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The Role of Subcompartments of the Golgi Complex in Protein Intracellular Transport [and Discussion]

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The role of subcompartments of the Golgi complex in protein intracellular transport

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[Plates 1 and 2]

The functioning of the Golgi complex in protein intracellular transport is most simply understood in terms of its being composed of a sequence of functionally distinct subcompartments. For example, the influence of perturbation of cellular Na^{+}–K^{+} balance on the transport of secretory and membrane glycoproteins is to greatly slow their passage from relatively proximal to relatively distal subcompartments. To further the understanding of the nature of these subcompartments a rat IgM myeloma has been subjected to analytical subcellular fractionation. Fractions selectively enriched in distinct Golgi-associated activities have been prepared and their membrane proteins compared with those of rough microsomal fractions. The subfractionation is extensive and suggests the possibility of obtaining well resolved Golgi subfractions. Myeloma cells stained intracellularly with Concanavalin A– and wheatgerm agglutinin–peroxidase conjugates show distinct labelling patterns. Concanavalin A stains the entirety of the rough endoplasmic reticulum as well as the proximal face of the Golgi stack. Wheatgerm agglutinin stains the distal face of the stack of Golgi cisternae. The staining patterns are not due to immunoglobulin as they are also observed in myeloma variants that fail to synthesize immunoglobulin.

Introduction

(a) Background

The transport of secretory proteins from the rough endoplasmic reticulum (r.e.r.) to the cell surface is mediated by smooth-surfaced vesicles and the elongated stacked cisterna of the Golgi complex. These several membranes interact successively in such a way as to accomplish rapid and efficient transport, allowing a given protein to reach the surface roughly 7–20 min after biosynthesis. The mechanisms operating along this pathway are largely obscure and in fact we even lack systematic compositional information about these distinct or partly distinct classes of membranes (Tartakoff 1980, 1982).

The importance of analysing these Golgi-associated transport operations is heightened by the realization that transport is highly selective: in addition to the secretory proteins, lysosomal proteins and numerous membrane proteins also pass by way of the Golgi complex. Many of these proteins deviate from the secretory path and avoid delivery to the cell surface.

It has been clear both to anatomists and histochemists that there is considerable heterogeneity among the several cisternae of the Golgi complex. For example, more proximal cisternae (oriented toward the transitional elements of the r.e.r.) can be selectively stained by over-osmication, while relatively distal cisternae are histochemically positive for uridine disphosphatase. It is my conviction that to understand the mechanism of transport we must learn the composition of the membranes of the Golgi complex and its subcompartments, we must learn to separate the subcompartments and ultimately to put them back together again.

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(b) *Choice of tissue*

It is customary to isolate subcellular fractions from the rat liver, yet especially for the particular job at hand the liver is not altogether suitable. The cell population of the liver is mixed, its Golgi complex is not highly organized, and large quantities of a potential low-density contaminant – the smooth endoplasmic reticulum – are present. Moreover, the hepatocyte has the unfortunate habit of producing a wide range of secretory proteins. The presence of such abundant secretory protein content within subcellular fractions poses an overwhelming problem: although procedures are known for eliminating such content, even the staunchest advocates of such procedures must admit that the extractions are incomplete (Howell & Palade 1982; Kreibich & Sabatini 1974). Thus in an isolated fraction it is impossible to discriminate rigorously between those proteins that are part of the cell's transport machinery and those that are being transported. Moreover, the secretory lipoproteins diminish the inherent density of the elements they traverse. In fact, this latter property of the liver has been exploited so as to recover highly purified Golgi subfractions of very low buoyant density (Morré 1971; Ehrenreich *et al.* 1973), which have been of major importance, especially for the study of the terminal sugar transferases. Nevertheless it is my feeling that we have to get away from such a preparative approach to the subcellular fractionation of the Golgi complex and that an analytical, all-inclusive approach would ultimately prove to be more powerful (de Duve 1971).

For these several reasons I have undertaken to study the Golgi complex and Golgi-enriched subcellular fractions of a myeloma. Homogeneous cell populations can be obtained in gram quantities and are ideally suited for rapid *in vitro* manipulations, pulse-chase labelling, viral infection, etc. Only a single secretory protein is present, thereby greatly reducing the danger of the secretory content's obscuring the analysis of the membrane proteins of subcellular fractions. Furthermore, the cells are so-called 'non-regulated' secretory cells (i.e. they secrete continuously without storing their secretory product), and are therefore relatively simple from the cytological point of view. In addition, since the biosynthesis of the carbohydrate of immunoglobulin (Ig) has been extensively studied, one can use selected stages of carbohydrate maturation as potential markers of compartments involved in intracellular transport. We work with an IgM-secreting rat myeloma (IR202, obtained from H. Bazin) because IgM is rather rich in carbohydrate and because by working with a rat tissue we expect to be able to take advantage of much of what is known of the enzymology and antigenicity of other rat tissues, including the liver. Most of the experiments I shall describe in this paper make use of myeloma cells grown as an ascitic tumour. In this form they devote about 12 % of their protein biosynthesis (10 min, [^{35}S]methionine pulse label) to secretory IgM production. We have no evidence for the presence of surface Ig on these cells. The cells do not contain visible viral particles.

(c) *Specific goals*

I wish to exemplify several distinct experimental approaches to the analysis of the structure and function of the Golgi complex of this myeloma. The first approach is pharmacological and makes use of the carboxylic ionophore monensin to perturb the Golgi complex. The second involves performing analytical subcellular fractionation in such a way as to spread Golgi elements in density gradients, and then examining the polypeptide composition of such subcellular fractions. The Golgi-enriched fractions are compared with rough microsomal fractions and with comparable fractions isolated from the rat liver. I shall then turn to the use of ultra-

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structural cytochemistry to localize lectin-binding sites, which are characteristic of the r.e.r. or Golgi subcompartments. Throughout, the emphasis will be on the divisibility of the Golgi complex into subcompartments and their possible role(s) in intracellular transport.

Pharmacological approach

The idea of attempting to perturb Golgi structure and function by manipulating cellular Na^+ and K^+ levels grew from some incomplete and scattered observations in the ultrastructural literature and from the realization that many cells must be able to tolerate alterations in Na^+–K^+ balance. The most dramatic agent employed is the carboxylic ionophore monensin, which appears to be universally effective on eukaryotic cells. I shall summarize its principal effects, which are published (Tartakoff 1983), and illustrate a few in the system at hand.

In the presence of 0.1–1.0 μM monensin, cells rapidly lose K^+ and gain a roughly stoichiometric amount of Na^+. Intracellular pH changes are brief and modest, there is no effect on ATP levels or Ca^{2+} efflux, and protein synthesis continues at a normal or near-normal rate. What is especially striking is that within no more than a few minutes the intracellular transport of both secretory and plasma membrane proteins ceases. Such an effect on IgM secretion can easily be observed when a 5 min [^{35}S]methionine pulse is followed by a 2 h chase incubation in control or monensin-containing medium. The presence of monensin reduces the amount of extracellular immunoprecipitable [^{35}S]Ig by *ca.* 90 %. Both autoradiographic and subcellular fractionation experiments show that in the presence of monensin newly synthesized proteins continue to exit from the r.e.r. at a normal rate and overaccumulate within the Golgi complex.

Available information indicates that each Ig heavy chain bears five or six asparagine-linked carbohydrate units and that the oligosaccharides of secreted Ig are fully resistant to endoglucosaminidase H. This resistance is normally acquired during passage through the Golgi complex due to the concerted action of glycosidases and terminal sugar transferases. It is worth pausing to recognize the high efficiency of this oligosaccharide maturation process, because an individual pentameric IgM molecule in the r.e.r. bears a total of 50–60 immature (endoglucosaminidase H-sensitive) oligosaccharide units.

We have enquired whether the IgM that is caused to accumulate in the presence of monensin has completed the normal maturation of its carbohydrate. The protocol that we have employed is a [^{35}S]methionine pulse–chase experiment, where the chase is either in control medium or in medium supplemented with monensin, or with the phenylhydrazone uncoupler of oxidative prosphorylation, CCCP, which arrests exit of Ig from the r.e.r. For comparison, samples of cells labelled in the presence of tunicamycin are also analysed. Each sample of immunoprecipated Ig is reduced and alkylated, divided in two, and incubated without or with endoglucosaminidase H. Figure 1 illustrates the sodium dodecyl sulphate polyacrylamide gel electrophoretic mobility of the labelled heavy chains, as revealed by autoradiography. In the pulse-labelled sample the mobility is slightly in advance of that of secreted heavy chains. All the chase intracellular samples are of similar mobility to the pulse-labelled sample (except after tunicamycin treatment, used to indicate the mobility of carbohydrate-free heavy chains). Upon endoglocosaminidase treatment, *all* the intracellular samples can be seen to be sensitive to their enzyme, as indicated by their mobility becoming almost as rapid as that of carbohydrate-free chains. The extracellular sample is unaffected. Since the control chase sample is fully sensitive, the intracellular pool of Ig with mature carbohydrate must be quite small.

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These observations, and others, show that secretory Ig carbohydrate maturation is greatly retarded in the presence of the ionophore. We have obtained analogous data bearing on the transport and oligosaccharide maturation of two plasma-membrane proteins, surface Ig and histocompatibility antigens. Because a number of control experiments show that the terminal sugar transferases are still active, we consider this effect to be ascribable to topographic, not

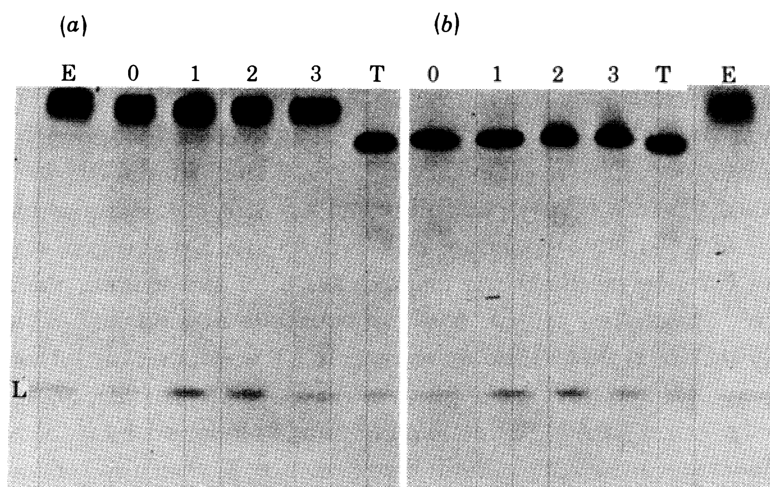

Figure 1. Evaluation of secretory Ig heavy chain carbohydrate maturation by use of endoglucosaminidase H. Ascitic IR 202 myeloma cells were pulse-labelled for 5 min with [^{35}S]methionine, washed, and either chilled (0) or returned to non-radioactive medium for 2 h at 37 °C. Among the reincubated samples, one was incubated without the addition of any perturbant of intracellular transport (1), one was incubated in the presence of 1 μM monensin (2), and one was incubated in the presence of 10 μM CCCP (3). An additional sample of cells (T) was preincubated for 1 h in tunicamycin (1 μg ml^{-1}), pulse-labelled, washed, and chilled at once. In each case, intracellular [^{35}S]Ig was recovered by immunoprecipitation and in condition (1) an extracellular sample (E) was recovered as well. Each sample was divided into halves, reduced and alkylated and incubated overnight at pH 5.5, 37 °C, without (*a*) or with (*b*) endoglucosaminidase H (30 μM ml^{-1}). Samples were then analysed by standard sodium sulphate polyacrylamide gel electrophoresis – autoradiography procedures. Because the enzyme removes high-mannose carbohydrate units, but not mature complex units, it allows a rough discrimination between polypeptides bearing the two classes of carbohydrate unit. It is clear that all the intracellular samples are sensitive to the enzyme, which implies that they bear immature carbohydrate units. By contrast, the extracellular sample is resistant. After enzyme treatment the intracellular samples still have a slightly slower mobility than the carbohydrate-free sample (T), presumably because the enzyme leaves the most internal glucosamine residue on the polypeptide chain and the heavy chains of IgM bear a total of five or six carbohydrate units. L, Ig light chains.

enzymological, considerations. Thus we are forced to think in terms of the Golgi's being composed of at least a pair of subcompartments: a proximal one that newly synthesized proteins can continue to enter in the presence of monensin, and a more distal one that is relatively inaccessible in the presence of monensin and in which maturation to endoglucosaminidase H resistance normally occurs. Because a number of authors have employed monensin to study the intracellular transport of other macromolecules it is possible to ascribe additional post-translational modifications to these two Golgi subcompartments, defined by reference to monensin's action. Thus a 'confined function model' of the Golgi complex can be considered in which the proximal compartment is the site of phosphorylation of lysosomal enzymes and lipid addition to viral envelope glycoproteins, whereas the distal compartment is responsible for the completion of the oligosaccharide maturation of asparagine-linked oligosaccharides and the sulphation of proteoglycans (Tartakoff 1982, 1983).

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In addition to interrupting intracellular transport, monensin has another striking effect: it causes a massive dilation of Golgi elements, which nevertheless remain closely adherent one to the next. It is not clear what causal relation, if any, exists between this dilation and the interruption of transport; however, the mere ultrastructural observation in its own right implies that the membranes of Golgi cisternae have a unique property (probably related to ion permeability) that distinguishes them from the membranes of the endoplasmic reticulum. This unique property deserves to be identified.

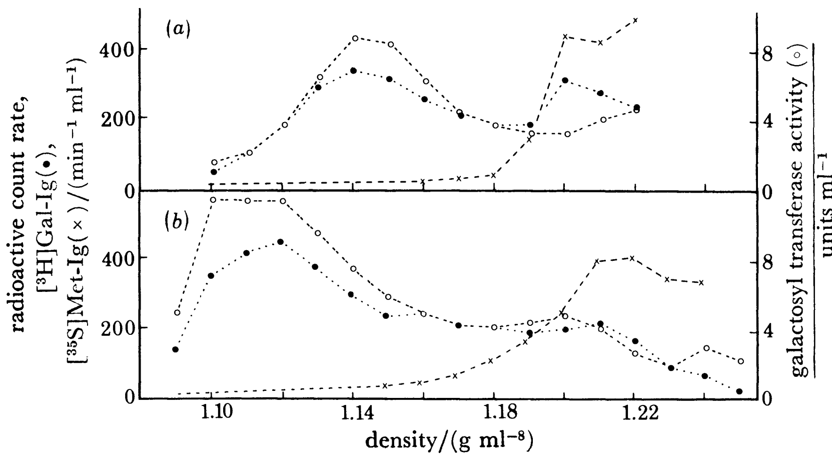

FIGURE 2. Comparative subcellular fractionation of myeloma cell postnuclear supernatants on (*a*) mannose-containing and (*b*) sucrose-containing gradients. Cells were labelled for 1 min with [^{35}S]methionine or for 5 min with [^{3}H]galactose and then mixed, homogenized, and briefly centrifuged to remove debris. The resulting supernatant was fractionated in parallel on two gradients, centrifuged overnight to equilibrium. Radioactive Ig was immunoprecipitated from each fraction and galactosyl transferase activity was measured as in Howell *et al.* (1978). In both gradients the [^{35}S]Ig is entirely recovered at densities greater than 1.18 g ml^{-1}. Coincident peaks of [^{3}H]Ig and galactosyl transferase are observed in lower-density regions. It is not known why a proportion of these activities is also recovered in denser fractions; it is possibly because the Golgi elements within which they reside bear extensive, clathrin-containing coats (coated vesicles normally sediment to *ca.* 55% sucrose). The higher equilibrium density of the Golgi markers in mannose gradients is suggested to be a reflection of the ability of mannose (unlike sucrose) to penetrate the membranes.

Subcellular fractionation

In light of the well established structural and histochemical heterogeneity of the stack of Golgi cisternae, and with the monensin data in mind, we have sought to isolate Golgi-enriched subcellular fractions and if possible subfractions. Our first concern has been to ensure that bound ribosomes remain attached to the r.e.r. and to this end we have taken two precautions: (1) just before chilling and homogenization, cells are treated with cycloheximide, which is also included in the homogenate, and (2) the fractionation is performed in the presence of Mg^{2+}. After the elimination of nuclei and debris, as a basic assessment of the adequacy of the conditions of isopycnic sedimentation we have sought to localize three parameters: (1) Ig labelled with a 1 min pulse of [^{35}S]methionine (to mark the rough microsomes), (2) Ig labelled with a 5 min pulse of [^{3}H]galactose (which should mark Golgi elements), and (3) galactosyl transferase activity measured with ovomucoid as acceptor (again a Golgi marker). We have performed such fractionation on sucrose gradients, yet the procedure of choice for the time being involves

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fractionation on mannose gradients. There are several motivating considerations: (1) in sucrose gradients the peaks of galactosyl transferase are routinely quite broad, (2) the published electron micrographs of Golgi fractions isolated in the presence of sucrose suggest that they are osmotically compressed (Ehrenreich *et al.* 1973), (3) if one performs subcellular fractionation in the presence of non-penetrating solutes, one must expect that both the size and the intrinsic density of the particles will influence their isopycnic sedimentation (de Duve 1965), and (4) it is known

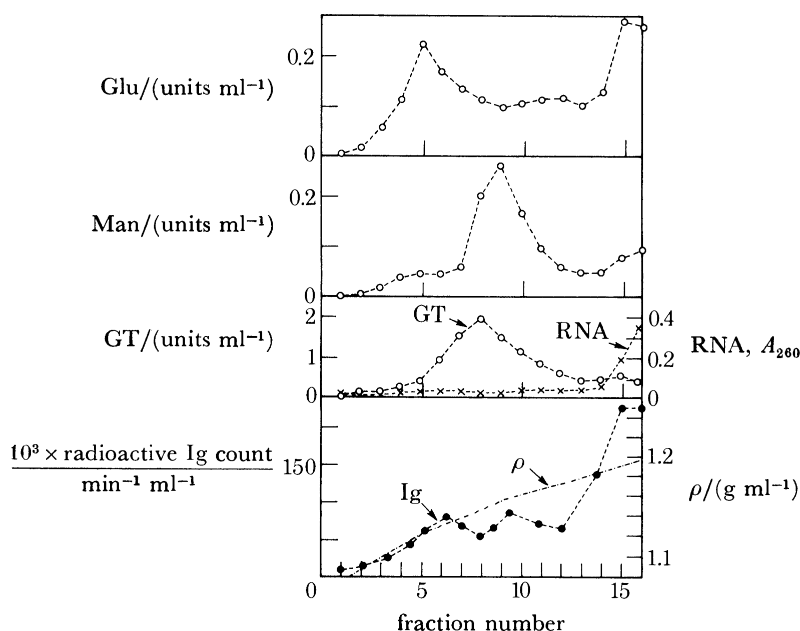

FIGURE 3. Fractionation of total microsomal fractions on mannose gradients. Non-radioactive . itic myeloma cells or cells labelled for 60 min with [^{35}S]methionine were homogenized and centrifuged to eliminate nuclei, mitochondria (detected by succinate dehydrogenase activity), and lysosomes (detected by endocytosed peroxidase activity). The resulting 'post-mitochondrial supernatants' were sedimented to collect total microsomal fractions, which were fractionated by overnight centrifugation on parallel continuous mannose gradients. The gradients were analysed for immunoprecipitable [^{35}S]Ig, RNA, galactosyl transferase activity (GT), mannosidase activity (Man) and glucosidase activity (Glu). Unlike pulse-labelled [^{35}S]Ig (figure 2), the 60 min labelled [^{35}S]Ig has gained access to compartments of relatively low density and accumulates sufficiently to give a pair of peaks centred near 1.13 and 1.16 g ml^{-1}. Three distinct Golgi-associated enzyme activities are also recovered in the upper part of the gradient. The non-coincidence of peaks of GT activity and [^{35}S]Ig indicates that a partial resolution of Golgi elements has in fact been achieved.

that mannosidase activity is present within Golgi membranes (Tabas & Kornfeld 1978, 1979 Tulsiani *et al.* 1977), and thus the mannosidase-bearing or more distal Golgi elements must be permeable to mannose; if not, they would swell. Figure 2 illustrates comparative isopycnic sedimentation on mannose and sucrose gradients. It is clear that in both cases the rough microsomes can be effectively eliminated, that the resolution of Golgi-associated activities is superior in the mannose gradient, and that their density is greater than in the sucrose gradient, presumably owing to the permeation of mannose. As anticipated, the peak of transferase activity is coincident with Ig pulse-labelled with [^{3}H]galactose. There is no indication of rupture and leakage of labelled Ig. Moreover, electron microscopic examination of the fractions centered at 1.13 or 1.16 g ml^{-1} shows that the fractions are largely composed of closed smooth-surfaced vesicles. There is no indication that they have undergone osmotic compression.

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We have made use of such mannose gradients to analyse total microsomal fractions from cells labelled for 60 min with [^{35}S]methionine or [^{3}H]2-D-mannose. When labelled Ig is recovered by immunoprecipitation, three peaks are seen, centred at densities of *ca.* 1.13, *ca.* 1.16, and greater than 1.19 g ml^{-1} (figure 3). Essentially all of the labelled Ig recovered from these gradient regions is sensitive to endoglucosaminidase H. Hence they correspond to relatively

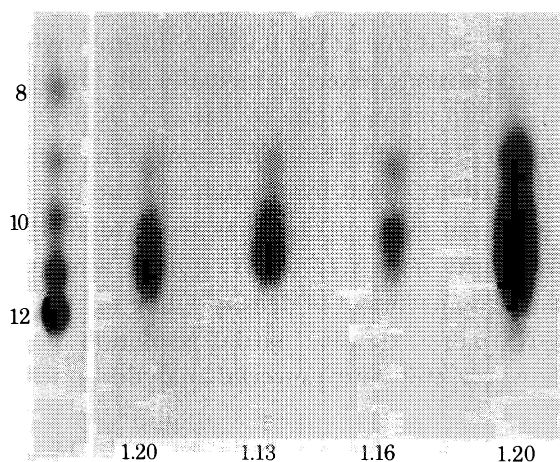

FIGURE 4. Analysis of [^{3}H]mannose-labelled Ig oligosaccharides recovered from mannose gradients. Myeloma cells were labelled for 60 min with [^{3}H]2-D-mannose in the presence of glucose (0.1 mg ml^{-1}) and fractionated to provide three peaks of labelled Ig cosedimenting with the peaks of [^{35}S]Ig illustrated in the bottom panel of figure 3. Each sample of [^{3}H]Ig recovered by immunoprecipitation was reduced, alkylated, treated with endoglucosaminidase H, desalted and fractionated by thin-layer chromatography on silica gel followed by fluorography. At the left is a family of [^{3}H]mannose-labelled oligosaccharides released from dolichol by weak acid hydrolysis and endoglucosaminidase H. The sizes of the species were assessed by comparison with dolichol and viral glycoprotein-derived standards obtained from Dr M. Snider (Massachusetts Institute of Technology) and Dr R. Datema (Giessen). A labelled [^{3}H]oligosaccharide sample recovered from the bottom of the gradient (1.20 g ml^{-1}) is run twice for comparison with material recovered from fractions centred at 1.13 and 1.16 g ml^{-1}. These rough microsomal oligosaccharides range in size from 11 to 9 sugar residues and are therefore considered to include $GlcNAc_1Man_9Glc_1$, $GlcNAc_1Man_9$ and $GlcNAc_1Man_8$. As suggested by Godelaine *et al.* (1982), species such as $Glc_1Man_8GlcNAc_1$ may also be present. The principal oligosaccharide obtained from [^{3}H]Ig of density 1.13 and 1.16 g ml^{-1} is somewhat smaller than the most abundant rough microsomal species and is therefore thought to lack glucose residues. In addition, a faint spot in the nonasaccharide region continues to be present.

proximal stations in Ig intracellular transport. We have enquired to what extent these three samples of labelled Ig can be ordered along the path of intracellular transport. To analyse the size of incomplete Ig oligosaccharides recovered from each gradient region, we have reduced and alkylated [^{3}H]mannose-labelled immunoprecipitates, treated with endoglucosaminidase H, and fractionated the released oligosaccharides by thin-layer chromatography (Holmes & O'Brien 1979). Figure 4 shows a fluorogram of such a chromatogram. At the level of rough microsomes, the sensitive oligosaccharides range in size from 11 to 9 sugar residues, which we provisionally consider to be $GlcNAc_1Man_9Glc_1$, $GlcNAc_1Man_9$, and $GlcNAc_1Man_8$. The last of these species has also been detected in thyroglobulin recovered from thyroid rough microsomes (Godelaine *et al.* 1982), thus showing that one mannose residue (as well as the glucose residues) can be removed before entry into the Golgi complex. In the two lighter density gradient regions, the largest of these species is less evident, suggesting that glucose removal has been completed. Nevertheless, the principal conclusion to be drawn is that extensively trimmed

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species (e.g. $GlcNAc_1Man_5$ or $GlcNAc_1Man_3$) must be of such transient existence that they cannot serve as markers of subcompartments, given the currently available fractionation procedures. This point might be re-explored making use of a myeloma variant that lacks selected terminal sugar transferases.

Figure 3 also shows the distribution of RNA, which is highly concentrated at the bottom of the gradient, and two other Golgi-associated enzyme activities implicated in the processing of oligosaccharides of glycoproteins: glucosidase (which is also recovered in the rough microsomal fraction) and mannosidase, both measured at pH 6 with *p*-nitrophenyl-α-D-glycoside substrates. We expect that these assay conditions detect principally the glucosidase AB of Grinna & Robbins (1979) and the α-1–3, 1–6 mannosidase of Tabas & Kornfeld (1979). Both activities have previously been detected in preparative Golgi fractions of rat liver, and the glucosidase has been detected at high specific activity in rat liver rough microsomes.

We conclude that upon exit from the r.e.r., Ig gains access to lower density glycosidase-rich gradient regions ranging in density from 1.12 to 1.17 g ml^{-1}, acquires galactose and possibly other terminal sugars within a compartment of density 1.15 g ml^{-1}, and quickly exits from the cell. We have accomplished a more extensive partial resolution of Golgi elements than has previously been reported; however, still more powerful analytical fractionation tools are clearly needed.

The subcellular fractionation scheme has also been evaluated by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis in the hope of seeing whether, despite the persistent overlap of Golgi-associated activities, certain generalizations might be made with respect to the polypeptide composition of these membranes. In order to simplify these gel patterns, somewhat, we have also treated individual gradient fractions with pH 11.2, 0.1 M Na_2CO_3 (Howell & Palade 1982) to extract a maximum of extrinsic protein, ribosomes, and content before loading the gels. Silver nitrate-stained gels are shown as figure 5. What is clear, concentrating on the pH 11 residue, is that the rough microsomes (best represented by the last two lanes on the right) are distinctly different from the continuum of fractions that range through the 1.16 and 1.13 g ml^{-1} density regions toward the top of the gradient. Moreover, there are certain bands that span most of the fractions that we have reason to identify as including Golgi elements (e.g. the band with molecular mass greater than 200 kDa indicated by an asterisk), and within this broad 'Golgi continuum' a number of subregions can be identified by their characteristic polypeptides. Although the resolution of such one-dimensional gels is limited, certain bands appear to be common to both the Golgi-enriched regions and the rough microsomal fraction.

Are these Golgi-enriched gradient regions representative of the Golgi complex of another cell? Which are the polypeptides essential to the basic transport operations of the Golgi complex, as opposed to the particular operations of a single cell type? To attempt to answer these questions, three myeloma gradient fractions (density 1.13, 1.16 and over 1.19 g ml^{-1}) have been extracted at pH 11 and compared with liver fractions extracted in parallel. The liver rough microsomal fraction was isolated by a procedure designed to maximize yield without compromising purity (Adelman *et al.* 1973); however, the Golgi-enriched fractions are all preparative, selected according to their very low density (due especially to their content) and the high specific activity of terminal sugar transferases (Ehrenreich *et al.* 1973; Leelevathi as cited by Tabas & Kornfeld 1979). As indicated by the asterisks in figure 6, a number of bands are shared between tissues. These obviously are the best candidates for further investigation and

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will be of particular interest with respect to raising antisera against the underlying components of the organelle.

Lectin-binding proteins

I wish now to shift emphasis and instead of looking at the totality of the proteins associated with the Golgi complex I shall focus on two largely distinct classes of proteins, those that bind Concanavalin A (Con A) (and therefore contain α-linked mannose-rich oligosaccharides unsubstituted at C-3, C-4 and C-6 (Kornfeld & Ferris 1975)) and those that bind wheatgerm

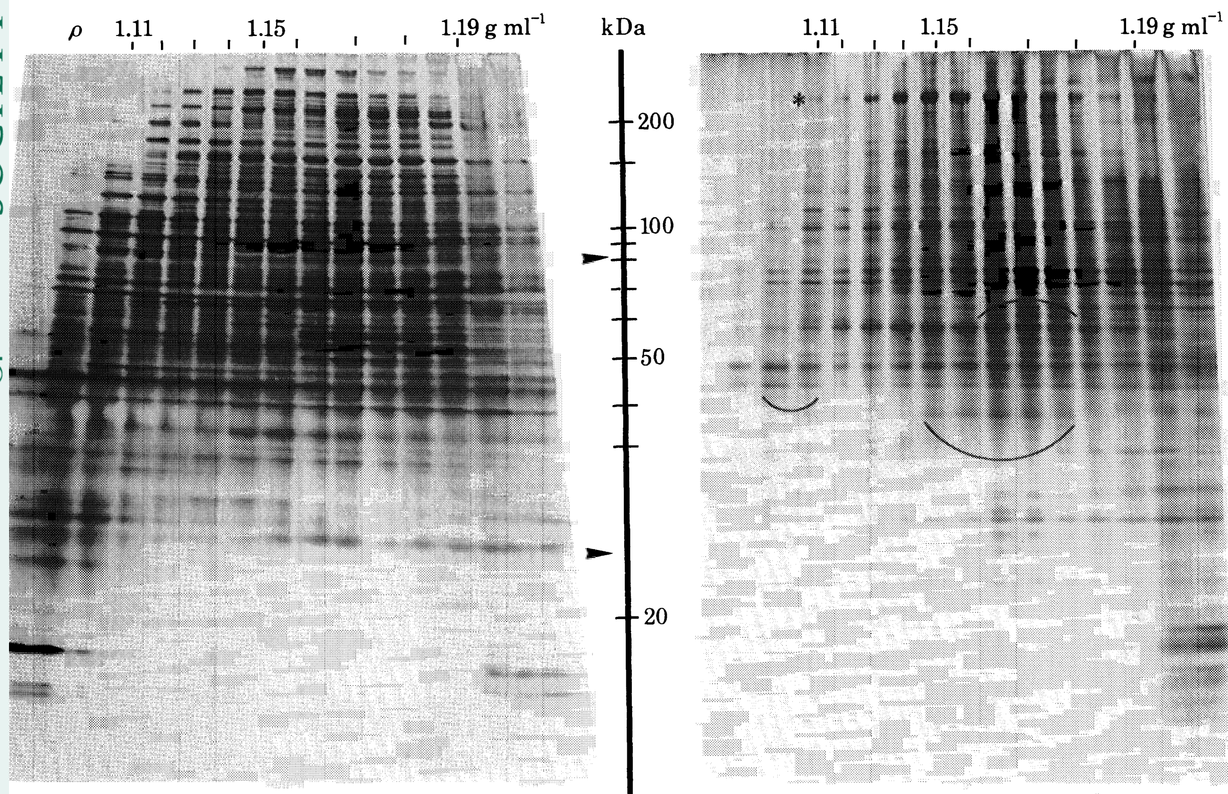

Figure 5. SDS polyacrylamide gel electrophoretic analysis of total microsomal fractions after resolution on continuous mannose gradients. Gradients identical to those illustrated in figure 3 were sampled at regular intervals and the successive fractions were analysed by gel electrophoresis followed by staining with silver nitrate. In the panel at the right the samples were extracted at pH 11.2 according to Howell & Palade (1982) before analysis. The mobilities of Ig heavy and light chains are indicated by the arrowheads along the molecular mass axis. These Ig chains are not conspicuous on a mass basis, although after biosynthetic labelling they predominate. In the right-hand panel the asterisk indicates a major protein common to all gradient regions including Golgi elements. The arcs indicate regions of the gradients with characteristic polypeptide composition, which are therefore thought to correspond to distinct membrane populations.

agglutinin (WGA) (and therefore bear terminal sialic acid or *N*-acetylglucosamine (GlcNAc) residues (Bhavanandan & Katlic 1979)). It has recently been reported that such lectins conjugated to fluorochromes give distinct staining patterns of the cytoplasm of fibroblasts and that WGA is selective for the Golgi complex, as judged both from the localization of fluorescence and the influence of monensin on the staining pattern (Virtanen *et al.* 1980).

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We have repeated these observations on the rat myeloma and extended them to the ultrastructural level, making use of the saponin-permeabilization procedure originated by Ohtsuki *et al.* (1978). When cytocentrifuged, air-dried, fixed cells are stained with fluorescein or rhodamine-conjugated lectins, one can readily show that essentially all of the cell cytoplasm is Con A-positive whereas the staining with WGA is largely restricted to a single perinuclear zone coincident with histochemically detected uridine diphosphatase.

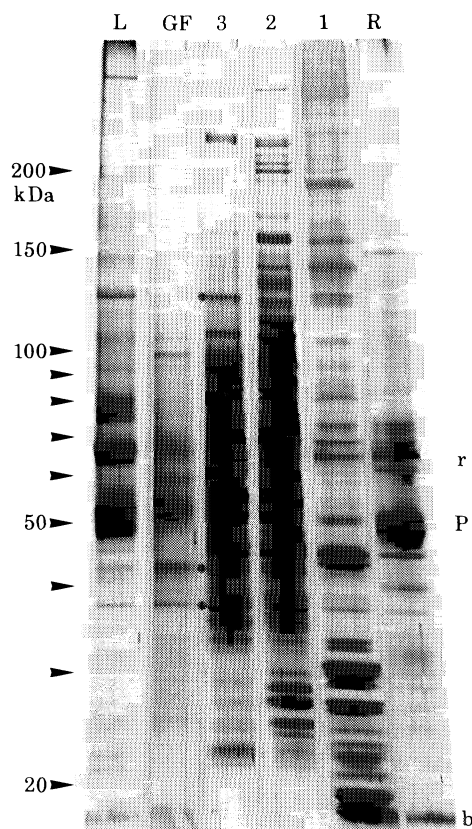

FIGURE 6. SDS polyacrylamide gel electrophoretic comparison of the protein composition of microsomal subfractions of the myeloma with corresponding conventional rat liver fractions, all after extraction at pH 11.2 Samples (1), (2), and (3) are derived from the mannose gradient illustrated in figure 3, (1) from the bottom of the gradient, (2) from the region at 1.16 g ml^{-1}, and (3) at 1.13 g ml^{-1}. The sample labelled R is a liver rough microsomal fraction, GF a Golgi fraction isolated (without ethanol intoxication) according to Ehrenreich *et al.* (1973) and L a Golgi fraction isolated according to Leelavathi as reported by Tabas & Kornfeld (1979). At the extreme right are indicated the expected mobilities of several rat liver microsomal proteins, the ribophorins (r), the cytochromes P_{450} (P) and cytochrome b_5 (b). The stars indicate proteins that are largely shared among the myeloma and liver 'Golgi fractions'.

At the ultrastructural level the staining patterns are more striking. We use lectin conjugates coupled through the carbohydrate moiety of peroxidase (Nakane & Kawaoi 1974) and find that permeabilization is very extensive because all cells (and, in the case of Con A–horseradish peroxidase (HRP), all r.e.r. profiles) are labelled. In figure 7, plate 1, one sees a typical cell stained with Con A–HRP. The reaction product is restricted to the r.e.r. and the perinuclear cisterna, where one may note the presence of (unstained) nuclear pores. The extensive staining of the r.e.r. is consistent with staining of myeloma homogenates reported by Hirano *et al.* (1972)

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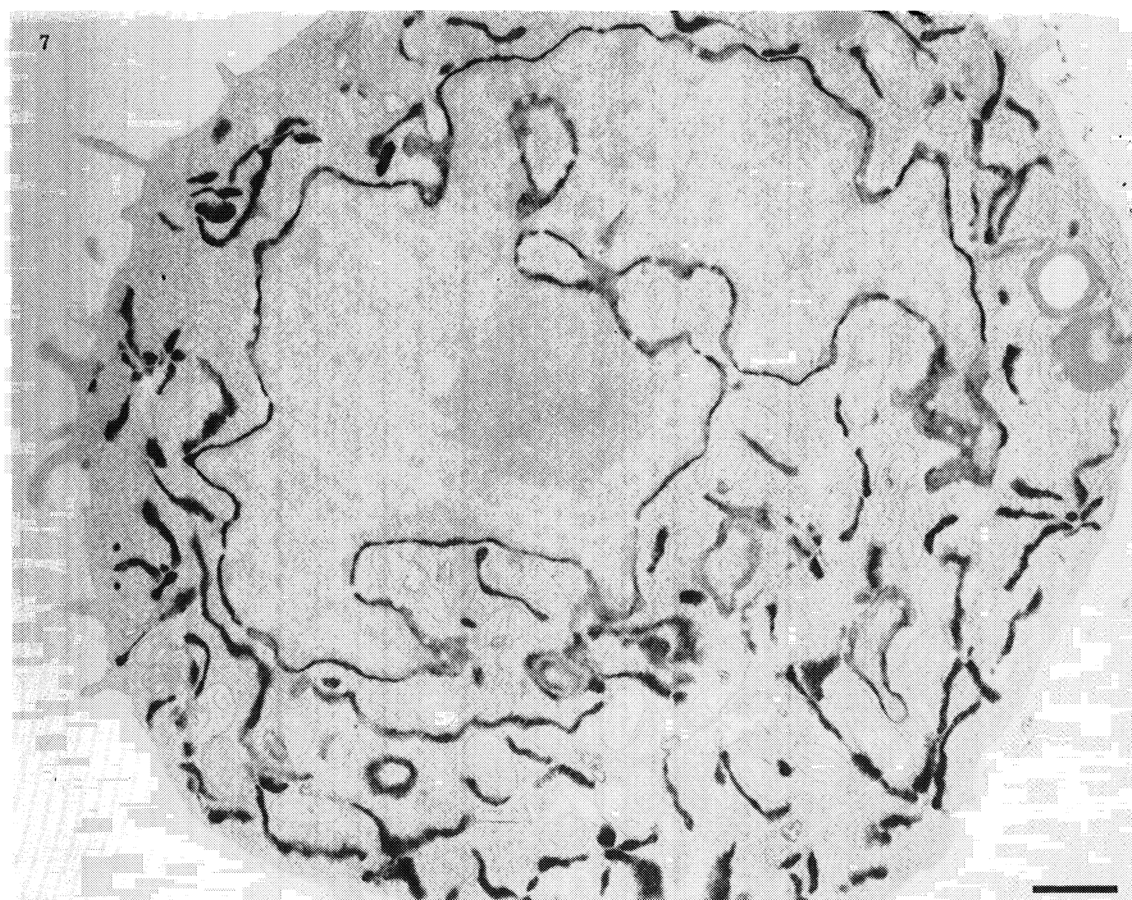

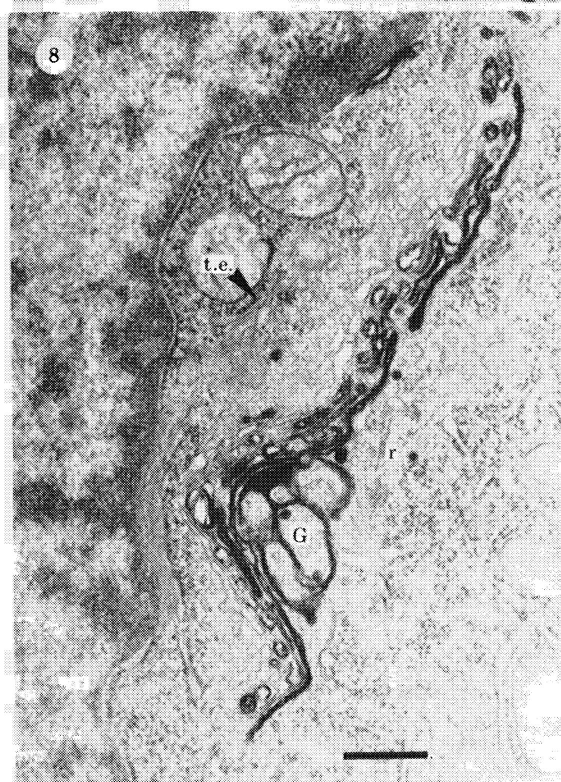

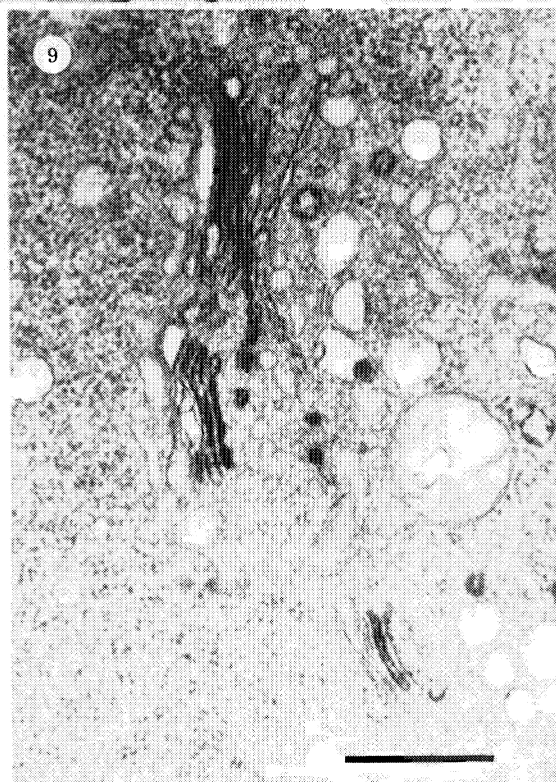

FIGURES 7–9. For description see overleaf.

(*Facing p.* 182)

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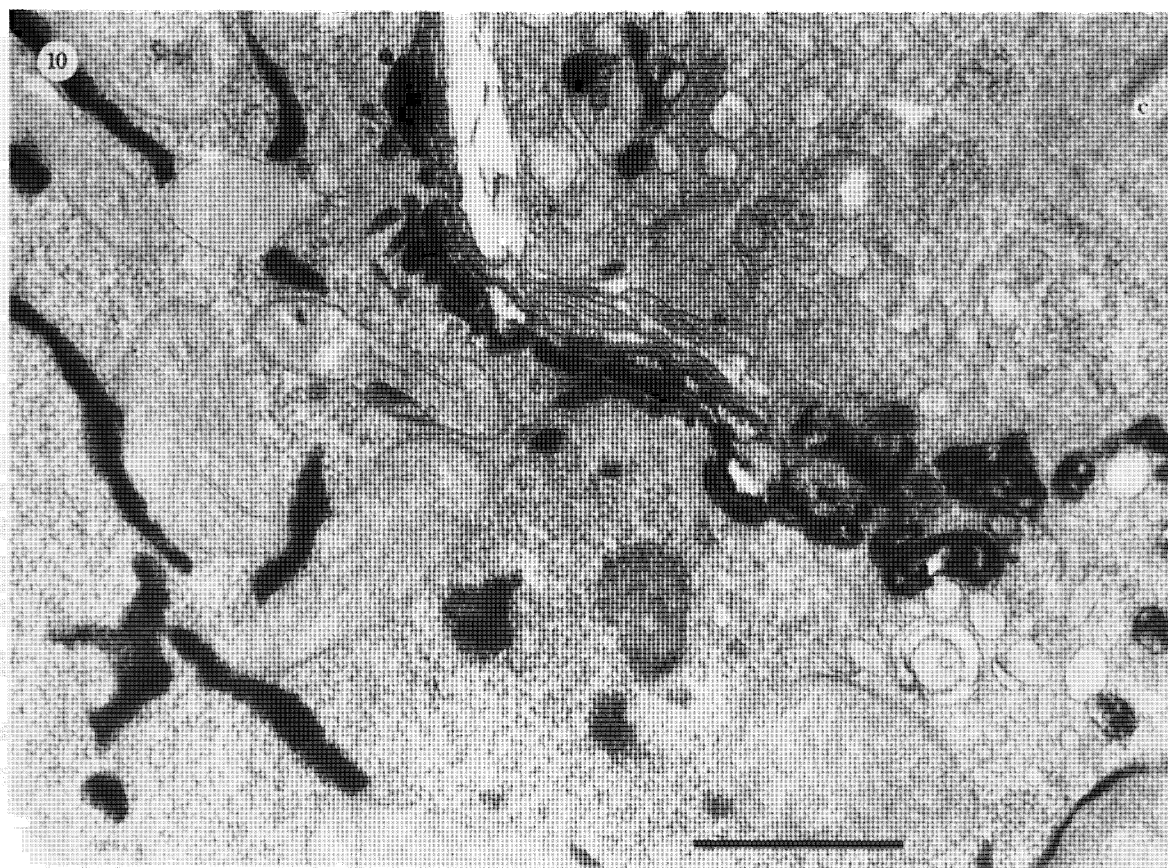

FIGURES 7–10. Thin sections of the rat myeloma (figures 7, 8 and 10) or the non-producing mouse myeloma Sp 2/0 Ag 14 (figure 9) stained with peroxidase-conjugated lectins in the presence of saponin. Cells are stained with Con A–HRP in figures 7 and 10 and with WGA–HRP in figures 8 and 9. Figure 7 illustrates the uniform intense staining of the cisternal space of the r.e.r. by Con A. The cell surface is unstained, in keeping with the very poor Con A agglutinability of these cells. Figure 8 shows that WGA is highly selective for the Golgi complex and that one face of the stack of cisternae is preferentially stained. This face is near 'G.e.r.l.-like' elements (G) and 'rigid lamellae' (r) and appears opposite the face juxtaposed to transitional elements of the r.e.r. (t.e.). Figure 9 illustrates a comparable staining gradient in a myeloma variant that no longer synthesizes Ig. Figure 10 shows that Con A stains a portion of the Golgi complex in addition to the r.e.r. The face stained is closely apposed to the r.e.r. and is hence provisionally identified as the proximal face, i.e. the face that is not stained with WGA. c, Centriole; bar = 0.5 μm.

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and the studies of Rodriguez Boulan *et al.* (1978*a*, *b*) of the luminally disposed class of rough microsomal Con A-binding glycoproteins of the rat liver.

The staining pattern with WGA–HRP is altogether different and is largely restricted to the Golgi stack, associated vesicles and multivesicular bodies. In fact, one has the impression (figure 8, plate 1) that it is only one face of the Golgi stack that is stained and that this is the face opposite the transitional elements of the r.e.r. and including 'G.e.r.l.'-like elements.

The material stained with both lectins is not simply the secretory Ig, because staining of non-producing variants of myelomas (e.g. Sp 2/0 Ag 14 of the mouse or YB 2/3.0 Ag 1.2.3 of the rat obtained from Dr Y. Argon and Dr C. Milstein) give similar staining patterns (figure 9, plate 1).

Our present biochemical analysis of WGA-binding proteins recovered from mannose gradients indicates the presence of a complex class of sialoglycoproteins ranging in molecular mass from 70 to over 200 kDa. Thus, the presence of WGA-binding sites in an anatomically distal portion of the Golgi stack correlates with the presence of terminal sugar tranferases (Roth & Berger 1982) and the above-mentioned inferences based on the use of monensin.

Finally, returning to the cells stained with Con A–HRP, there is an additional important point. In figure 7 only the r.e.r. is stained; however, when one examines the Golgi stack in such cells (figure 10, plate 2) it is clear that again one face of the stack is stained, and this time it appears to be the face closest to the r.e.r. Thus, as recently suggested on the basis of other evidence (Howell *et al.* 1978; Ito & Palade 1978; Rothman 1981), there may be r.e.r. components that are common to the Golgi stack. What is novel is that the present data suggest that they are confined to the anatomically proximal face.

Conclusion

In conclusion, the stack of cisternae of the Golgi complex shares certain features with the r.e.r. and may be subdivided by a number of means. Several altogether distinct approaches may be used to reveal this heterogeneity: perturbation by ionophores, subcellular fractionation, and ultrastructural cytochemistry. By cross-correlating the data obtained by these multiple approaches, convergent and therefore self-reinforcing conclusions should be obtained. Such a systematic mapping of the Golgi complex is still lacking but should ultimately serve as a basis for understanding the functions of this pivotal organelle.

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Discussion

T. Feizi (*Division of Communicable Diseases, Clinical Research Centre, Harrow, U.K.*). Although the SP 2/0 and YB 2 myeloma cells are not secreting immunoglobulin, they are undoubtedly synthesizing and glycosylating a number of other cellular proteins. Could it be that the differing staining patterns of the proximal and distal faces of the Golgi apparatus with lectins represent reactions with 'passenger' glycoproteins rather than structural components of the Golgi membranes?

A. M. Tartakoff. No quantitative data are available allowing comparison of the relative mass of secretory and for example lysosomal glycoproteins present within the Golgi complex. Nevertheless, the wild-type myeloma cells are highly specialized toward Ig biosynthesis and one would therefore suspect that most Golgi 'passenger' glycoproteins in the wild-type are Ig. In the mutants that fail to synthesize Ig other 'passenger' glycoproteins surely predominate; however, it is not yet known to what extent they, as opposed to 'resident' glycoproteins, account for the lectin staining patterns.

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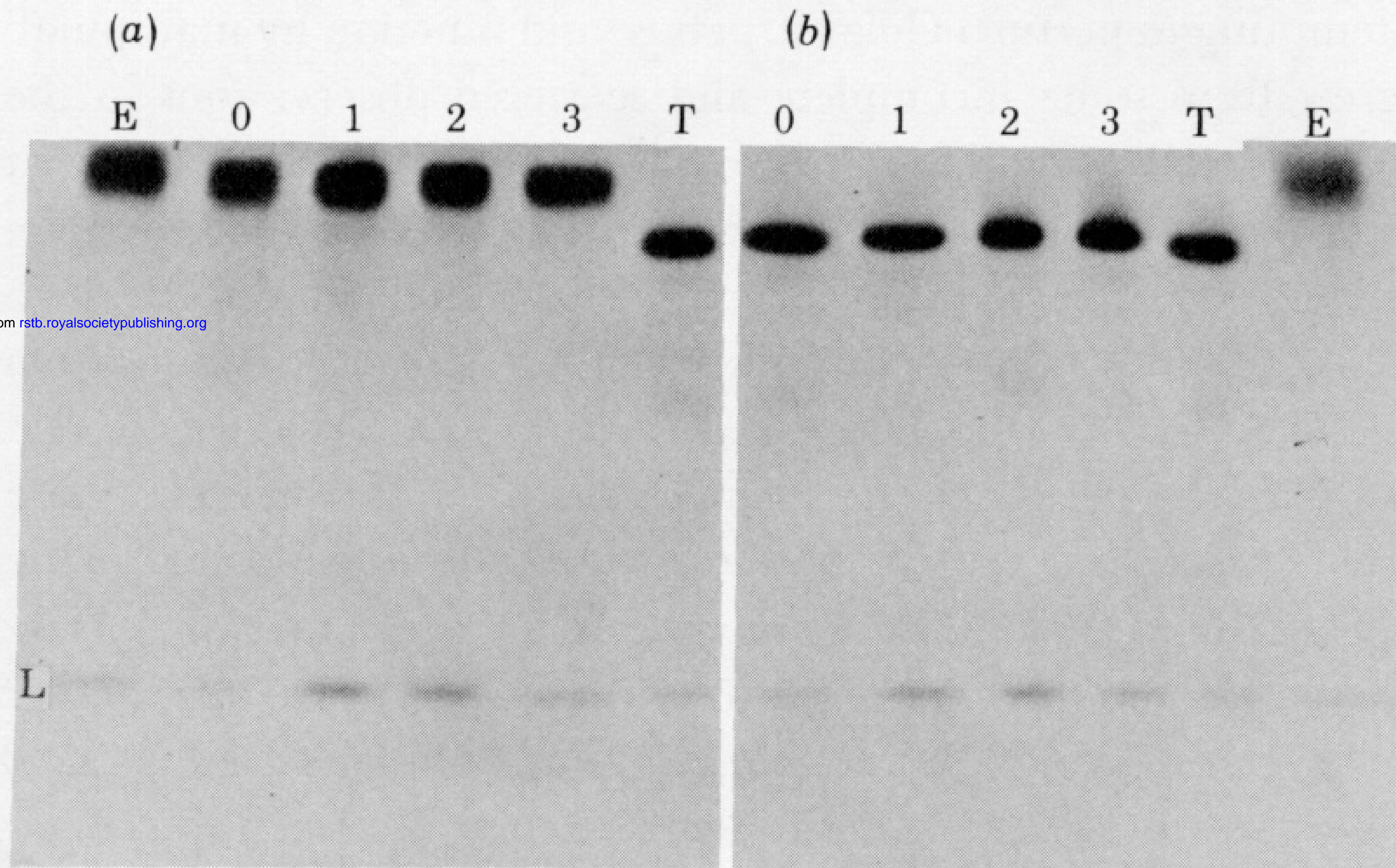

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FIGURE 1. Evaluation of secretory Ig heavy chain carbohydrate maturation by use of endoglucosaminidase H. Ascitic IR 202 myeloma cells were pulse-labelled for 5 min with [^{35}S]methionine, washed, and either chilled (0) or returned to non-radioactive medium for 2 h at 37 °C. Among the reincubated samples, one was incubated without the addition of any perturbant of intracellular transport (1), one was incubated in the presence of 1 μM monensin (2), and one was incubated in the presence of 10 μM CCCP (3). An additional sample of cells (T) was preincubated for 1 h in tunicamycin (1 μg ml^{-1}), pulse-labelled, washed, and chilled at once. In each case, intracellular [^{35}S]Ig was recovered by immunoprecipitation and in condition (1) an extracellular sample (E) was recovered as well. Each sample was divided into halves, reduced and alkylated and incubated overnight at pH 5.5, 37 °C, without (*a*) or with (*b*) endoglucosaminidase H (30 μM ml^{-1}). Samples were then analysed by standard sodium sulphate polyacrylamide gel electrophoresis – autoradiography procedures. Because the enzyme removes high-mannose carbohydrate units, but not mature complex units, it allows a rough discrimination between polypeptides bearing the two classes of carbohydrate unit. It is clear that all the intracellular samples are sensitive to the enzyme, which implies that they bear immature carbohydrate units. By contrast, the extracellular sample is resistant. After enzyme treatment the intracellular samples still have a slightly slower mobility than the carbohydrate-free sample (T), presumably because the enzyme leaves the most internal glucosamine residue on the polypeptide chain and the heavy chains of IgM bear a total of five or six carbohydrate units. L, Ig light chains.

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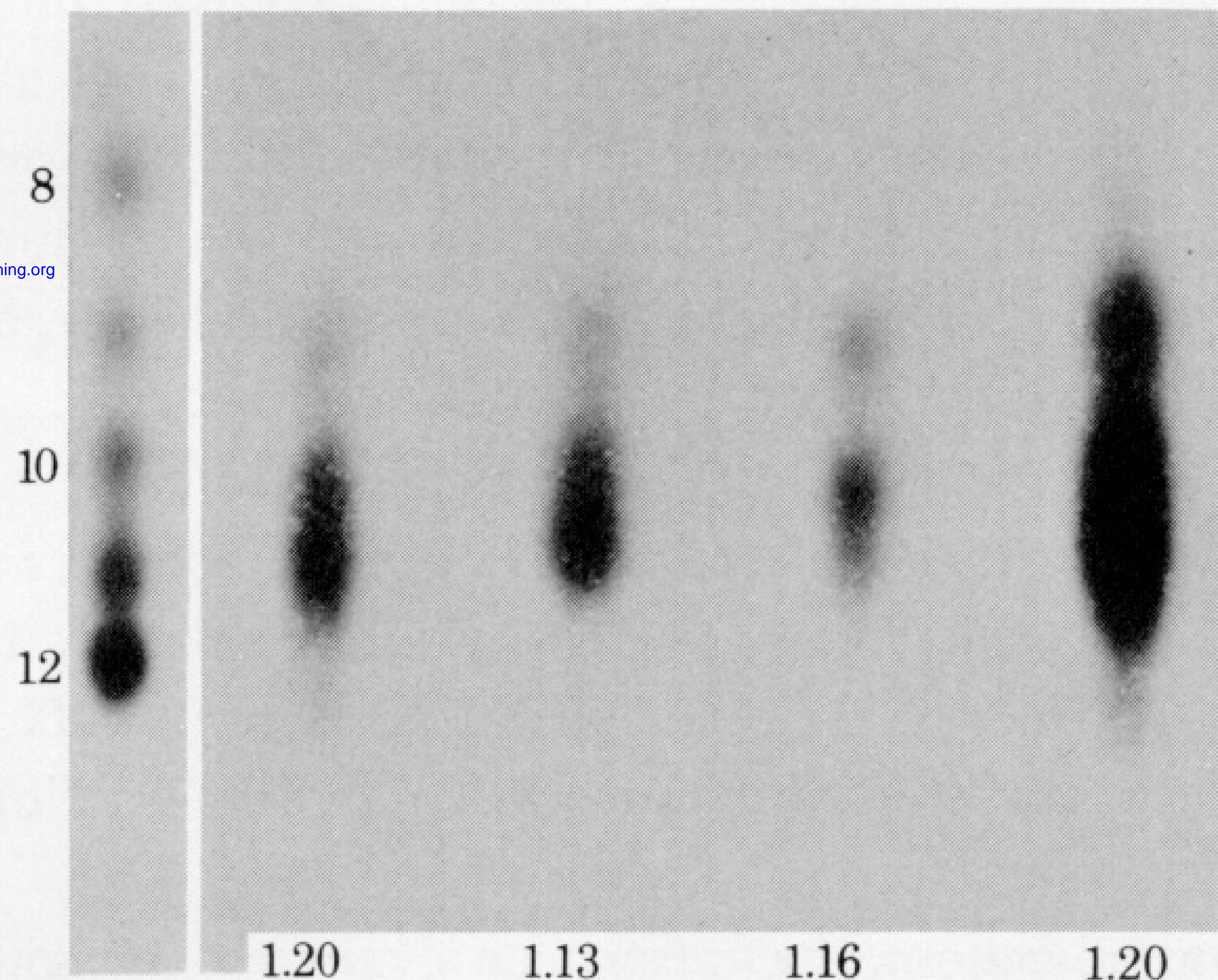

Figure 4. Analysis of [^{3}H]mannose-labelled Ig oligosaccharides recovered from mannose gradients. Myeloma cells were labelled for 60 min with [^{3}H]2-D-mannose in the presence of glucose (0.1 mg ml^{-1}) and fractionated to provide three peaks of labelled Ig cosedimenting with the peaks of [^{35}S]Ig illustrated in the bottom panel of figure 3. Each sample of [^{3}H]Ig recovered by immunoprecipitation was reduced, alkylated, treated with endoglucosaminidase H, desalted and fractionated by thin-layer chromatography on silica gel followed by fluorography. At the left is a family of [^{3}H]mannose-labelled oligosaccharides released from dolichol by weak acid hydrolysis and endoglucosaminidase H. The sizes of the species were assessed by comparison with dolichol and viral glycoprotein-derived standards obtained from Dr M. Snider (Massachusetts Institute of Technology) and Dr R. Datema (Giessen). A labelled [^{3}H]oligosaccharide sample recovered from the bottom of the gradient (1.20 g ml^{-1}) is run twice for comparison with material recovered from fractions centred at 1.13 and 1.16 g ml^{-1}. These rough microsomal oligosaccharides range in size from 11 to 9 sugar residues and are therefore considered to include $GlcNAc_1Man_9Glc_1$, $GlcNAc_1Man_9$ and $GlcNAc_1Man_8$. As suggested by Godelaine *et al.* (1982), species such as $Glc_1Man_8GlcNAc_1$ may also be present. The principal oligosaccharide obtained from [^{3}H]Ig of density 1.13 and 1.16 g ml^{-1} is somewhat smaller than the most abundant rough microsomal species and is therefore thought to lack glucose residues. In addition, a faint spot in the nonasaccharide region continues to be present.

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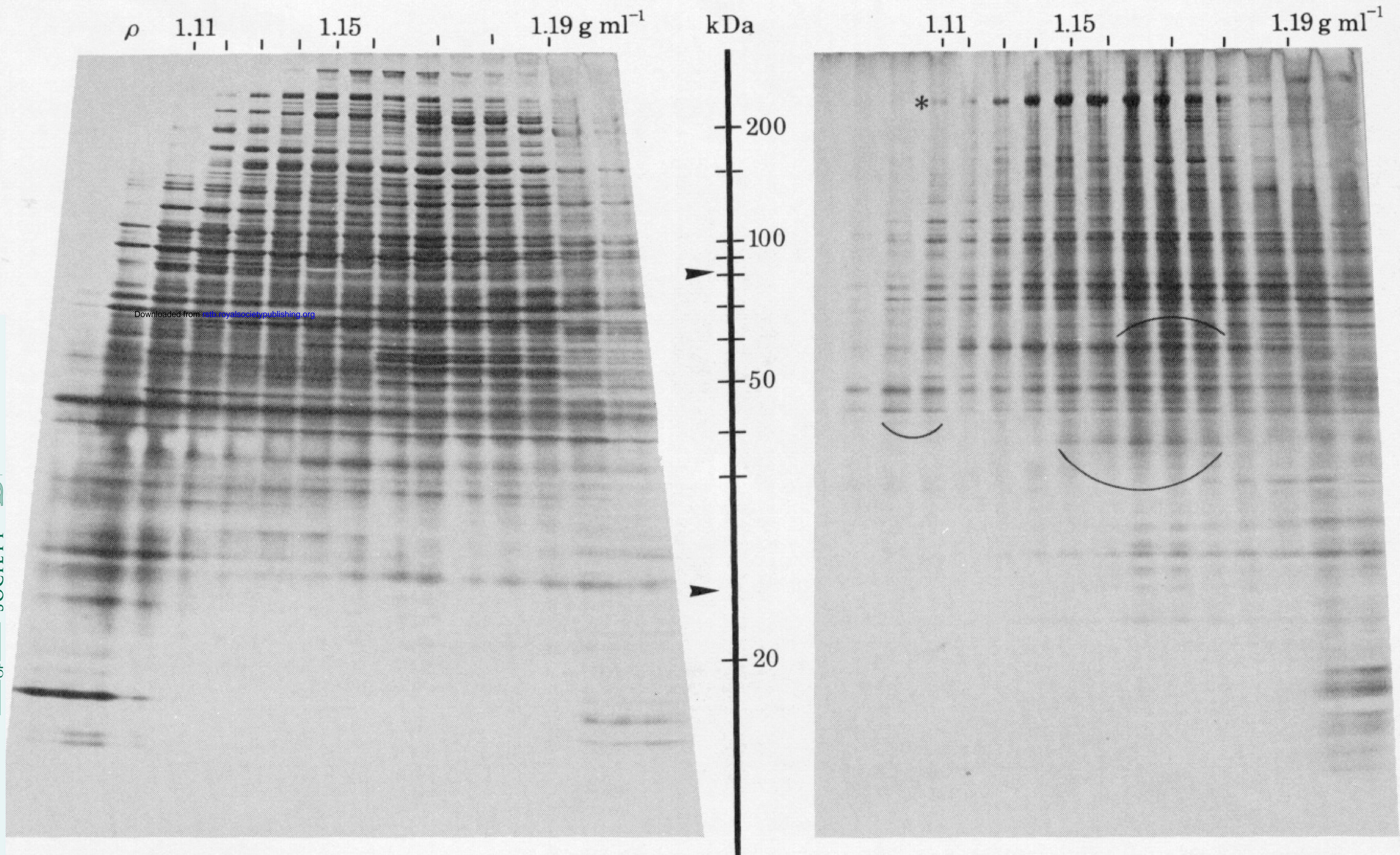

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FIGURE 5. SDS polyacrylamide gel electrophoretic analysis of total microsomal fractions after resolution on continuous mannose gradients. Gradients identical to those illustrated in figure 3 were sampled at regular intervals and the successive fractions were analysed by gel electrophoresis followed by staining with silver nitrate. In the panel at the right the samples were extracted at pH 11.2 according to Howell & Palade (1982) before analysis. The mobilities of Ig heavy and light chains are indicated by the arrowheads along the molecular mass axis. These Ig chains are not conspicuous on a mass basis, although after biosynthetic labelling they predominate. In the right-hand panel the asterisk indicates a major protein common to all gradient regions including Golgi elements. The arcs indicate regions of the gradients with characteristic polypeptide composition, which are therefore thought to correspond to distinct membrane populations.

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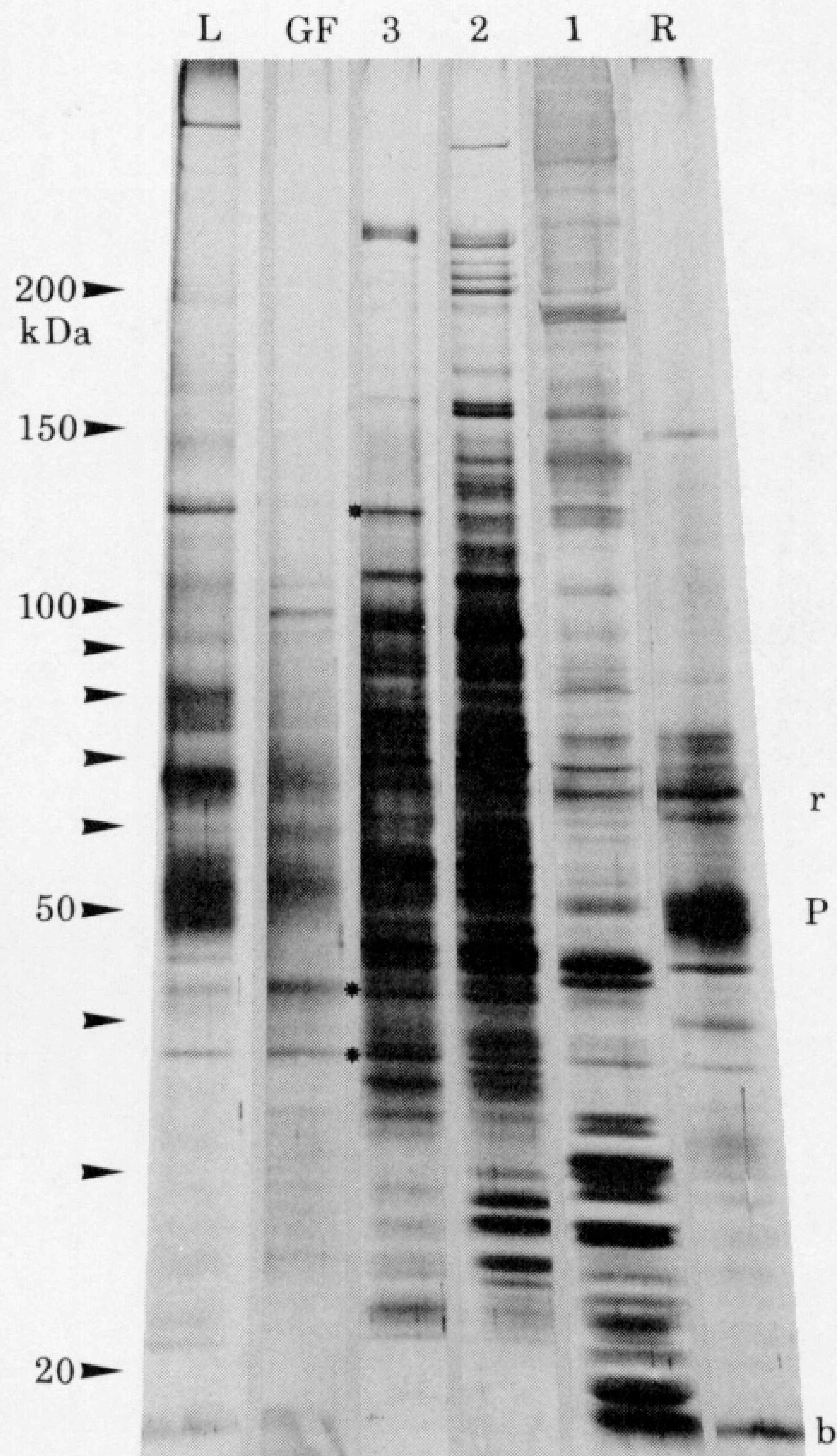

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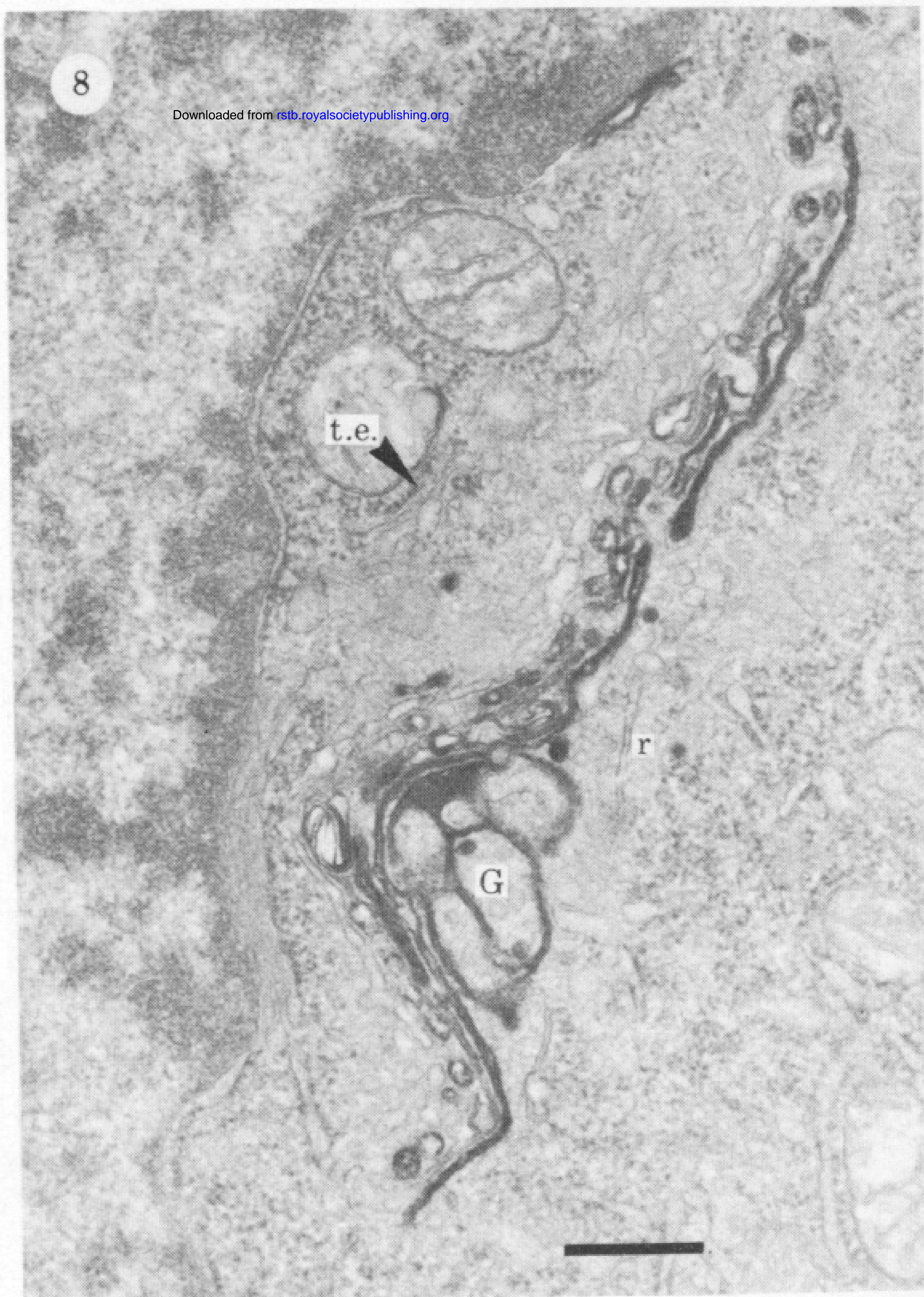
FIGURE 6. SDS polyacrylamide gel electrophoretic comparison of the protein composition of microsomal subfractions of the myeloma with corresponding conventional rat liver fractions, all after extraction at pH 11.2 Samples (1), (2), and (3) are derived from the mannose gradient illustrated in figure 3, (1) from the bottom of the gradient, (2) from the region at 1.16 g ml^{-1}, and (3) at 1.13 g ml^{-1}. The sample labelled R is a liver rough microsomal fraction, GF a Golgi fraction isolated (without ethanol intoxication) according to Ehrenreich *et al.* (1973) and L a Golgi fraction isolated according to Leelavathi as reported by Tabas & Kornfeld (1979). At the extreme right are indicated the expected mobilities of several rat liver microsomal proteins, the ribophorins (r), the cytochromes P_{450} (P) and cytochrome b_5 (b). The stars indicate proteins that are largely shared among the myeloma and liver 'Golgi fractions'.



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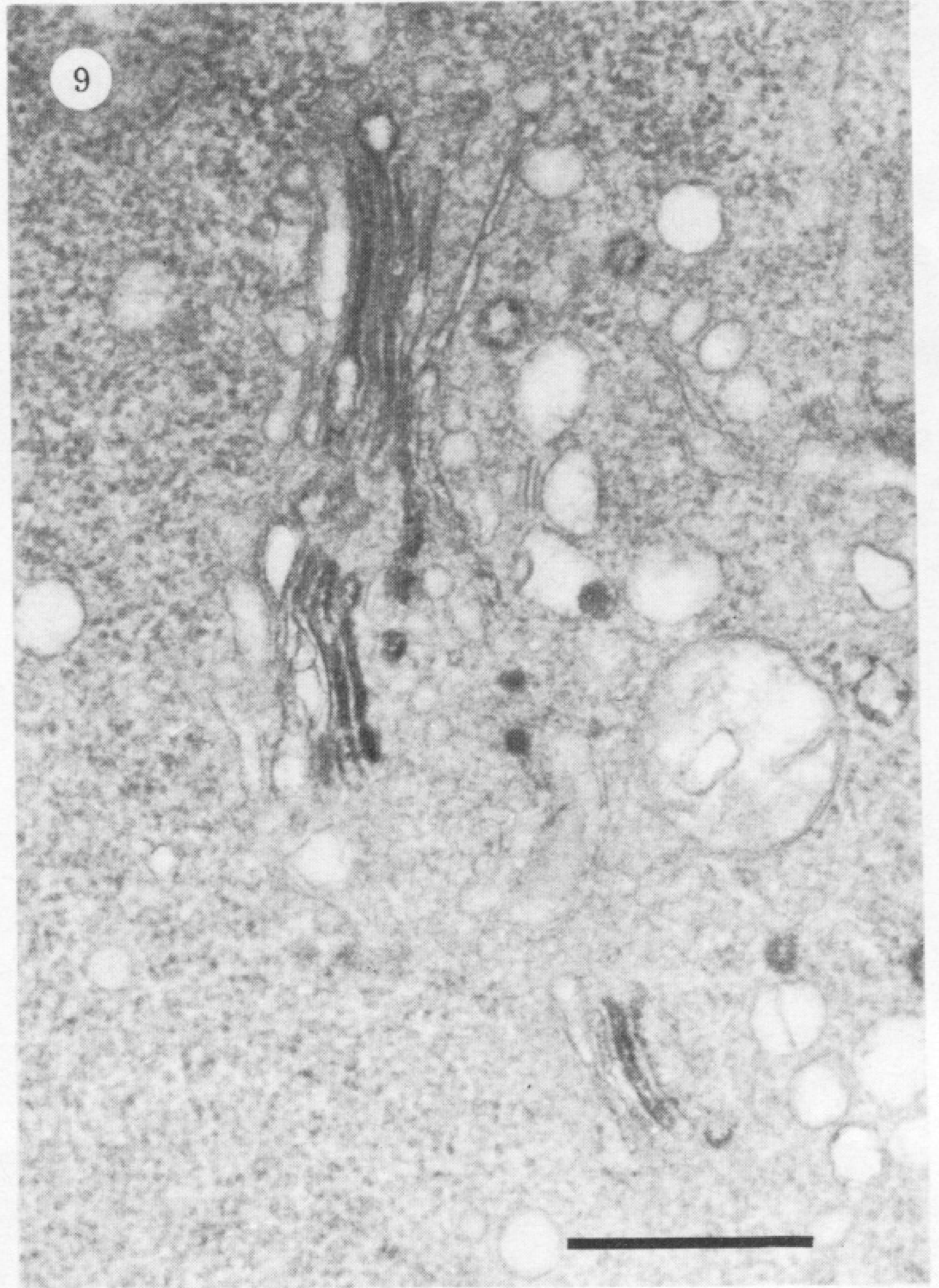

FIGURES 7–9. For description see overleaf.

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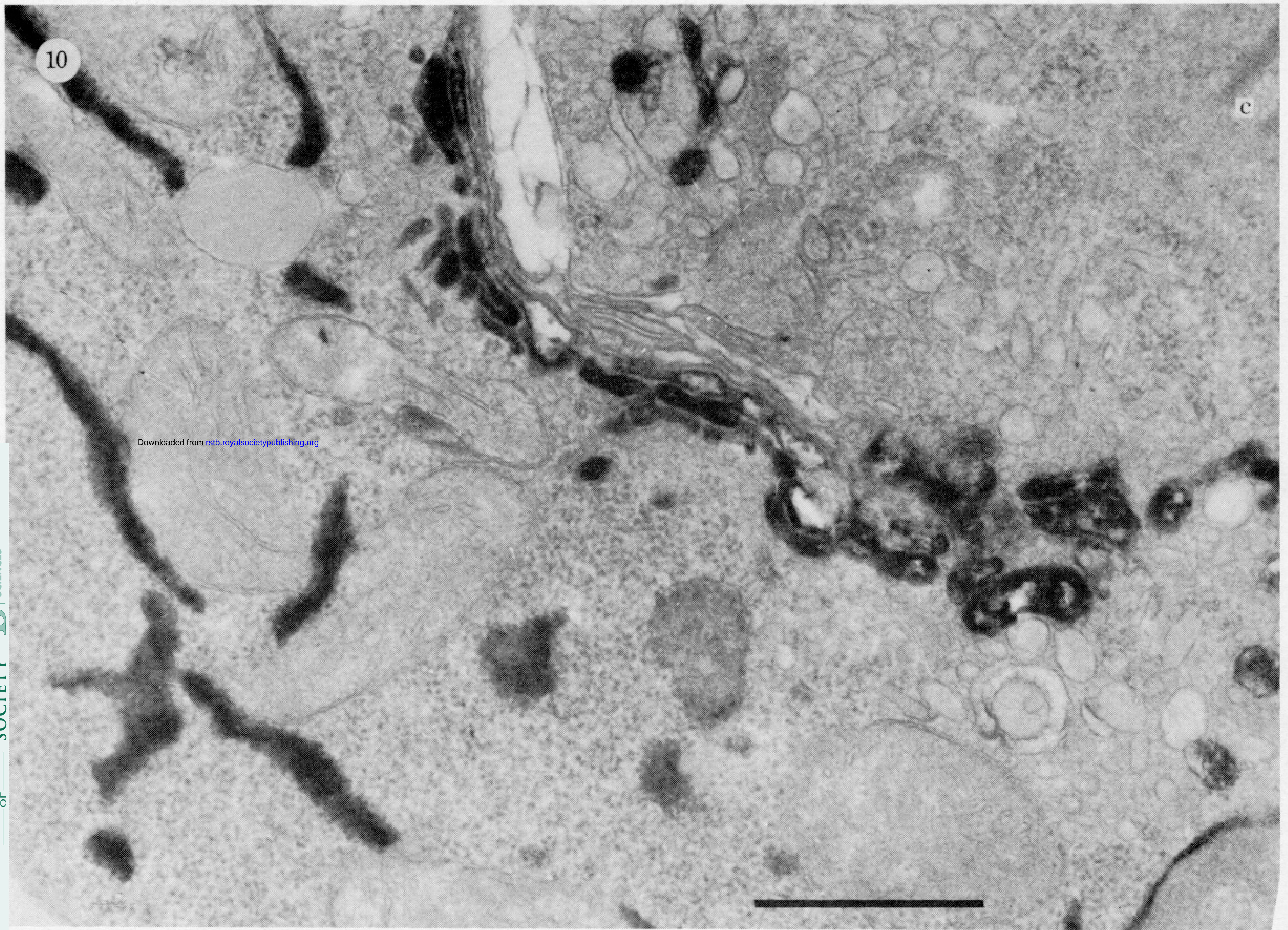

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FIGURES 7–10. Thin sections of the rat myeloma (figures 7, 8 and 10) or the non-producing mouse myeloma Sp 2/0 Ag 14 (figure 9) stained with peroxidase-conjugated lectins in the presence of saponin. Cells are stained with Con A–HRP in figures 7 and 10 and with WGA–HRP in figures 8 and 9. Figure 7 illustrates the uniform intense staining of the cisternal space of the r.e.r. by Con A. The cell surface is unstained, in keeping with the very poor Con A agglutinability of these cells. Figure 8 shows that WGA is highly selective for the Golgi complex and that one face of the stack of cisternae is preferentially stained. This face is near 'G.e.r.l.-like' elements (G) and 'rigid lamellae' (r) and appears opposite the face juxtaposed to transitional elements of the r.e.r. (t.e.). Figure 9 illustrates a comparable staining gradient in a myeloma variant that no longer synthesizes Ig. Figure 10 shows that Con A stains a portion of the Golgi complex in addition to the r.e.r. The face stained is closely apposed to the r.e.r. and is hence provisionally identified as the proximal face, i.e. the face that is not stained with WGA. c, Centriole; bar = 0.5 μm.