycopeptides. Sulfate radioactivity in chondroitin sulfate and heparan sulfate was determined after digestion of the glycosaminoglycans with chondroitinase ABC, and separation of the resulting disaccharides from the undegraded heparan sulfate by gel filtration on Sephadex G-25. Because our samples did not contain any significant amounts of dermatan sulfate (as shown by the identical results obtained with chondroitinase ABC and AC), the [^{35}S]disaccharides can only originate from chondroitin sulfate A and C.

Biochemical Assays

Protein was determined by the method of Lowry et al. (15), using bovine serum albumin as standard. Total protein radioactivity was measured on washed TCA precipitates as described previously (20). The radioactivity of GH and PRL is that recovered in the corresponding bands of SDS disc gels (20). The total sulfated carbohydrate radioactivity is that which remained in the fractions after extensive dialysis. RNA was assayed according to Schmidt and Tannhauser (26). Phospholipids were assayed according to Ames (1) after extraction and purification according to Folch et al. (5). The following techniques were used for enzyme assays: 5′-nucleotidase: Widnell and Unkeless (33); NADH and NADPH-cytochrome *c* reductases: Sottocasa et al. (29); NADH-ferricyanide reductase: Takesue and Omura (30).

Materials

[^{35}S]inorganic sulfate and D-[6-^{3}H]glucosamine were purchased from the Radiochemical Centre (Amersham, England); ^{125}I from Sorin (Saluggia, Italy); Con A and 5′-adenosine monophosphate from Sigma Chemical Co. (St. Louis, Mo.); NADH, NADPH, and cytochrome *c* (from horse heart, grade 1) from Boehringer (Mannheim, Germany); chondroitinase ABC from Miles Laboratories Inc. (Elkhart, Ind.). Bovine GH (NIH-GH-B18) and PRL (NIH-P-B4) were the kind gift of Dr. A. Parlow, Harbor General Hospital (Torrance, Calif.). All other chemicals were reagent grade.

RESULTS

The weight of the anterior pituitary of adult cows is between 2 and 3 g. Parenchymal cells are arranged in groups separated by well developed fibrous septa, often infiltrated by adipose tissue. The latter is also present as a thick layer around the gland. The light microscope appearance of pituitary slices after in vitro incubation is shown in Fig. 2. Most cells appear well preserved, although the proportion of damaged cells is slightly increased as compared to nonincubated slices (not shown).

By electron microscopy (Fig. 3) PRL cells (4) were recognized as a major component of the gland. In comparison to their better studied counterparts in the rat, these cells contain more numerous large dense granules (250–450 nm in diameter). A few of the granules are irregular in shape, while most appear spherical. The rough-surface endoplasmic reticulum is less developed than in the rat, while the Golgi complex is well organized in multiple parallel stacks. Membrane-free granule cores, in various stages of dissolution, are often seen in pocket indentations of the plasmalemma, especially at the vascular pole of cells (Fig. 3, insets).

Characterization of the Cell Fractions Isolated from the Bovine Pituitary Gland

All of the particulate fractions isolated according to our centrifugation scheme (Fig. 1) were studied by both electron microscopy and biochemical analyses. The present report is, however, restricted to the data concerning the postnuclear and final supernatants and the cytologically well-defined fractions containing organelles involved in the intracellular transport of PRL (i.e., microsomes, PRL-G, and the subfractions isolated from the latter: MLG and lubrol supernate).

ELECTRON MICROSCOPY: The PRL-G fraction (Fig. 4) was purified from the heterogeneous postnuclear pellet, containing secretory granules (most of large size), mitochondria,

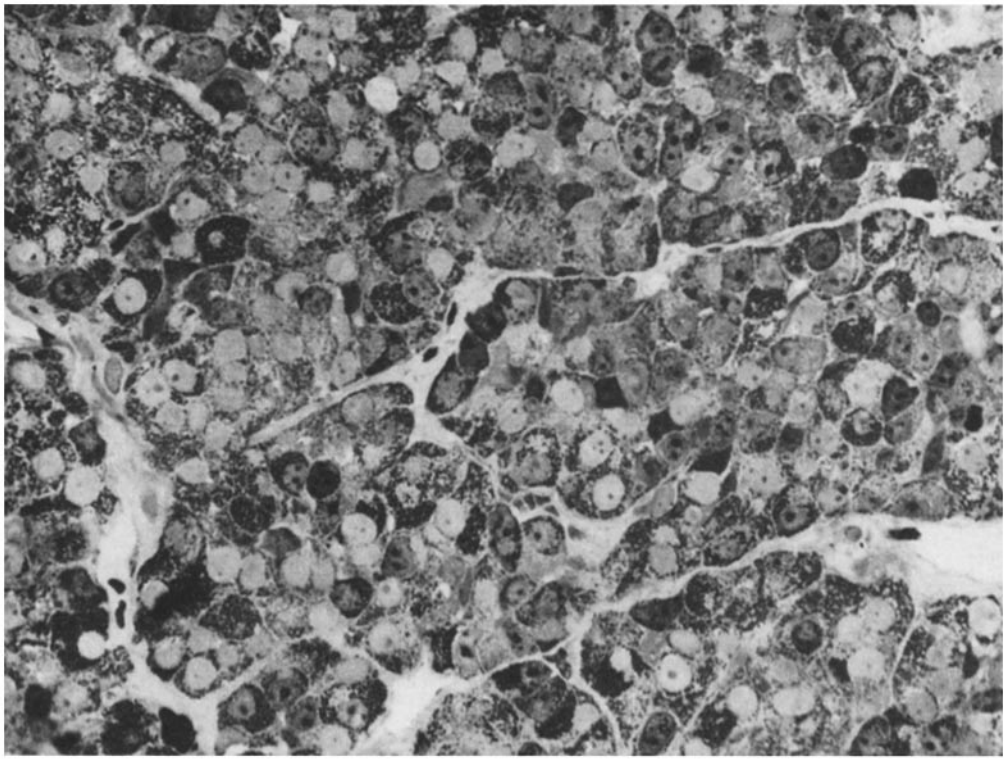

FIGURE 2 Light microscope appearance of a pituitary tissue slice incubated in vitro for 45 min. Thick septa of connective tissue, containing dilated blood vessels, separate groups of parenchymal cells, many of which are heavily granulated; nuclei are large and their chromatin appears evenly dispersed. The cytoplasm density is variable; only very few cells appear clearly damaged. × 160.

large rough-surfaced microsome vesicles and cisternae, and smooth membrane vacuoles and sheets. This pellet was resuspended in a medium containing 1 M KCl, 5 mM $MgCl_2$, and 0.5 mM puromycin, kept at 0°C for 15 min and then heated at 37°C for 10 min. This treatment modifies the buoyancy of rough-surfaced microsomes (by detaching ribosomes) and results in the lysis of most GH granules, while leaving PRL granules relatively unaffected (35). The PRL-G fraction (Fig. 4) isolated from the incubated postnuclear pellet by discontinuous sucrose gradient flotation (Fig. 1), contains large secretory granules similar to those described within PRL cells (Fig. 3). In some of these granules the content has a uniform dense texture, while in many others it appears stippled by clear dots; the content of a few granules appears as disorganized masses of threadlike appearance. The state of the limiting membrane is also quite variable. A few granules are wrapped by a continuous, well-preserved membrane, while in many others the membrane contour is discontinuous or only partial. Furthermore, many membrane-bound vesicles and membrane sheets lie between the granules. Some of these membranes are continuous with portions of granule membranes still remaining *in situ*, others are probably ghosts resulting from granule disruption, and some might be caused by contaminants. However, recognizable contaminant organelles were very rarely seen.

The MLG fraction (Fig. 5) was obtained by Lubrol PX treatment of the PRL-G fraction and recovery by gradient sedimentation of the nonsolubilized material. It is composed of dense granules, either totally devoid of the limiting membrane or retaining a few small membrane fragments. In some MLG the contour is sharp, while in many others it has a typical moth-eaten appearance. Other MLG appear partially or totally disassembled into amorphous masses of dense filaments.

The microsomal fraction (not shown) was found to be composed primarily of vesicles, most of which were covered by attached ribosomes. Other components include smooth flat cisternae, membrane sheets, sparse mitochondria, and some small secretory granules.

TOTAL PROTEIN, HORMONES, PHOSPHOLIPIDS, RNA AND ENZYME ACTIVITIES: The biochemical characterization of the pituitary fractions is reported in Table I. The postnuclear supernate contains ~75% of protein and nearly 90% of PRL present in the gland (not shown in the table). Approximately half of the PRL, along with 17.7% of the protein in the postnuclear supernate, was recovered in the PRL-G fraction. The latter contains also GH (~8% of the hormone present in the postnuclear supernate) and relatively low levels of phospholipids and RNA. NADPH-cytochrome *c* reductase activity (a microsomal marker in liver [27]) is undetectable in the PRL-G fraction, while the activities of 5′-nucleotidase and NADH-cytochrome *c* reductase are fairly low (sp act: ~25 and 10% of the postnuclear supernate, respectively). The significance of the data concerning NADH-cytochrome *c* reductase is, however, not clear, as the recovery of this activity was always incomplete, probably because of its rapid inactivation during cell fractionation.

It can be calculated from the data in Table I that ~72% of the protein and nearly 79% of PRL originally present in the isolated PRL-G fraction were recovered in the MLG fraction after treatment with 0.2% Lubrol PX and reisolation by density gradient centrifugation. The remainder was found in the supernate (Lubrol supernate). The concentration of phospholipids was reduced to one-fourth of that found in intact PRL granules. The distribution of NADH-cytochrome *c* reductase could not be determined because this activity is strongly in-

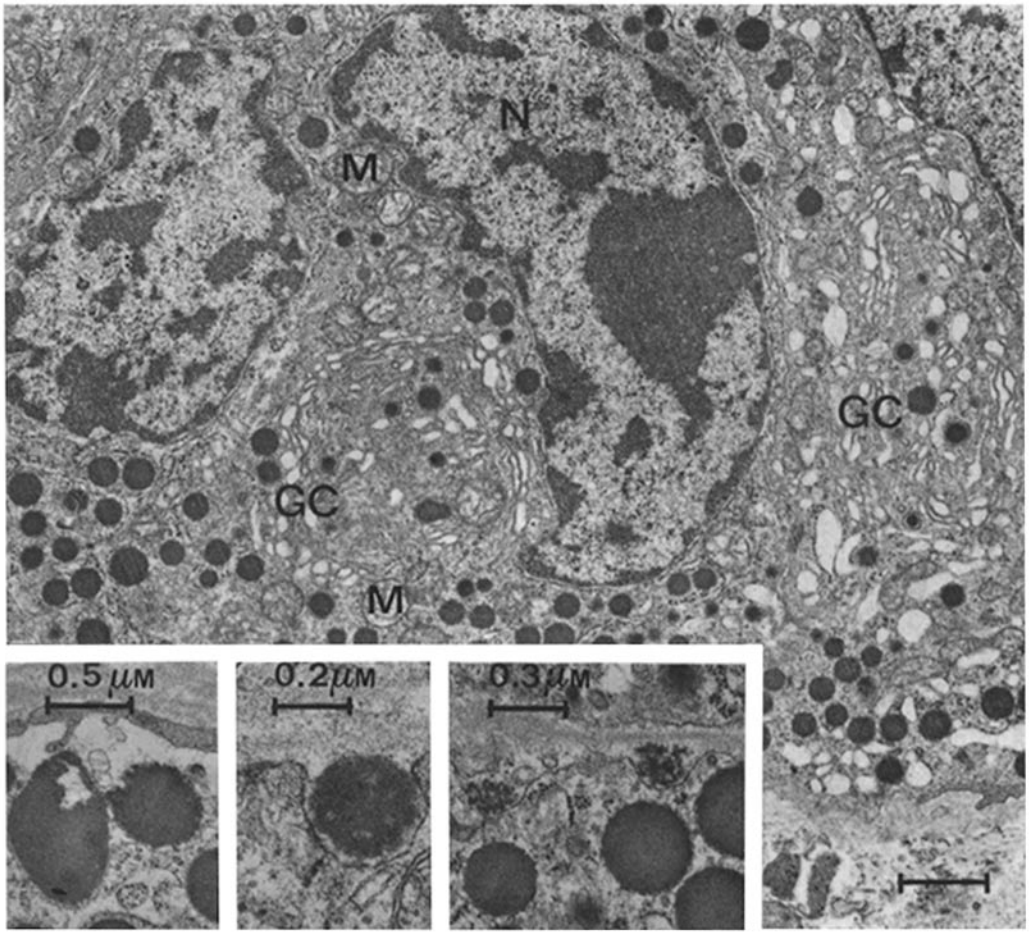

FIGURE 3 The field illustrates representative bovine mammotrophs, which contain large, dense, homogeneous granules, surrounded by closely apposed limiting membranes. Their Golgi complex (*GC*) is well developed and includes immature granules. The rough-surfaced endoplasmic reticulum is relatively sparse. *M*, mitochondria; *N*, nucleus. × 11,300. The *insets* show PRL granules in various stages of solubilization, after their discharge by exocytosis. × 23,100; × 49,500; × 31,500. Note that discharged granules closely resemble the isolated MLG shown in Fig. 5. Unless otherwise indicated, the bar in this and in the following figures corresponds to 1 μm.

hibited by the detergent. However, when ferricyanide is used as electron acceptor, the inhibition is only ~40%. Of the recovered NADH-ferricyanide reductase activity, ~62% was found in the Lubrol supernate and the remainder in MLG. In terms of specific activity, the MLG value was ~5 and 1.5% of that in the postnuclear supernate and total microsomes, respectively (not shown in the table). The total microsomal fraction appears enriched (with respect to the postnuclear supernate) in phospholipids (approximately threefold), 5′-nucleotidase (nearly fivefold), NADPH-cytochrome *c* reductase (over sixfold), and RNA (fourfold). The concentration of GH is also considerable, while that of PRL is relatively low.

SDS PAGE: Analyses on SDS-10% polyacrylamide slab gels (Fig. 6*A*) revealed that GH and PRL are the major protein components of all the fractions investigated. In addition, as many as 50 other polypeptides, with apparent mol wt varying from 140,000 to 15,000 daltons, were resolved from both the postnuclear supernate and total microsomes. A much more simple situation was found in MLG, where only 13 bands appeared localized in the gel region between 80 and 45,000 daltons. Some of these are not typical of the MLG fraction because they are also present, and sometimes prominent, in other fractions. However, at least one band (which migrates as a peptide of ~72,000 daltons (labeled by arrow in Fig. 6*A*; band 9*a* in Fig. 7), is enriched in the MLG pattern. The SDS PAGE pattern of the Lubrol supernate is also quite simple, with a dense band corresponding to PRL and a few poorly resolved minor bands in the region between 65,000 and 50,000 daltons. Of such bands, two (horizontal arrows in Fig. 6*A*) are enriched in comparison with the MLG fraction. Two bands migrating faster than PRL were also observed. One of these comigrates with GH.

The distribution of glycoproteins in SDS 7.5% polyacrylamide gels was investigated by ^{125}I-Con A binding. As can be seen in Fig. 6*B*, several positive bands were revealed in all fractions. The microsomes gave a very strong reaction, with over 20 clearly positive bands; moreover, a heavy positive band was present at the gel front. In the MLG fraction and the corresponding lubrol supernate there were fewer prominent bands and the reaction at the gel front was faint. A comparison of the fluorography and Coomassie Blue patterns (Fig. 7) shows a good correspondence in the 60,000- to 30,000-dalton region of the microsome gel. In contrast, several Con A-positive bands separated in the high molecular weight region of the MLG gel have only faint counterparts in the stained pattern.

Finally the stains all method was used with SDS polyacrylamide disc gels (Fig. 8). By this procedure acidic glycoconjugates such as glycosaminoglycans and sialo- and sulfated gly-

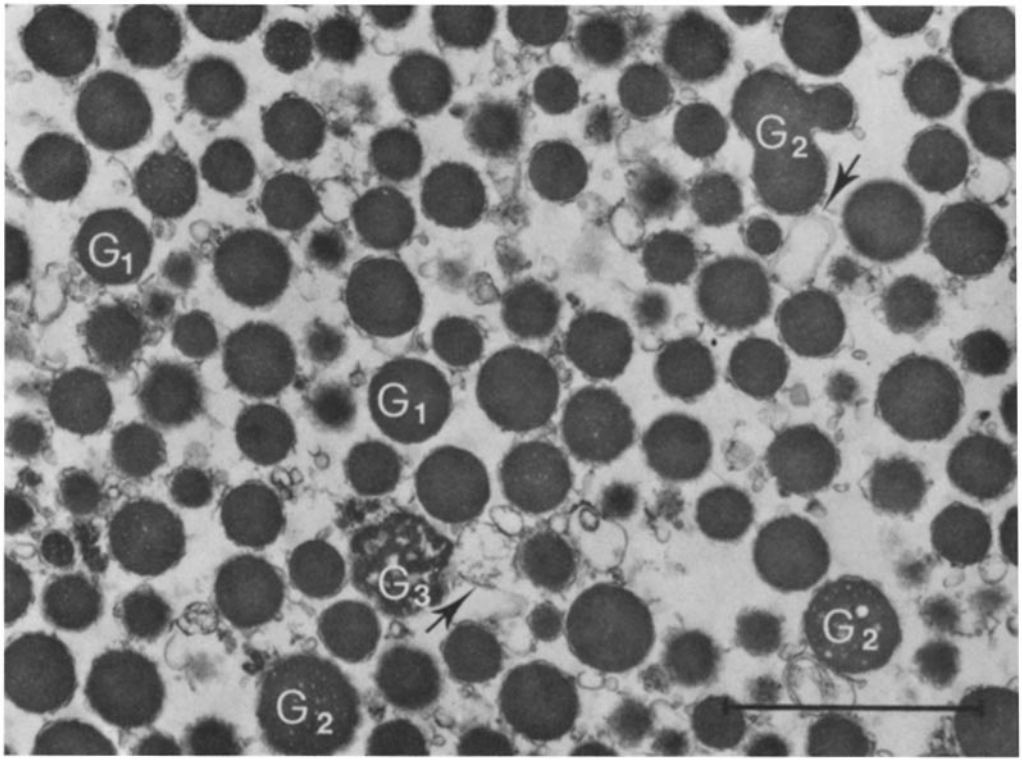

FIGURE 4 Representative field of a PRL-G pellet. This fraction is composed essentially of large, dense granules, most of which are spherical, while some have typical irregular shapes. Many granules have a compact internal structure (G_1), while others (G_2) appear stippled by white dots, which probably result from an initial disarrangement of the granule content. In a few granules (G_3) the damage has proceeded to a partial disruption of the content. The state of the limiting membrane is also variable. In some granules the membrane is well preserved, while in others it is interrupted and partially detached, or entirely lacking. Membrane vesicles and sheets lie between the granules. At least some of these probably originate from disrupted PRL granules (arrows). Recognizable contaminants are rarely encountered. × 34,300.

coproteins, are stained blue, while other proteins stain pink. In microsomes the pattern is composed exclusively of pink and purple bands. The latter color might result from the overlapping of pink and blue bands. In the postnuclear supernate and postnuclear pellet a faint blue band appears. This blue band is greatly enriched in MLG, where it corresponds to band *9a* of the Coomassie Blue-stained pattern (Fig. 7). In addition, a slightly more rapidly migrating minor blue band also appears in MLG. No such bands were observed in the lubrol supernate.

DISTRIBUTION AND LABELING OF GLYCOSAMINOGLYCANS AND GLYCOPROTEINS: Subcellular fractions were prepared from pituitary slices labeled in vitro with sodium [^{35}S]sulfate or D-[6-^{3}H]glucosamine as described in Materials and Methods.[2] Soluble fractions were extensively dialyzed to remove labeled precursors and then lyophilized. The possibility that the ^{3}H labeling of the fractions was caused by the metabolism of glucosamine to amino acids was excluded by the observation that in SDS PAGE the radioactivity was distributed throughout the gels, with very little label recovered in the bands of PRL and GH (not shown).

The distribution of nondialyzable radioactivity in the subcellular fractions, calculated on a protein basis, varied depending on the precursor used (Table II). With slices labeled for 30 min and then chased for 15 min, the ^{35}S macromolecular radioactivity in the PRL-G fraction was nearly 60% of that found in the postnuclear supernate, while that in total microsomes was ~220%. The corresponding values for D-[6-^{3}H]glucosamine-labeled macromolecules are 90 and 280%, respectively.

When the PRL-G fraction was treated with 0.2% Lubrol PX, ~40% of the ^{35}S and 30% of the ^{3}H nondialyzable radioactivity was solubilized, and the rest remained with the MLG. The nature of the labeled macromolecules recovered in the latter fraction was also investigated. ^{35}S counts were found to be nearly equally distributed between glycopeptides (~55%) and glycosaminoglycans (~45%). In contrast, the ^{3}H radioactivity was mostly in glycopeptides (glycosaminoglycans ≅2%). These data demonstrate that the bovine MLG fraction contains glycosaminoglycans in addition to glycoproteins, and that at least some of the latter are sulfated.

The glycosylated macromolecules of pituitary fractions were characterized biochemically with the exception of the Lubrol supernate, for which insufficient material was available. The glycosaminoglycan composition is given in Table III. It is apparent that the amounts of glycosaminoglycans are very low in all fractions. This is particularly true for MLG, whose

[2] When D-[6-^{3}H]glucosamine was used as precursor, in all fractions investigated a large proportion (70–75%) of the incorporated radioactivity was recovered after pronase treatment in the form of low molecular size glycopeptides or oligosaccharides which were retarded during the step of gel filtration on Sephadex G-15 (used for desalting the pronase-digested samples). Although the identity of this labeled material is not clear, we know from previous studies that the glucosamine-labeled glycopeptides prepared from adult rat brain are eluted almost entirely in the void volume from a column of Sephadex G-15 (18), and that similar low molecular size material was not observed in pituitary subcellular fractions labeled with [^{35}S]sodium sulfate.

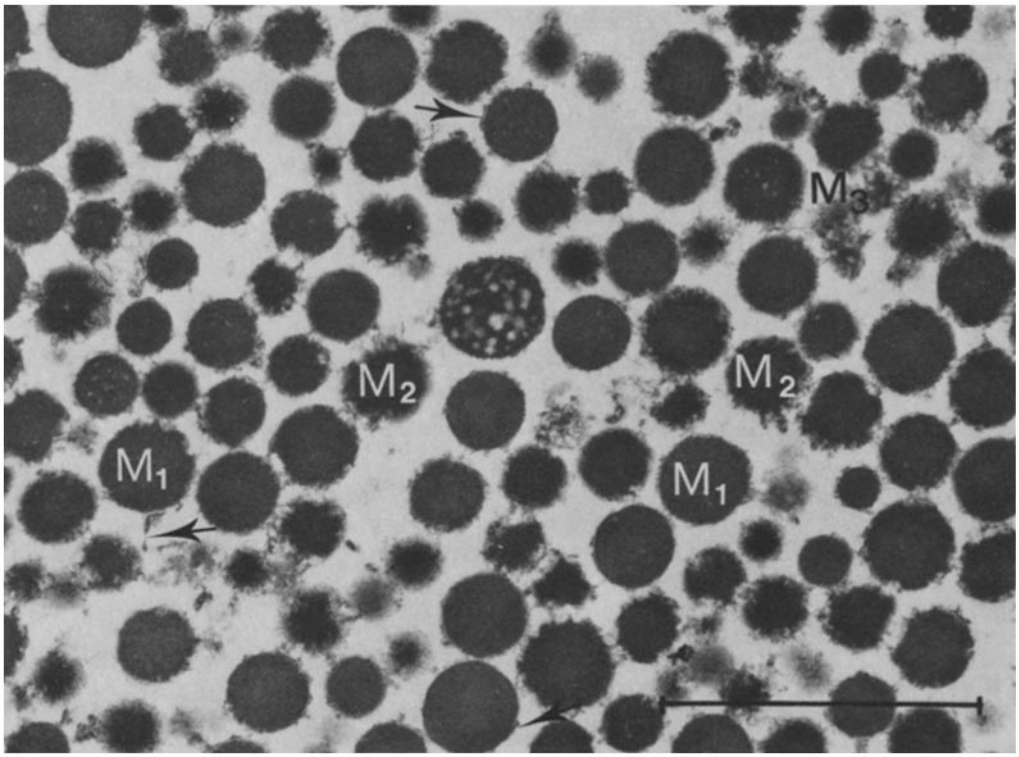

FIGURE 5 Representative field on an MLG pellet. Except for the absence of the membrane and the irregular, often moth-eaten appearance of their contour, many granule cores shown here (M_1) appear very similar to the intact PRL granules illustrated in Fig. 4. Others, however, are more damaged (M_2) with marked unwinding of the material located superficially to yield twisted filaments which often mix with those of surrounding partners. Finally some MLG have been converted to amorphous masses (M_3), where distinct granule cores are no longer recognizable. Note that most membranes (both those bounding the granules as well as those intermingled among them) have disappeared. The few recognizable membrane fragments are labeled by arrows. × 42,400.

TABLE I

Chemical and Enzyme Composition of Subcellular Fractions Isolated from Bovine Pituitary

Sample	Protein*	PRL‡	GH‡	Phospho-lipids‡	RNA‡	NADPH-cytochrome *c* reductase§	NADH-cytochrome *c* reductase§	5′-nucleotidase‖
Postnuclear supernate	50.00	288	130	191	23.5	2.32	186	2,175
Total microsomes	4.60	60	114	592	91.8	14.55	518	10,495
PRL-G	8.85	800	60	141	4.9	<0.1	20	575
Lubrol supernate	2.68	571	<40	×	–	<0.5	××	×××
MLG	6.38	874	66	38	5.0	<0.1	××	×××

×, Lubrol PX interferes with the phosphate assay; ××, the activity is strongly inhibited by Lubrol PX; ×××, the activity is erratically activated by Lubrol PX. The values of phospholipids, RNA, and enzyme activities are averages of two experiments; those of protein, GH and PRL, of three experiments.

* mg/g, fresh weight.

‡ μg/mg protein.

§ nmol of cytochrome *c* reduced/min/mg protein.

‖ μg Pi released/20 min/mg protein.

glycosaminoglycan level is ~55% of that of the postnuclear supernate. The highest concentration was found in total microsomes (145% of that in the postnuclear supernate). The relative proportion of the different glycosaminoglycans among the fractions is also quite similar. In all cases heparan sulfate is the major component, together with chondroitin sulfate and hyaluronic acid in progressively lower concentration.

The distribution of glycoproteins (Table IV) is analogous in some respects to that of glycosaminoglycans. In MLG the concentrations of *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid are 41, 27, and 42% of those found in the postnuclear supernate. The corresponding values in microsomes are 107, 87, and 187%. Considerable amounts of these sugars were found in final supernate. Additional data on the sugar composition of the glycoproteins are available for the MLG, in which mannose, galactose, and fucose are also present. Finally, data on the specific radioactivity of glycoprotein hexosamine and sialic acid of the various fraction isolated from D-[6-^{3}H]glucosamine-labeled slices are reported in Table V. The specific radioactivity of both these sugars was high in the MLG fraction. In contrast, in microsomes the specific radioactivity of hexosamine was high, while that of sialic acid was distinctly lower.

Radioautography and Mixing Experiments

The analytical data indicate that the MLG fraction contains a relatively low concentration of glycoproteins and glycosa-

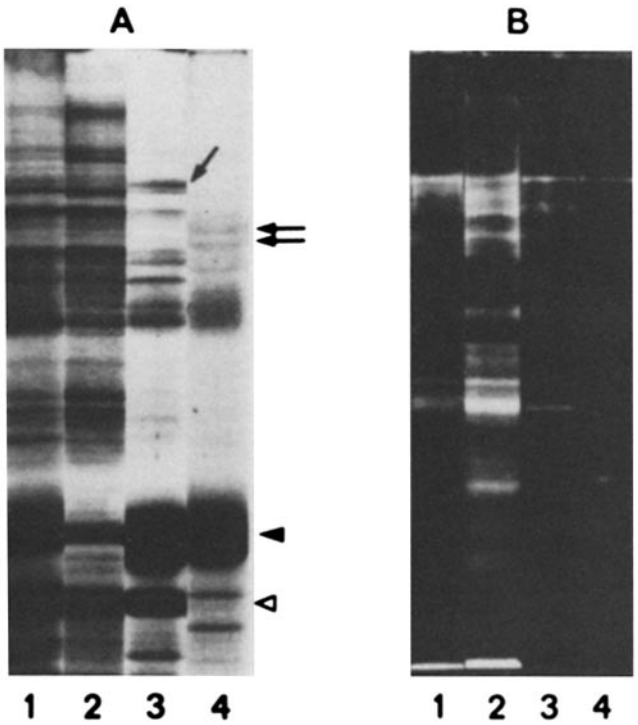

FIGURE 6 (*A*) SDS slab gel (10% in acrylamide) stained with Coomassie Blue and dried under vacuum; *1*, postnuclear supernate; *2*, microsomes; *3*, MLG; *4*, Lubrol supernate. About 80 μg of protein was applied in each trough. The position of the PRL and GH bands is indicated by closed and open arrowheads, respectively. The small oblique arrow points to a minor band, which is prominent in MLG, with migrating rates corresponding to a peptide of ~72,000 daltons. The horizontal small arrows point to two polypeptides enriched in the Lubrol supernate (mol wt ~65,000). (*B*) Fluorography of an SDS (7.5% in acrylamide) slab gel treated with ^{125}I-Con A. The pituitary fractions analyzed (~80 μg) are as follows: *1*, postnuclear supernate; *2*, microsomes; *3*, MLG; *4*, Lubrol supernate. To produce visible patterns for all fractions, it was necessary to expose each gel for different times. The figure is a composite of several prints of a single gel. Exposure times were 2 h for microsomes, 6 h for postnuclear supernate, and 16 h for MLG and Lubrol supernate. On polyacrylamide gels of this porosity, GH and PRL migrate with the running front.

minoglycans, and that the composition of these complex carbohydrates is similar to that in other cell fractions. Therefore, the possibility must be considered that these complex carbohydrates are not components of the PRL granule matrix itself, but are caused by artifacts, such as contamination of the MLG fraction and/or adsorption of soluble molecules during the homogenization and cell fractionation procedures.

To investigate the first possibility, MLG pellets isolated from slices labeled with D-[6-^{3}H]glucosamine or [^{35}S]sulfate were studied by electron microscope radioautography. With both tracers the radioactivity grains were associated with typical granule cores, whether they were completely free of their limiting membrane or still retained small membrane remnants (Fig. 9). Moreover, no concentration of the label was found in those areas of the pellets containing occasional membrane fragments intermingled with MLG. Statistical analysis of the results revealed a <1% probability that the association of radioactivity with MLG was fortuitous (Table VI). Moreover, radioautography of the intact tissue confirmed that PRL granules are labeled with [^{35}S]sulfate (Fig. 10) and suggested therefore that the complex carbohydrates recovered in the MLG

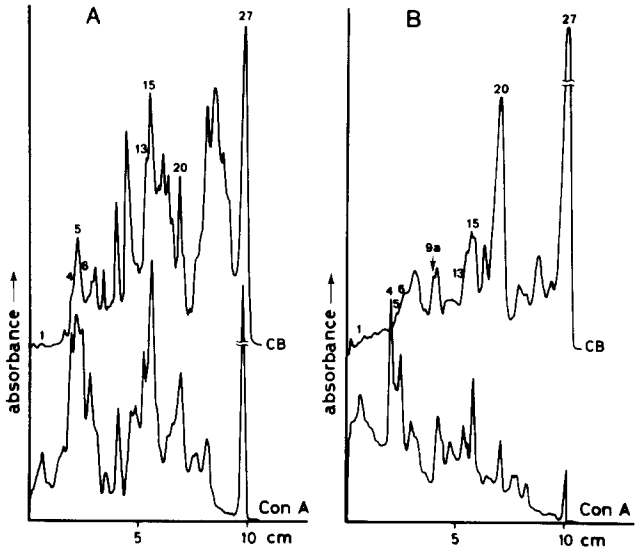

FIGURE 7 Densitometric tracing of Coomassie Blue (*CB*) stain and ^{125}I-Con A fluorography patterns of microsomes (*A*) and MLG (*B*) fractions in SDS slab gels (7.5% in acrylamide). The major separated bands of the microsomes stained with Coomassie Blue were numbered successively from the origin to the front. The same numbers were given to the Coomassie Blue-stained bands of the MLG and microsome patterns as to the fluorography bands of both fractions with the same electrophoretic mobility. The 72,000-dalton band of MLG (Coomassie Blue stain) which is missing in microsomes is labeled *9a*. 80 and 130 μg of protein was used for microsomes and MLG, respectively.

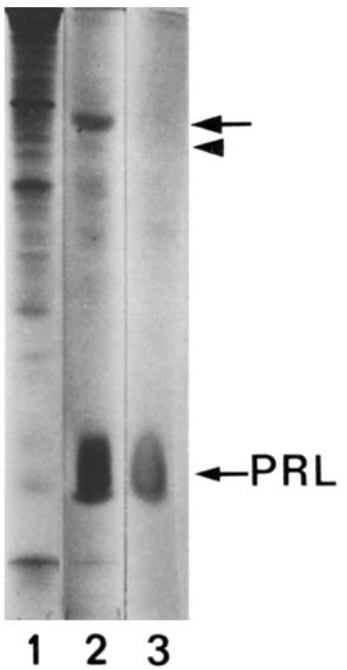

FIGURE 8 Stains all treatment of SDS disc gels of pituitary fractions (~350 μg). *1*, microsomes; *2*, MLG; *3*, Lubrol supernate. The microsome pattern (*1*) includes a number of bands stained either pink or purple. In contrast, MLG (*2*) shows only two major bands. One is pink and corresponds to PRL; the other is blue and corresponds to the ~72,000-dalton band labeled *9a* in Fig. 7 (arrow). A second barely visible band is located just ahead of the first (arrowhead). No such blue bands appear in the gels of Lubrol supernate (*3*).

TABLE II

Distribution of Nondialyzable Radioactivity in Subcellular Fractions Isolated from Bovine Pituitary

Sample	^{35}S radioactivity	^{3}H radioactivity
	cpm/mg protein	
Postnuclear supernate	40,320	3,850
Total microsomes	90,300	10,800
PRL-G	22,980	3,510
MLG	19,120	3,250

Tissue slices were labeled for 30 min with [^{35}S]sulfate or D-[6-^{3}H]glucosamine and then chased in nonradioactive medium for an additional 15 min. Data shown are averages of three (^{35}S) and two (^{3}H) experiments.

TABLE III

Distribution of Glycosaminoglycans in Subcellular Fractions Isolated from Bovine Pituitary

Fraction	Hyaluronic acid	Heparan sulfate	Chondroitin sulfate
	*μmol/100 mg of LFDW**		
Postnuclear supernate	0.016	0.083	0.057
	(10)	(53)	(37)
Total microsomes	0.043	0.097	0.084
	(19)	(43)	(38)
Final supernate	0.018	0.060	0.041
	(15)	(50)	(35)
MLG	0.010	0.045	0.031
	(12)	(52)	(36)

Lipid-free subcellular fractions were digested with pronase and glycosaminoglycans precipitated by cetylpyridium chloride. After separation of sulfated glycosaminoglycans from hyaluronic acid by differential precipitation with the same detergent, individual glycosaminoglycans were quantitated by measuring glucosamine (hyaluronic acid and heparan sulfate) and galactosamine (chondroitin sulfate). Data on MLG are averages of two experiments; for the other fractions only one assay was carried out. Figures in parentheses are the percentages of total glycosaminoglycans.

* μmol of hexosamine/100 mg of lipid-free dry weight.

TABLE IV

Distribution of Glycoproteins in Subcellular Fractions Isolated from Bovine Pituitary

Fraction	N-acetylglucosamine	N-acetylgalactosamine	Sialic acid
	*μmol/100 mg of LFDW**		
Postnuclear supernate	1.275	0.399	0.744
Total microsomes	1.363	0.346	1.390
Final supernate	2.775	0.821	1.238
MLG‡	0.522	0.109	0.314

Data on MLG are averages of two experiments; for the other fractions only one assay was carried out. Lipid-free subcellular fractions were digested with pronase and glycosaminoglycans removed by cetylpyridinium chloride precipitation. Hexosamines were determined by using the amino acid analyzer, neutral sugars by automated ion exchange chromatography of their borate complexes, after hydrolysis of the glycopeptide for 3 h in 2N trifluoroacetic acid at 100°C.

* LFDW: lipid-free dry weight

‡ Fucose, 0.072; Mannose, 0.373; Galactose, 0.449 μmol/100 mg of LFDW.

fraction cannot be largely accounted for by artifactual adsorption. This conclusion was confirmed by the results of the mixing experiments reported in Table VII. A set of pituitary slices was labeled for 180 min with [^{35}S]sulfate, then homogenized and fractionated according to the usual scheme. The recovery of labeled macromolecules is reported in column *A*.

TABLE V

Specific Activity of Glycoprotein Hexosamine and Sialic Acid in Subcellular Fractions Isolated from Bovine Pituitary

Sample	Hexosamine	Sialic acid
	cpm/μmol	
Postnuclear supernate	59,840	61,120
Total microsomes	92,880	46,800
MLG	93,760	96,000

Tissue slices were labeled in vitro with D-[6-^{3}H]glucosamine for 30 min and then chased in nonradioactive medium for an additional 15 min. Sugar assays were as in Table IV.

A small aliquot of the final supernate obtained from these slices, dialyzed extensively to remove the tracer, was then added to the 0.32 M sucrose used to homogenize a set of nonradioactive pituitary tissue. Thus, the radioactivity in the second homogenate was entirely accounted for by exogenously added soluble ^{35}S macromolecules and its distribution in particular fractions could only be caused by adsorption artifacts. The data of column *B* indicate that indeed these artifacts occur to a considerable extent, because only 43% of the exogenous soluble counts were recovered in the final supernate and the remaining 57% was redistributed. If the assumption is made that the behavior of the ^{35}S-labeled soluble macromolecules used as tracer is representative of the behavior of the soluble complex carbohydrate mixture present in pituitary homogenates, an attempt can be made to evaluate the adsorption artifacts for individual fractions. Thus, the size of the soluble adsorbing pool will correspond to the ^{35}S radioactivity recovered in the final supernate multiplied by 2.38 (=100/43). In the experiment illustrated in column *A*, this value corresponds to 18.5% of the total radioactivity present in the homogenate. If one considers that the MLG fraction did adsorb 1% of this soluble pool (column *B*), it can be concluded that in the biosynthetically labeled fraction, only the 0.185% of the total incorporated radioactivity is caused by adsorption and should therefore be subtracted from the radioactivity recovered in the fraction (4.9% of the homogenate) to correct for the artifact.

DISCUSSION

The findings reported in this paper confirm and extend results on the rat pituitary, the system in which the isolation of MLG was originally developed. In these previous studies glycosaminoglycans and glycoproteins were shown to be present in the PRL granule matrix, but their characterization was incomplete and not quantitative (7). Many features previously described for rat PRL granules are also shared by their bovine counterparts, such as their ability to survive removal of their limiting membrane by treatment with Lubrol PX. However, the bovine granules are distinctly less stable than those of the rat. Therefore, although most of the PRL originally present in the total cow granules was recovered in well-preserved MLG, a sizable quantity was solubilized together with the membranes and appeared in the Lubrol supernate. MLG and Lubrol supernate are therefore not pure fractions, but represent granule subfractions, one enriched in content, the other in membrane material. It is therefore not surprising that the SDS PAGE analysis of these two preparations demonstrated a considerable overlapping. However, of the components revealed by the various staining techniques used, some were prominent in the detergent-solubilized material, others in the MLG fraction, suggesting a localization in the PRL granule membrane and matrix,

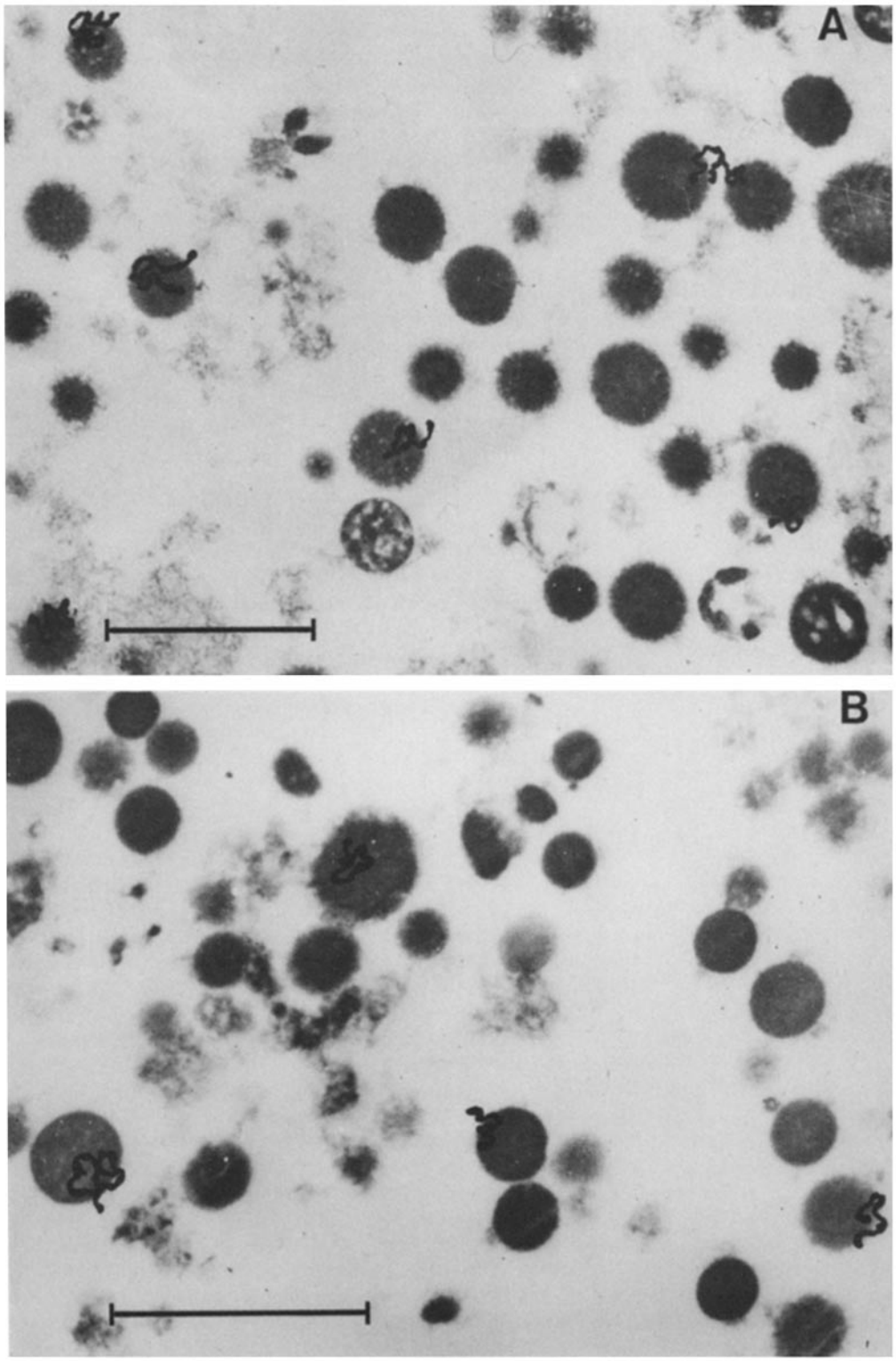

FIGURE 9 Electron microscope radioautography of MLG pellets obtained from pituitary slices labeled in vitro for 30 min with either D-[6-^{3}H]glucosamine (*A*) or [^{35}S]sulfate (*B*), and then incubated for 15 min in nonradioactive medium. With either radioactive precursor the silver grains observed in the autoradiograms appear located on granule cores, including those entirely free of limiting membranes. Exposure: *A*, 30 d; *B*, 15 d. *A*, × 31,000; *B*, × 38,500.

respectively. In particular, the localization in the matrix seems to apply to the blue bands revealed by stains all as well as to at least some sulfated complex carbohydrates. In fact, only 40% of the nondialyzable [^{35}S]radioactivity of total PRL granules was solubilized by the Lubrol treatment together with ~20% of PRL, while the rest remain associated with MLG.

Besides MLG and Lubrol supernate, which originate primarily from mammotroph cells, we also analyzed fractions

which include material from all pituitary cell types, namely the postnuclear supernate and total microsomes. The heterogeneity of these two fractions with respect to both Coomassie Blue staining and Con A binding was expected because they contain membranes of different type and origin (each of which might have a different protein and complex carbohydrate composition) as well as soluble components. In particular, glycosylated secretion products and their precursors are known to be present in various pituitary cell types, and are therefore expected to be recovered in the postnuclear and microsomal fractions. It should, however, be mentioned that the subunits of the pituitary glycoprotein hormones (thyroid-stimulating hormone, follicle-stimulating hormone, and luteinizing hormone), because of their relatively low molecular weight, would all migrate at front of the 7.5% SDS polyacrylamide gels we used and therefore should not appear as discrete bands in the Con A pattern.

The study of complex carbohydrates also revealed striking similarities among pituitary fractions. Thus, all the Con A-positive bands present in MLG are also present in other fractions, including microsomes, which contain little or no PRL granules. Moreover, the biochemical analysis of glycoproteins and glycosaminoglycans revealed that the relative amounts of hyaluronic acid, heparan sulfate, and chondroitin sulfate are quite similar in MLG and microsomes, as are those of *N*-acetylglucosamine, *N*-acetylgalactosamine, and sialic acid. Taken together, these findings raise the question as to whether the complex carbohydrates recovered in the MLG fraction are really components of the PRL granule matrix, or whether they result entirely from artifacts, such as contamination and adsorption of soluble components. However, our control experiments do not support this latter interpretation. Radioautography of MLG pellets isolated from pituitary slices labeled with [^{35}S]sulfate or D-[6-^{3}H]glucosamine ruled out the possibility that the radioactivity found in the fraction was caused by highly labeled contaminants. Additional evidence against cross-contamination comes from a comparison of MLG and microsomes. The latter, being the most highly labeled fraction, are also the most likely source for an artifactual labeling of MLG. In fact, we found that the ^{125}I-Con A patterns of microsomes and MLG overlap only in part and that in MLG isolated from D-[6-^{3}H]glucosamine-labeled slices the specific activity of hexosamines is approximately the same as that in the microsomes, but the specific activity of sialic acid is quite different. Moreover, the results of the mixing experiment indicate that although under our experimental conditions the pituitary organelles do adsorb small amounts of soluble ^{35}S macromolecules, in the case of MLG this adsorption can account for only a minor fraction of the recovered sulfated complex carbohydrates. This conclusion is also supported by the radioautographic ^{35}S labeling of PRL granules in intact mammotroph cells, a finding which was also obtained recently

TABLE VI

Distribution of Autoradiographic Grains in Membraneless PRL Granule Pellets Isolated from D-[6-^{3}H]Glucosamine or [^{35}S]-Sulfate-labeled Bovine Pituitary Slices

	D-[6-^{3}H]Glucosamine	[^{35}S]Sulfate
	grains/100 μm²	
MLG	18.7 ± 5.9*	15.4 ± 4.8*
Extragranular space	6.4 ± 3.2‡	3.7 ± 0.9‡
Background	4.8 ± 1.9	2.9 ± 0.8

Tissue slices were labeled in vitro for 30 min and then chased in nonradioactive medium for an additional 15 min.

* $P < 0.01$ vs. extragranular space.

‡ $P > 0.1$ vs. background.

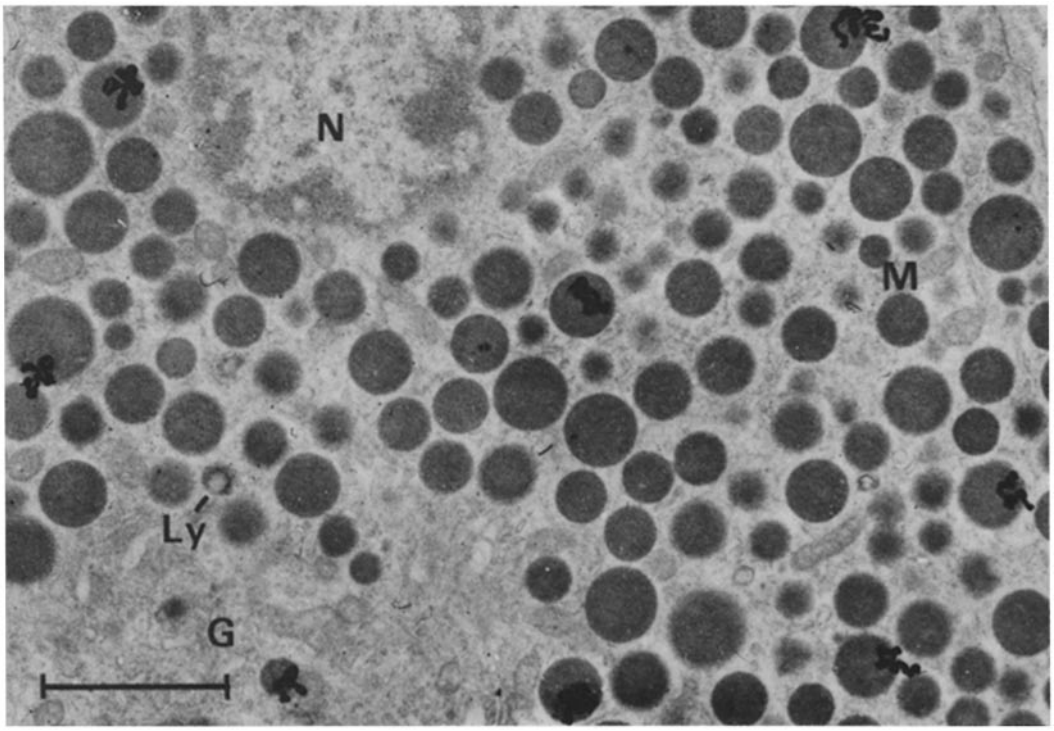

FIGURE 10 Electron microscope radioautography of a bovine mammotroph cell in pituitary slices labeled for 30 min with [^{35}S]-sulfate and then incubated for 15 min in nonradioactive medium. Fixation with 1% OsO_4. Silver grains are seen over PRL granules both in the large Golgi complex (*G*) and in the rest of the cytoplasm. *N*, nucleus; *M*, mitochondrion; *Ly*, lysosome. Exposure: 15 d. × 25,000.

TABLE VII

Distribution of ^{35}S-labeled Macromolecules among Pituitary Cell Fractions Isolated from Slices Labeled for 180 min with [^{35}S]Sulfate (A) or from Nonradioactive Tissue Homogenized in the Presence of ^{35}S-labeled Final Supernate (B)

	^{35}S-labeled macromolecules	
Cell fractions	A Labeled bio-synthetically*	B Redistributed from ^{35}S-labeled final supernate‡
	% of total homogenate	
Postnuclear supernate	75.0	72.0
Total microsomes	10.2	7.9
Final supernate	7.8	43.0
MLG	4.9	1.0

* Pituitary slices (~50 mg protein) were labeled in vitro with [^{35}S]sulfate, then homogenized and fractionated. Total macromolecular radioactivity of the homogenate was 9.2×10^6 cpm.

‡ Nonradioactive pituitary tissue (~50 mg protein) was homogenized in 0.32 M sucrose containing dialyzed ^{35}S-labeled postnuclear supernate (0.1 mg protein, 25,250 cpm, obtained in the experiment illustrated in column *A*) and then fractionated.

by Rosenzweig and Farquhar working on dissociated rat pituitary cells (24).

We can therefore conclude that a mixture of complex carbohydrates is segregated within PRL granules. Two important features of this mixture should be mentioned. The first is its low concentration. From the data of Tables I, III, and IV it can be calculated that, on a molar basis, MLG contain approximately 6- and 50-fold more PRL (mol wt ~22,000) than hexosamines in glycopeptides and glycosaminoglycans, respectively. It seems interesting that also in the other protein-secreting granule type so far analyzed, the zymogen granule of the pancreas, the concentration of sulfated carbohydrates was found to be very low (22). The different situation reported for complex carbohydrates in chromaffin granules of the adrenal medulla might be only apparent because in this system two glycoproteins, chromogranin A and dopamine-β-hydroxylase, are the major macromolecular secretion products (6, 10). The low concentrations of complex carbohydrates found in PRL granules seem incompatible with the idea previously proposed (9) that in these organelles the insolubilization of the matrix is caused by the direct binding of all hormone molecules to the negatively charged residues of glycosylated polyanions.

Another interesting feature of complex carbohydrates of PRL granules is their heterogeneity. Glycoproteins, some of which are sulfated, constitute the major portion of these glycoconjugates. Although our previous studies on rat mammotrophs had already indicated the presence of sulfated glycoproteins in PRL granules (7), both the complexity of the pattern and the extent of glycoprotein sulfation now revealed in the bovine granules were unexpected. Moreover, bovine PRL granules were found to contain small amounts not only of heparan sulfate and chondroitin sulfate, but also of hyaluronic acid. While the presence of the first two glycosaminoglycans had already been reported in rat PRL granules as well as in other secretory granules of different origin, the latter was never observed in organelles of this type.

In the following paper we report kinetic studies carried out on bovine pituitary slices doubly labeled with [^{35}S]sulfate and [^{3}H]leucine (11). The results obtained support the conclusion that glycosaminoglycans and glycoproteins are components of PRL granules themselves rather than of contaminants of the MLG fraction, and give some further insight on the possible functional significance of these macromolecules within secretory granules.

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REFERENCES

1. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8:115–118.
2. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium labelled proteins and nucleic acid in polyacrylamide gels. *Eur. J. Biochem.* 46:83–88.
3. Caro, L. G. 1962. High resolution autoradiography. II. The problem of resolution. *J. Cell Biol.* 15:189–199.
4. Dacheux, F., and M. P. Dubois. 1976. Ultrastructural localization of PRL, GH and LH by immunocytochemical techniques in the bovine pituitary. *Cell Tissue Res.* 174:245–260.
5. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* 226:497–509.
6. Geissler, D., A. Martinek, R. U. Margolis, R. K. Margolis, J. A. Skrivanek, R. Ledeen, P. König, and H. Winkler. 1977. Composition and biogenesis of complex carbohydrates of ox adrenal chromaffin granules. *Neurosci.* 2:685–693.
7. Giannattasio, G., and A. Zanini. 1976. Presence of sulfated proteoglycans in prolactin secretory granules isolated from the rat pituitary gland. *Biochim. Biophys. Acta.* 439:349–357.
8. Giannattasio, G., A. Zanini, and J. Meldolesi. 1975. Molecular organization of rat prolactin granules. I. In vitro stability of intact and "membraneless" granules. *J. Cell Biol.* 64:246–251.
9. Giannattasio, G., A. Zanini, and J. Meldolesi. 1975. Meccanismi di trasporto endocellulare, di "storage" e di "release" dell'ormone somatototropo e della prolattina nell'ipofisi di ratto. *In* Atti delle Giornate Endocrinologiche Senesi. E. E. Müller and S. E. Piazzi, editors. I. S. V. T. Sclavo, Siena, Italy. 29–43.
10. Giannattasio, G., A. Zanini, and J. Meldolesi. 1979. Complex carbohydrates of secretory organelles. *In* Complex Carbohydrates of the Nervous Tissue. R. U. Margolis and R. K. Margolis, editors. Plenum Press, New York. 327–345.
11. Giannattasio, G., A. Zanini, P. Rosa, J. Meldolesi, R. K. Margolis, and R. U. Margolis. 1980. Molecular organization of prolactin granules. III. Intracellar transport of sulfated glycosaminoglycans and glycoproteins of the bovine prolactin granule matrix. *J. Cell Biol.* 86:273–279.
12. King, L. E., and M. Morrison. 1976. The visualization of human erythrocyte membrane proteins and glycoproteins in SDS polyacrylamide gel electrophoresis employing a single staining procedure. *Anal. Biochem.* 71:223–230.
13. Kronquist, K. E., A. Elmahdy, and R. A. Ronzio. 1977. Synthesis and subcellular distribution of heparan sulfate in the rat exocrine pancreas. *Arch. Biochem. Biophys.* 182: 188–196.
14. Lee, Y. C., J. F. McKelvy, and D. Lang. 1969. Rapid automatic analysis of sugar components of glycoproteins. II. Neutral sugars. *Anal. Biochem.* 27:567–574.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265–275.
16. Maizel, V. J. 1971. Polyacrylamide gel electrophoresis of viral proteins. *Methods Virol.* 5: 179–246.
17. Margolis, R. K., and R. U. Margolis. 1970. Sulfated glycopeptides from rat brain glycoproteins. *Biochemistry.* 9:4389–4396.
18. Margolis, R. K., R. U. Margolis, C. Preti, and D. Lai. 1975. Distribution and metabolism of glycoproteins and glycosaminoglycans in subcellular fractions of brain. *Biochemistry.* 14:4797–4804.
19. Meldolesi, J., N. Borgese, P. De Camilli, and B. Ceccarelli. 1978. Cytoplasmic membranes and the secretory process. *In* Membrane Fusion. G. Poste and G. N. Nicolson, editors. Elsevier North-Holland, Inc., New York. 509–627.
20. Meldolesi, J., D. Marini, and M. L. De Monte-Marini. 1972. Studies on in vitro synthesis and secretion of growth hormone and prolactin. I. Hormone pulse-labeling with radioactive leucine. *Endocrinology.* 91:802–808.
21. Palade, G. E. 1975. Intracellular aspects of the process of protein secretion. *Science (Wash. D. C.).* 189:347–358.
22. Reggio, H., and J. C. Dagorn. 1978. Ionic interactions between bovine chymotrypsinogen A and chondroitin sulfate ABC. *J. Cell Biol.* 78:951–957.
23. Reggio, H. A., and G. E. Palade. 1978. Sulfated compounds in the zymogen granules of the guinea pig pancreas. *J. Cell Biol.* 77:288–314.
24. Rosenzweig, L. J., and M. G. Farquhar. 1980. Sites of sulfate incorporation into mammotrophs and somatotrophs of the rat pituitary as determined by quantitative electron microscopic autoradiography. *Endocrinology.* In press.
25. Rostas, J. A. P., P. T. Kelly, and C. W. Cotman. 1977. The identification of membrane glycoproteins in polyacrylamide gels. A rapid method using [^{125}I]labeled lectins. *Anal. Biochem.* 80:366–372.
26. Schmidt, G., and S. J. Tannhauser. 1945. A method for the determination of deoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. *J. Biol. Chem.* 161: 83–89.
27. Slaby, F., and M. G. Farquhar. 1980. Characterization of rat somatotroph and mammotroph secretory granules: presence of sulfated molecules. *Mol. Cell Endocrinol.* In press.
28. Smith, R. E., and M. G. Farquhar. 1966. Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. *J. Cell Biol.* 31:319–347.
29. Sottocasa, G. L., B. Kuylenstierna, L. Ernster, and A. Bergstrand. 1967. An electron transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. *J. Cell Biol.* 32:415–438.

30. Takesue, S., and T. Omura. 1970. Solubilization of NADH-cytochrome b_5 reductase from liver microsomes by lysosomal digestion. *J. Biochem. (Tokyo).* 67:259–268.
31. Weibel, E. R. 1969. Principles for morphometry in electron microscopic cytology. *Int. Rev. Cytol.* 26:235–302.
32. Whur, P., A. Herscovics, and C. P. Leblond. 1969. Radioautographic visualization of the incorporation of galactose-^{3}H and mannose-^{3}H by rat thyroids in vitro in relation to the stages of thyroglobulin synthesis. *J. Cell Biol.* 43:289–311.
33. Widnell, C. C., and J. C. Unkeless. 1968. Partial purification of a lipoprotein with 5′-nucleotidase activity from membranes of rat liver cells. *Proc. Natl. Acad. Sci. U. S. A.* 61: 1050–1058.
34. Zanetta, J. P., I. G. Morgan, and G. Gombos. 1975. Synaptosomal plasma membrane glycoproteins: fractionation by affinity chromatography on concanavalin A. *Brain Res.* 83: 337–348.
35. Zanini, A., and G. Giannattasio. 1973. Isolation of prolactin granules from rat anterior pituitary glands. *Endocrinology.* 92:349–357.
36. Zanini, A., G. Giannattasio, and J. Meldolesi. 1974. Separation of rat pituitary growth hormone and prolactin by SDS polyacrylamide gel electrophoresis. *Endocrinology.* 94: 594–598.