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DIFFERENTIAL SECRETION OF PROTEINS AND GLYCOPROTEINS BY LIVERS OF IMMATURE AND ADULT RATS

EFFECT OF ANTIMICROTUBULE DRUGS*

SALMAN AZHAR and EVE REAVEN†

Department of Medicine, Stanford University School of Medicine, and Geriatric Research, Education and Clinical Center, Veterans Administration Medical Center, Palo Alto, CA 94304, U.S.A.

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Abstract—This study was initiated to re-examine reported differences in the action of antimicrotubule agents on plasma protein secretion from livers of immature versus adult rats. The aim was (1) to determine the composition and to monitor the secretion of various plasma proteins and glycoproteins from liver slices labeled *in vitro* with specific amino acids and sugar residues, and (2) to correlate observed differences in secretion of these proteins with structural changes in the hepatocytes of the different aged animals. For the most part, slices of liver from fetal (term), neonatal (4- to 5 days old), and adult rats (70 days old) were incubated with radioactive amino acids or various tritiated sugars specific for N-linked core oligosaccharide and/or N-linked terminal oligosaccharide chains. Our findings indicate that liver slices of fetal and neonatal rats are efficient in synthesizing plasma proteins including fully glycosylated glycoproteins. The secretion of glycosylated and nonglycosylated proteins believed to be processed through Golgi complexes was inhibited to the same extent (~70–80%) by antimicrotubule agents, regardless of the age of the host animal. However, other proteins and glycoproteins secreted by livers of immature rats were found to be relatively insensitive (i.e. inhibited to only 30–40%) to the action of various antimicrotubule drugs. The glycoproteins were found to lack N-linked terminal sugars (although the glycoproteins did contain N-linked core sugars), and it is likely that the drug-insensitive proteins bypassed critical glycosylating sites in the Golgi compartment prior to release. Overall, these findings support earlier data showing that antimicrotubule drugs have a special impact on Golgi-associated events in liver cells. To what extent these findings are related to the action of microtubules remains to be seen.

Over the years, evidence has accumulated to suggest that antimicrotubule agents have a special impact on Golgi structure and function in liver. Initially, antimicrotubule agents were shown to block the hepatic release of various plasma proteins [1–4]. Subsequently, the structure of the Golgi apparatus in hepatocytes was found to be extremely sensitive to the action of antimicrotubule drugs [4, 5]. For example, ~80% of hepatocyte Golgi cisternal membranes are uniquely disrupted after treatment of rats with these agents [4]. Also, *in vivo* treatment of rats with colchicine or vinblastine specifically inhibits the activity of various hepatic Golgi-associated terminal glycosyltransferases (although endoplasmic reticulum-associated sugar transferases and several other non-Golgi enzymes are unaffected [6]). Finally, antimicrotubule agents were shown to restrict the incorporation of certain precursor phospholipids into the endoplasmic reticulum and/or Golgi-forming membranes (i.e. microsomal and GF_3 Golgi fractions), and to retard the flow of newly synthesized membranes from one subcellular compartment to another [7]. The mechanism responsible for these events linking antimicrotubule drugs to alterations in Golgi structure and function has never been defined.

It was of some interest, therefore, to learn that the liver of immature rats is less sensitive to the action of antimicrotubule agents than the liver of adult rats. Reports from Kaufman *et al.* [8, 9] indicated that the release of both glycosylated and nonglycosylated proteins from liver slices of fetal and neonatal rats declines between 30 and 40% when treated with colchicine (or other microtubule drugs), whereas the secretion of these same proteins from liver slices of adult rats declines between 70 and 80% under identical circumstances. The authors concluded that participation of microtubules in liver plasma protein secretion is not fully active during hepatic development.

In the current study we took advantage of the differential colchicine sensitivity of liver from immature and adult rats to further explore the relationship of antimicrotubule drugs to Golgi structure and secretory function. We assumed that plasma glycoproteins were glycosylated by liver cells during transport of the proteins through Golgi complexes

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† Address reprint requests to: Eve Reaven, Ph.D., Veterans Medical Center, GRECC/182B, 3801 Miranda Ave., Palo Alto, CA 93404.

[10–17] in both immature and adult rats, and we were interested in determining why the secretion of these glycoproteins was not identically reduced in both groups of animals following the use of various antimicrotubule agents [18–20]. The plan was to compare the secretion of a non-glycosylated protein, like albumin [18–20], with a glycosylated protein, like transferrin [21–23], which could be separately labeled in its peptide backbone (with amino acids), its N-linked core oligosaccharide chain (with glucosamine), and its N-linked terminal oligosaccharide chain (with specific N-terminal sugars). The proteins could be identified by specific immunoprecipitation, and glycoproteins could be further characterized by affinity chromatography using immobilized lectin columns of known sugar specificity.

MATERIALS AND METHODS

Materials

Animals. Non-fasted Sprague–Dawley rats were used in all experiments. Unless otherwise stated, the age of adult male rats was 70 ± 3 days (300–325 g). The age of fetuses was estimated from the time of mating (±0.5 day), and the rats were used at term (20.5 to 21.5 days). The age of neonatal animal was determined from the time of natural birth and was accurate to ± 0.5 day. In some experiments, 26-day (60–80 g), 42-day (160–180 g), 90-day (350–400 g) and 1-year-old male rats (600–650 g) were also employed.

Pregnant animals (20 ± 1 days) were anaesthetized with sodium thiamylal (Surital, 50 mg/kg body wt), and the uteri were exposed by laparatomy. Fetuses were removed from the uteri and killed by decapitation. The livers were excised, washed in cold Dulbecco's phosphate-buffered saline (NaCl, 137 mM; KCl, 2.7 mM; $CaCl_2$, 4.5 μM; $MgCl_2$, 2.5 μM; Na_2HPO_4, 8.1 mM; and KH_2PO_4, 1.5 mM; pH 7.3), blotted on filter paper, and weighed. Neonatal (5 ± 0.5 days), other postnatal, and adult animals were also killed by decapitation and the livers were treated similarly.

Chemicals and reagents. L-[4,5-^{3}H(N)]Leucine (sp. act. 40–60 Ci/mmol), *N*-acetyl-D-[^{3}H]mannosamine (sp. act. 22.7 Ci/mmol) and L-[6-^{3}H]fucose (sp. act. 56 Ci/mmol) were purchased from E.I. du Pont de Nemours & Co., NEN Research Products, Boston, MA. D-[6-^{3}H]Glucosamine HCl (sp. act. 25–40 Ci/mmol) was supplied by ICN Chemical and Radioisotope Division, Irvine, CA. L-[^{35}S]Methionine (sp. act. 1390 Ci/mmol), L-[2,3-^{3}H]aspartic acid (sp. act. 8–20 Ci/mmol), L-[4,5-^{3}H]lysine HCl (sp. act. 75–100 Ci/mmol), L-[2,4,6-^{3}H]phenylalanine (sp. act. 60–80 Ci/mmol), L-[U-^{14}C]alanine (sp. act. 150 mCi/mmol), L-[U-^{14}C]proline (sp. act. 250 mCi/mmol) and D-[6-^{3}H]galactose (sp. act. 20–40 Ci/mmol) were obtained from the Amersham Corp., Arlington Heights, IL. Colchicine, lumicholchicine, vinblastine, podophyllotoxin, glucose, galactose, fucose, glucosamine and pyruvate were the products of the Sigma Chemical Co., St. Louis, MO. Nocadazole and formaldehyde-fixed *Staphylococcus aureus* cells (Pansorbin) were purchased from the Aldrich Chemical Co., Milwaukee, WI, and the Calbiochem-Behring Corp., San Diego, CA, respectively. Wheat germ agglutinin (WGA)-agarose (7 mg lectin/ml gel) and concanavalin A (ConA)-agarose (7 mg lectin/ml gel) were supplied by Vector Laboratories, Inc., Burlingame, CA. Rabbit anti-rat albumin, rabbit anti-rat transferrin, chromatographically purified albumin and transferrin were obtained from Cappel Laboratories, Cochranville, PA. Endoglycosidase H (endo-β-*N*-acetylglucosaminidase H) was purchased from ICN Immuno Biologicals, Lisle, IL. Various amino acids were obtained from the United States Biochemical Corp., Cleveland, OH. All other reagents used were of analytical grade.

Methods

Composition of incubation medium. All incubations were carried out in Earle's balanced salt solution containing amino acids [24], glucose (1 mg/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml). The millimolar concentrations of amino acids present were as follows: L-alanine, 0.40; L-arginine·HCl, 0.36; L-asparagine, 0.30; L-aspartic acid, 0.45; L-cysteine (free base), 0.57; L-cystine·2HCl, 0.06 mM; L-glutamic acid, 1.02; L-glutamine, 2.40; glycine 0.67; L-histidine·HCl·H2O, 0.72; hydroxy-L-proline, 0.20; L-isoleucine, 0.19; L-leucine, 0.4; L-lysine·HCl, 1.1; L-methionine, 0.34; L-phenylalanine, 0.30; L-proline, 0.43; L-serine, 0.25; L-threonine, 0.63; L-tryptophan, 0.20; L-tyrosine, 0.22; and L-valine, 0.56.

Incorporation of labeled amino acids by liver (protein labeling). Liver slices prepared by the technique of Ballard and Oliver [25], and weighing ~225–250 mg, were preincubated in incubation medium for 30 min at 37° in the presence of CO_2:O_2 (5:95%). At the end of the preincubation, either [^{3}H]leucine, [^{35}S]methionine or other ^{3}H- or ^{14}C-labeled amino acids were added to appropriate vials. The final medium concentration of [^{3}H]leucine or other labeled amino acids was 0.1 mM (5 μCi/ml). At the end of the incubation (15–240 min), medium was removed and the slices were washed three times with incubation medium (containing a 10 mM concentration of the respective amino acid). The medium and washings were pooled and subsequently processed for determination of radioactivity. This fraction was considered to contain the secreted proteins.

Following incubation, the liver slices were homogenized in 2.5 ml of buffer A (10 mM Tris–HCl, 25 mM KCl, 1 mM EDTA, 0.15 M NaCl, 1 mM *p*-phenylmethylsulfonyl fluoride, 1 mM benzamidine and 100 units/ml aprotinin), and a suitable aliquot was taken to determine the incorporation of radioactivity into protein. The remaining homogenate in each case was made to 0.5% sodium deoxycholate and 0.5% Nonidet P-40 and incubated at 4° for 3 hr to release proteins sequestered within, or associated with, endomembranes [1, 26–28]. The samples were then cleared of nonsolubilized material by centrifugation at 100,000 *g* for 1 hr. The extracts containing solubilized proteins were collected, and suitable aliquots were used for the determination of total protein or specifically for albumin or transferrin protein content.

Isolation and assay of radioactive proteins. Radioactive proteins in total homogenates, cellular extracts (deoxycholate-Nonidet P-40 extract) or medium were prepared for counting as described by Kousvelari *et al.* [29]. Briefly, 100-μl aliquots of samples were vortex-mixed with 10 ml of 10% trichloroacetic acid at 4°. After standing for 30–60 min, each sample was filtered through a glass fiber disc (2.5 cm diameter, Reeve-Angel, Scientific Products, San Francisco), washed three times with 10% trichloroacetic acid, and dried, and radioactivity was determined by liquid scintillation spectrometry. The incorporation of radioactivity into newly synthesized cellular proteins or medium (secreted) proteins was expressed as picomoles of radioactive amino acid per milligram tissue protein.

Immunoprecipitation. Specific immunoprecipitation of albumin and transferrin was carried out using fixed *S. aureus* to precipitate antigen–antibody complexes [30]. The procedure used was a modification of the procedure of Owen *et al.* [31] and Redman *et al.* [32]. One- to two-milliliter aliquots of cellular extracts (deoxycholate-Nonidet P-40 extract) or medium were treated twice with 50 μl of 10% suspension of Pansorbin (*S. aureus* cells, 30 min each time at 4°) to preclear samples. Bacteria were removed by centrifugation at 12,000 *g* for 5 min. The supernatant fractions were then supplemented with excess amounts of antiserum specific for rat transferrin or albumin and incubated overnight at 4°, followed by the addition of 50 μl of 10% Pansorbin suspension for an additional hour. Following centrifugation (12,000 *g* for 5 min), the sedimented bacteria-immunoprecipitate pellets were washed five times with buffer A containing 0.5% Nonidet P-40 and sodium deoxycholate, once with buffer A containing 0.5% Nonidet P-40, and once with buffer A alone. The washed pellets were resuspended in 100 μl of sodium dodecyl sulfate (SDS)–sample buffer (without tracking dye) and heated for 4 min at 100°. After brief centrifugation, suitable aliquots were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) according to laemmli [33] or counted for radioactivity to determine the level of incorportion into the newly synthesized proteins. The titer and specificity of anti-albumin and anti-transferrin preparations were monitored routinely. All immunoprecipitations were carried out under conditions of antibody excess. The immunoprecipitates of albumin and transferrin were analyzed by SDS–PAGE [33] to show the specificity of precipitation.

Incorporation of [^{3}H]*glucosamine,* [^{3}H]*galactose,* [^{3}H]*fucose and* N*-acetyl-*[^{3}H]*mannosamine.* Carbohydrate labeling was carried out in glucose-free incubation medium containing 10 mM pyruvate (modified incubation medium). Liver slices (~225–250 mg) were preincubated for 30 min (unless otherwise stated) and then incubated with 50 μM (final concentration) [^{3}H]glucosamine (8 μCi/ml), [^{3}H]fucose (8 μCi/ml), [^{3}H]galactose (10 μCi/ml) or *N*-acetyl-[^{3}H]mannosamine (12.5 μCi/ml) for 15–240 min. At the end of the labeling period, the samples were processed as described above for protein labeling experiments.

Treatment of liver slices with antimicrotubule agents. To determine the effect of colchicine (lumicolchicine or other antimicrotubule agents) on protein and glycoprotein secretion, liver slices were pretreated for 30 min with or without 10 μM colchicine (unless otherwise stated). Following preincubation, slices were incubated with labeled amino acids or sugars for 15–240 min with continued colchicine treatment. At desired time intervals, samples were assayed to determine the incorporation of radioactivity into total protein (including glycoprotein), albumin and transferrin. Sugar incorporation was normally monitored in total protein fractions and in immunoprecipitated transferrin.

*Treatment with endo-β-*N*-acetylglucosaminidase H (endoglycosidase H).* Liver slices from neonatal and adult rats (200–250 mg) were rinsed with incubation medium without methionine and incubated with 1 ml of this medium for 1 hr at 37°. Subsequently the slices were incubated for 20 min with L-[^{35}S]methionine at 50 μCi/ml in methionine-free incubation medium, then washed with medium and reincubated for various times (50–120 min) in the presence of unlabeled methionine (2 mM). After incubation, the slices were homogenized, and transferrin was immunoprecipitated as described above. For digestion with endoglycosidase H, transferrin immunoprecipitates were dissociated from the fixed *S. aureus* by boiling in 0.1 M Tris–HCl buffer, pH 8.0, containing 1% SDS and 2-mercaptoethanol for 4 min as described by Owen *et al.* [31]. After removal of the bacteria by centrifugation, each sample was divided into two equal portions and incubated for 20 hr at 37° with agitation in the presence or absence of 10 mU/ml of endoglycosidase H [31]. To each sample 50 μg bovine serum albumin was added as carrier protein, and then total proteins were precipitated with an equal volume of 30% trichloroacetic acid for 2 hr on ice. The samples were centrifuged and pellets were washed once with absolute ethanol and once with ether, dried, and dissolved in sample buffer for SDS–PAGE. SDS–PAGE was carried out according Laemmli [33] using 5–20% linear polyacrylamide slab gels. After electrophoresis the gels were analyzed by fluorography [34, 35].

Lectin column chromatography. Columns of ConA-agarose and WGA-agarose (1 ml of settled gel) were used to fractionate highly glycosylated and partially glycosylated forms of transferrin secreted by liver slices of fetal, neonatal and adult rats. ConA-agarose columns were extensively washed with 60 bed volumes of buffer B [50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 1 mM $MnCl_2$] and then 100 bed volumes of buffer C (0.15 M NaCl–50 mM HEPES, pH 7.4). Similarly, WGA-agarose was washed extensively with buffer C (120–150 bed volume). The medium in each case was dialyzed against buffer C to remove the majority of radioactivity and traces of sugar and then applied to ConA-agarose or WGA-agarose at 4°. The samples were recycled through respective columns four to five times. After the final cycle, the flow was stopped for 60–90 min and then each column was washed with 6–8 column volumes of buffer C. Adsorbed material was eluted with 2–4 bed volumes of buffer

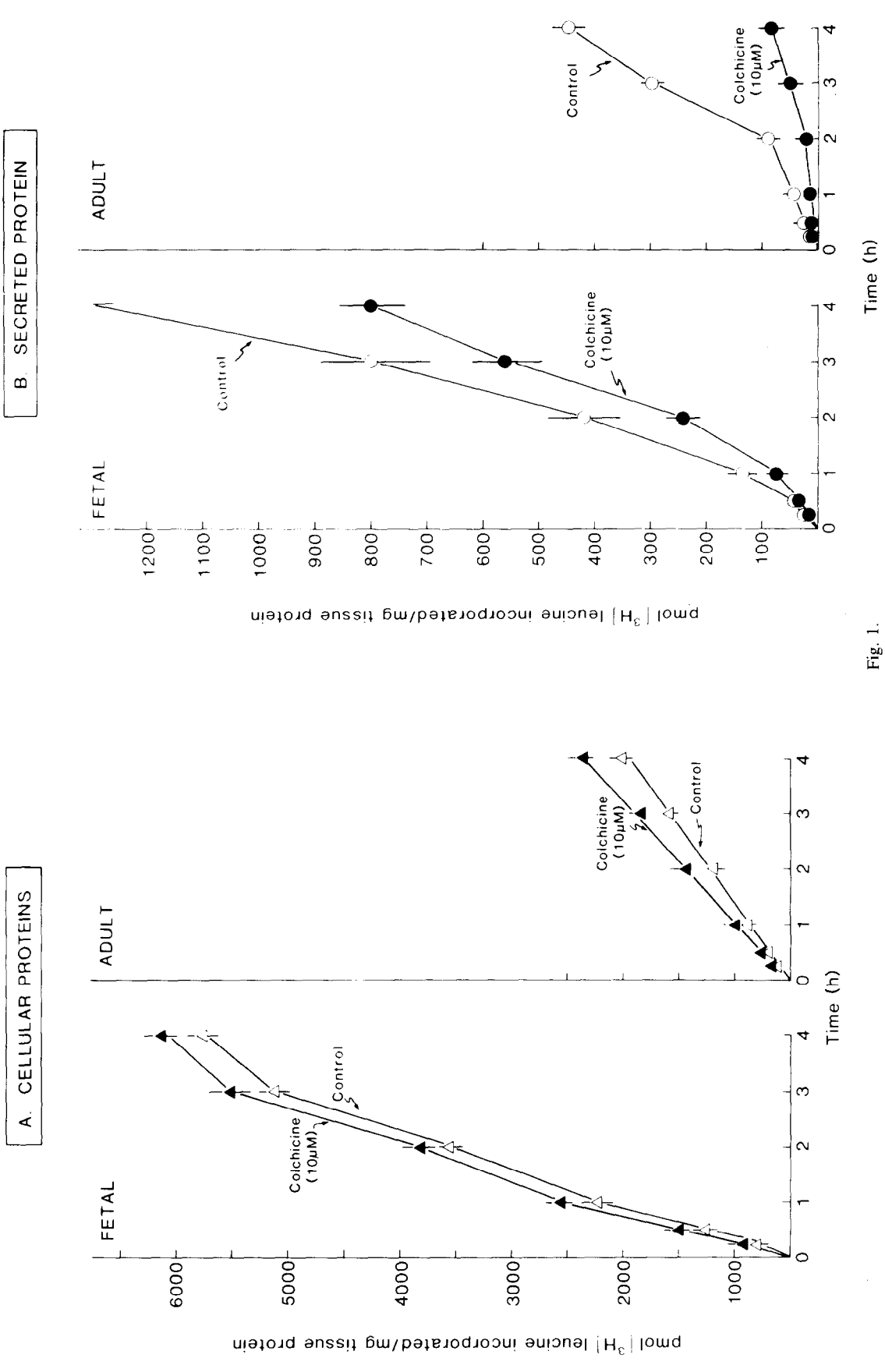
A. CELLULAR PROTEINS
FETAL
ADULT
Colchicine (10μM)
Control
pmol [3H] leucine incorporated/mg tissue protein
Time (h)
B. SECRETED PROTEIN
FETAL
ADULT
Control
Colchicine (10μM)
pmol [3H] leucine incorporated/mg tissue protein
Time (h)

Fig. 1.

C containing 0.3 M methyl-α-D-mannopyranoside (ConA-agarose) or 0.3 M *N*-acetyl-D-glucosamine (WGA-agarose). Pooled fractions of unadsorbed and adsorbed material (eluted with specific sugars [36, 37]) were subsequently processed for immunoprecipitation and quantitation of radioactive transferrin.

Radioactivity determination. The content of radioactivity in aqueous samples was determined by scintillation counting in Beta Phas (West Chem. Products). Dried glass fiber discs containing ^{3}H-, ^{14}C- or ^{35}S-labeled proteins were placed in scintillation vials and shaken with 1% SDS at 37° before addition of 10 ml of scintillation fluid. Radioactivity was determined in a Beckman LS-3801 scintillation spectrometer.

Assay of galactosyltransferase. Golgi galactosyltransferase activity was measured as described previously from this laboratory [6]. All assays were carried out under experimental conditions in which enzyme activity was linear with respect to enzyme protein concentration and incubation time.

Protein measurement. Protein content of various samples was determined by a modification of the procedure of Lowry *et al.* [38] as described by Markwell *et al.* [39].

Electron microscopy techniques. Livers of adult and neonatal rats were perfusion-fixed in order to obtain information on the *in vivo* content of hepatocyte Golgi complexes and associated microtubules. Livers of adult rats were flushed with 0.2 M cacodylate, then perfused (as previously described [4]) with 2% glutaraldehyde (in 0.15 M cacodylate buffer, pH 7.2, 22°) for 10 min, 2.2 ml/min. Neonatal rats were infused with buffer, followed by glutaraldehyde through the jugular vein (0.3 ml/min) for 10 min. After perfusion, the livers were excised, minced into fine pieces, and submerged in fixative overnight. Fixed and washed samples were postfixed for 1 hr in 1% osmium tetroxide (veronal buffer, pH 7.2, 22°) and stained *en bloc* for 45 min (2% uranyl acetate in veronal buffer, pH 5.5, 22°) before degradation in graded alcohols and embedment in Epon–Araldite plastic. Tissue blocks which showed portal areas were subsequently thin-sectioned and stained with uranyl acetate and lead citrate. Samples of liver slices (used in incubation experiments) were submersion-fixed with glutaraldehyde and processed as described above.

For assessment of Golgi complexes, ten nucleated hepatocytes chosen at random from one portal area of each of three rats were photographed at low magnification (2.7×) to accommodate the profile of the entire cell. These electron micrographs were photographically enlarged to 11,000× on 11 in. × 14 in. paper. Each Golgi region was identified on the print (with the aid of a magnifying lamp) and encircled with a colored pencil. To estimate the content of Golgi complexes in such cells, the ratio of cytoplasmic area occupied by Golgi complexes to total cytoplasm was measured in each cell using planimetric techniques (Bioquant Image Analysis System, R-M Biometrics, Nashville, TN).

To assess the relationship of microtubules to Golgi complexes [4], ten randomly selected nucleated hepatocytes from each animal were photographed at 16,000× at 12 and 6 o'clock positions relative to the nucleus; if no Golgi regions were present in these positions, two additional photographs of the cell were taken at 3 and 9 o'clock. These micrographs were enlarged photographically to 48,000×. Each Golgi region (Golgi cisternae and associated vesicles and vacuoles) was subsequently identified and encircled with a tight-fitting curve. A second line, separated from the first by 1 cm [4], was drawn following the contour of the first line. All microtubule lengths or cross-sections found within the total area enclosed by the second line were considered to be associated with the Golgi complex, and each microtubule segment was recorded as a single count regardless of its length. The number of microtubules counted was subsequently expressed as microtubule number per unit area Golgi cytoplasm.

RESULTS

Incorporation of [^{3}H]*leucine by liver tissue of immature and adult rats*

A series of experiments was carried out to examine the effect of colchicine given *in vitro* on the incorporation of [^{3}H]leucine into newly synthesized proteins and the secretion of these labeled proteins into the medium.

(1) [^{3}H]*Leucine incorporation into total protein.* Figure 1 shows the time curve of [^{3}H]leucine incorporation (± colchicine) into total tissue (1A) and secreted (1B) plasma proteins. In experiments without colchicine, incorporation of tritiated leucine into hepatic cellular proteins (1A) increased linearly with time. The rate of leucine incorporation was substantially greater in fetal than in adult liver. With liver from fetal rats, radioactivity appeared in the medium (1B) 15 min after exposure to the radioactive amino acids and increased in the medium in almost a linear manner with time over a 4-hr period. In contrast, in experiments with liver from adult rats, the appearance of radioactivity in the medium showed a distinct lag (60–90 min) before showing a linear increase (1B).

In the same experiments, the use of colchicine (10 μM) decreased the secretion of [^{3}H]leucine-labeled proteins and caused an equivalent increase in tissue-associated radioactivity (1B). The effect of colchicine on delaying the appearance of labeled

Fig. 1. Time-dependent incorporation of [^{3}H]leucine into cellular and secreted proteins in the presence and absence of colchicine. Liver slices were placed in 2.5 ml medium containing 0.1 mM leucine in the presence or absence of colchicine (10 μM). After preincubation for 30 min, 12.5 μCi of [^{3}H]leucine was added. At the time shown, the medium was removed and slices were homogenized in buffer A containing 0.5% sodium deoxycholate and Nonidet P-40. Homogenate and medium were precipitated with 10% trichloroacetic acid and processed for determination of radioactivity as described under Materials and Methods. Results are expressed as pmol radioactivity incorporated into cellular (A) or secreted (B) protein. Data represent the mean ± SE of four separate experiments.

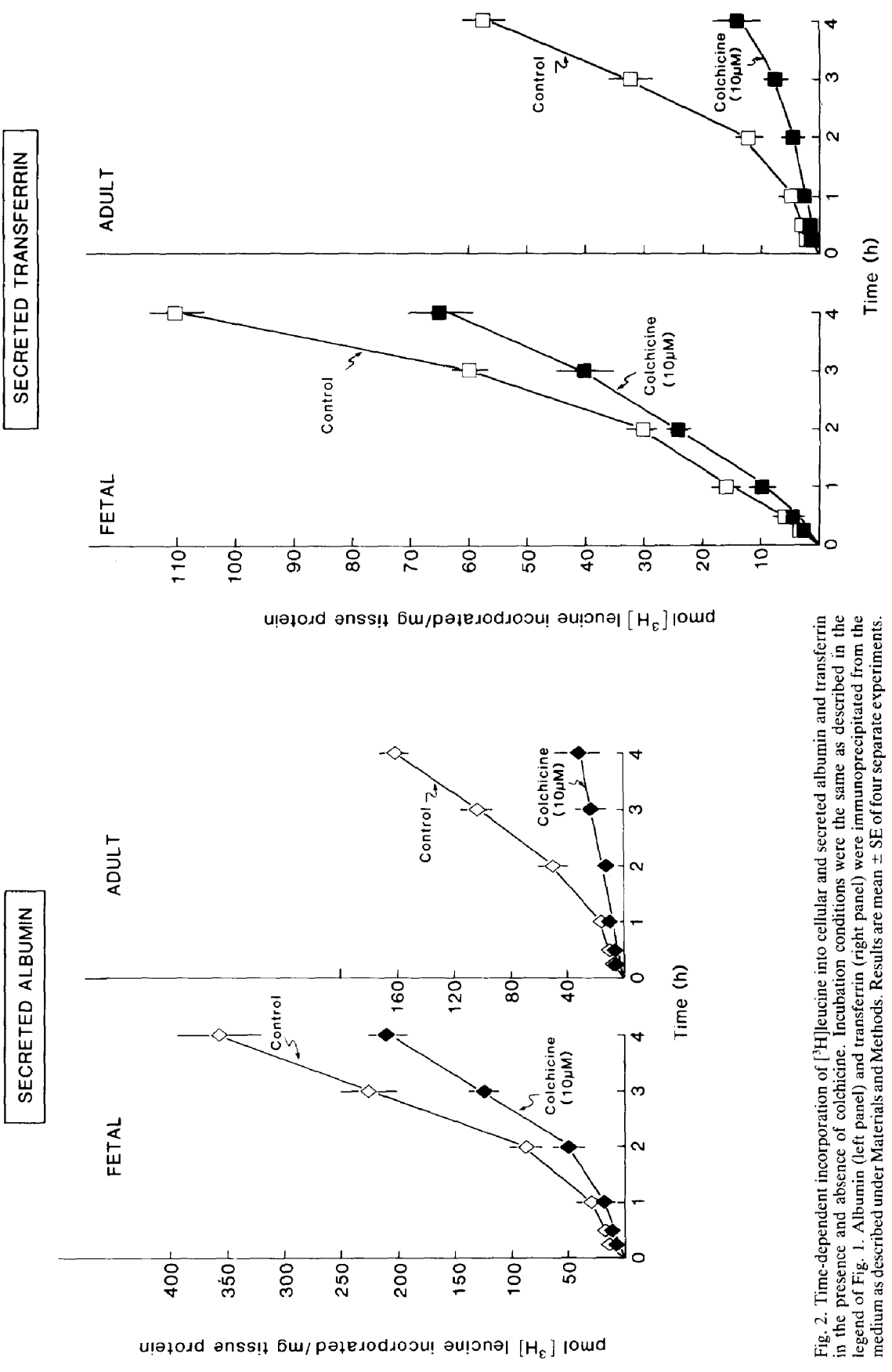

Fig. 2. Time-dependent incorporation of [^{3}H]leucine into cellular and secreted albumin and transferrin in the presence and absence of colchicine. Incubation conditions were the same as described in the legend of Fig. 1. Albumin (left panel) and transferrin (right panel) were immunoprecipitated from the medium as described under Materials and Methods. Results are mean ± SE of four separate experiments.

proteins in the medium was particularly pronounced in experiments with liver from adult animals: in these experiments colchicine treatment caused an 80–85% decrease in the amount of labeled protein which was secreted (in a 4-hr incubation period) as compared to only a 35–40% decrease in the amount of labeled protein secreted in similar experiments with liver from fetal rats (1B). However, colchicine at the concentration employed did not affect the total protein biosynthetic capacity of liver tissue from either fetal or adult rats, e.g. mean (± SEM) values for [^{3}H]leucine incorporated into total protein (slices + medium) from fetal rats with or without colchicine were 6954 (± 686) and 7024 (± 591) pmol/mg protein, whereas the values for samples from adult rats with or without colchicine were 1748 (± 146) and 1844 (± 131) pmol/mg protein respectively. For ease of presentation, subsequent figures will display only secretion data indicating the amounts of newly labeled proteins that were released by liver tissue under different conditions.

(2) [^{3}H]*Leucine incorporation into specific proteins.* To study the inhibitory effect of colchicine on the secretion of serum proteins in greater detail, experiments were carried out on two specific constituents of rat plasma proteins, transferrin and albumin. Liver slices from fetal and adult rats were labeled with [^{3}H]leucine as described above, and subsequently transferrin and albumin were immunoprecipitated from medium (or tissue homogenates) at different times ranging between 15 and 240 min. Figure 2 illustrates the increase of radioactivity found in both secreted proteins over a 4-hr incubation period. Although colchicine interfered with the secretion of albumin and transferrin in liver slices of both fetal and adult animals, the inhibition of secretion from adult tissues was substantially greater (80–85%) than that seen in the fetal tissues (~40%).

These results with colchicine are similar to those described for total protein secretion and suggest that liver tissue from fetal rats is less sensitive to the action of colchicine than is liver tissue of adult animals.

(3) *Specificity of colchicine effect.* To characterize further the unequal action of colchicine on liver slices from fetal and adult rats, additional experiments were conducted to determine the following: (a) the dose-dependent effect of colchicine on secretion of [^{3}H]leucine-labeled plasma proteins; (b) the effect of colchicine on secretion of proteins labeled with amino acids other than leucine; (c) the effect of colchicine on protein secretion from liver slices of rats on various ages; and (d) the effect of antimicrotubule agents, other than colchicine, on protein secretion from liver slices.

Figure 3 shows the effect of increasing concentrations of colchicine on the secretion of [^{3}H]leucine-labeled proteins during a 4-hr incubation period. Incubation of liver slices from adult rats with increasing concentrations of colchicine ranging from 0.1 to 50 μM led to a dose-dependent inhibition in protein secretion, reaching a plateau at 10 μM. The EC_{50} (the concentration required to produce half-maximal inhibition) was calculated to be 1.3 μM. A similar concentration–response curve for colchicine was observed when liver slices from fetal rats were substituted for those from adult animals. However, as shown before, colchicine was found to be less effective in inhibiting protein secretion in liver tissue from fetal rats, and a higher EC_{50} for colchicine (~3.5 μM) was observed in the fetal tissues.

This dissociation in colchicine action persisted when secretory proteins were radiolabeled with neutral (alanine), acidic (aspartic acid), basic (lysine), aromatic (phenylalanine), sulfur-containing (methionine) amino acids or imino acid (proline), instead of leucine, i.e. the differential ability of col-

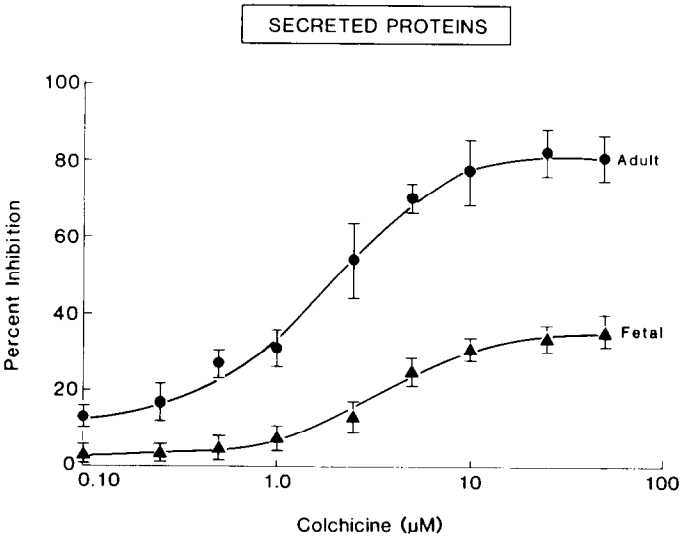

Fig. 3. Effect of various concentrations of colchicine on the secretion of [^{3}H]leucine-labeled proteins. Liver slices from fetal and adult rats were preincubated (30 min) with indicated concentrations of colchicine and then exposed to [^{3}H]leucine. After 4 hr, the medium containing secreted proteins was precipitated with trichloroacetic acid and measured for radioactivity. Results represent percent inhibition of secretion of total proteins and are mean ± SE of three experiments.

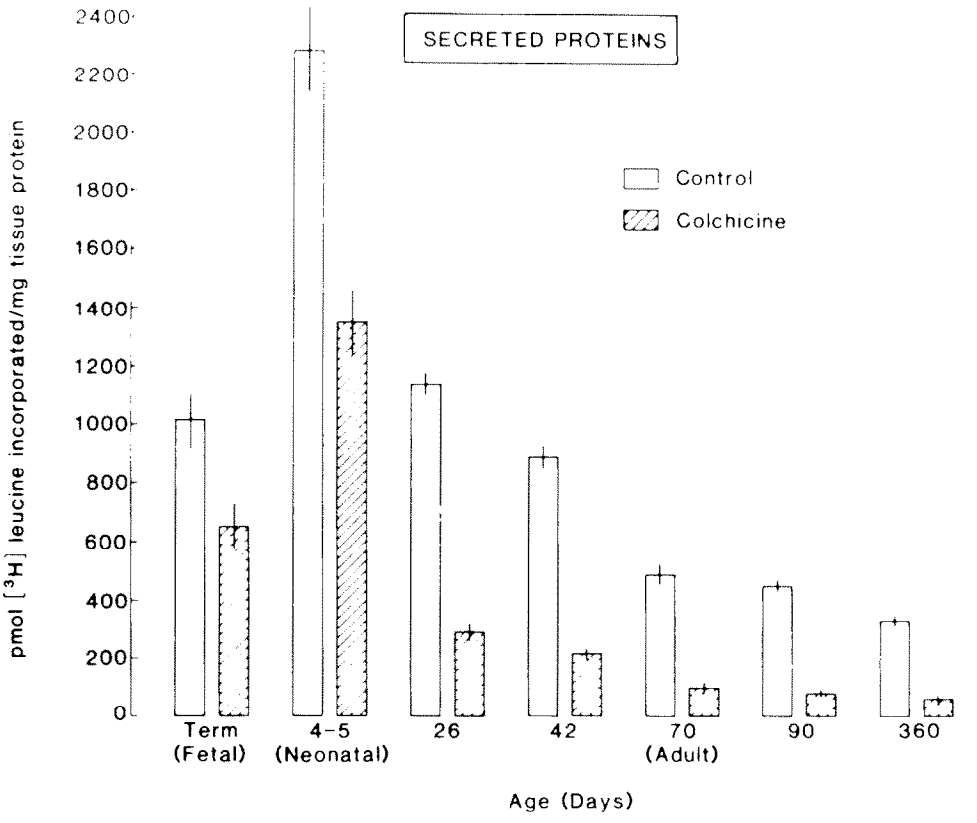

Fig. 4. Effect of colchicine on the secretion of [^{3}H]leucine-labeled proteins by liver slices from rats of different ages. Incubation conditions were the same as described in the legend of Fig. 1, except that liver slices were obtained from rats of different ages. Results are mean ± SE of five separate experiments. Incubations were carried out for 4 hr.

chicine to block protein secretion in fetal and adult liver was not confined to a specific type of radioactive amino acid used in labeling experiments (data not shown).

In another set of experiments, liver slices from rats of various ages were labeled with [^{3}H]leucine in the presence or absence of colchicine, and newly synthesized secreted proteins were measured as before. Figure 4 shows that, even without colchicine, large differences existed in the amount of newly synthesized proteins secreted by the liver samples from these rats. As noted earlier, liver from fetal rats incorporated and secreted more [^{3}H]leucine-labeled protein (per mg tissue protein) than did liver from adult (70 day) rats, and the data from Fig. 4 indicate that liver from neonatal rats was even more active in this regard than liver from fetal (term) animals. However, subsequently (as postnatal rats aged from 5 to 70 days), there was a stepwise decrease in the capacity of the liver to incorporate [^{3}H]leucine and secrete the labeled proteins. The effect of colchicine was to inhibit the release of ~35–40% of the newly synthesized proteins in fetal and neonatal rats; in tissues of older animals, regardless of age, this inhibition was in the range of 75–80%. Thus, liver from neonatal rats (like fetal rats) is relatively resistant to the action of colchicine when compared to liver from adult animals.

Table 1. Effects of various antimicrotubule drugs on the secretion of [^{3}H]leucine-labeled protein

	[^{3}H]Leucine incorporated (pmol/mg tissue protein)		
Antimicrotubule agents	Fetal	Neonatal	Adult
Control	1218 ± 146 (100)	2169 ± 240 (100)	473 ± 69 (100)
Podophyllotoxin (50 μM)	944 ± 63 (23)	1583 ± 192 (27)	206 ± 17 (66)
Nocadazole (25 μM)	738 ± 35 (39)	1432 ± 103 (34)	136 ± 3 (71)
Vinblastine (10 μM)	714 ± 38 (41)	1345 ± 98 (38)	148 ± 8 (68)
Colchicine (10 μM)	777 ± 41 (36)	1410 ± 116 (35)	102 ± 29 (79)
Lumicholchicine (10 μM)			480 ± 48 (100)

Results are mean ± SE of four separate experiments. Incubations were carried out for 4 hr. The numbers in parentheses represent percent inhibition due to the use of an antimicrotubule drug.

Table 1 shows that all the antimicrotubule agents used had the same basic action as colchicine on the livers tested, but podophyllotoxin (50 μM) was somewhat less effective than the other drugs in blocking the secretion of proteins. Lumicholchicine had no noticeable effect in these incubations.

Incorporation of ^{3}H-labeled sugars by proteins of liver tissue of immature and adult rats

We next examined whether the relative insensitivity to colchicine noted in immature liver could be demonstrated when the carbohydrate (instead of the amino acid) portion of the secreted protein was labeled. Figure 5 shows that liver slices of fetal rats incorporated ~4 times more glucosamine into total secreted proteins than did liver of adult rats (5A); similarly, liver of fetal rats incorporated ~4-fold more glucosamine into secreted transferrin (5B). Other data (not shown) indicate that the incorporation of other tritiated sugars (fucose, galactose and *N*-acetylmannosamine) followed the same pattern. Among the various sugars tested, the highest incorporation was observed with glucosamine, followed by sugars specific for terminal sugar residues such as *N*-acetyl mannosamine, (precursor or sialic acid [40]), galactose and, finally, fucose. The higher incorporation of [^{3}H]glucosamine into proteins was not surprising since it is a precursor for N-linked core sugars as well as a precursor for the terminal sugars, glucosamine and sialic acid [2]. The low fucose incorporation was probably due to the fact that it is quantitatively less significant in hepatic secretory proteins than the other sugars [41, 42].

To support the assumption that galactose, sialic acid and fucose are used exclusively as terminal sugars of *N*-asparagine-linked oligosaccharides, liver slices were pretreated with tunicamycin. This antibiotic has been utilized in the past as a specific inhibitor of *N*-asparagine-linked glycosylation [43, 44]. Thus, when N-glycosylation was blocked by tunicamycin treatment, no radioactive fucose, galactose or *N*-acetyl mannosamine was incorporated into secretory proteins or transferrin (data not shown). The lack of significant incorporation of sugars into albumin (a nonglycosylated protein) also excludes the possibility that sugar was metabolized *in vivo* into other compounds such as amino acids.

Effect of colchicine on ^{3}H-labeled sugar incorporation into secreted proteins. Figures 5 and 6 also show that colchicine interfered with the secretion of newly labeled glycoproteins (and transferrin) from liver slices of fetal, neonatal, and adult rats. However, unlike the situation with [^{3}H]leucine-labeled proteins, colchicine blocked the secretion of the [^{3}H]glucosamine-labeled proteins from liver slices of both types of immature liver and adult liver to an equal degree.

Table 2 gives data on the incorporation of several sugars (glucosamine, fucose and *N*-acetylmannosamine) ± colchicine into cellular, secreted and total (cellular + secreted) hepatic proteins of adult and fetal animals. These data make several points regarding the effect of colchicine on the tissues: (1) in each case, colchicine interfered with the secretion of the labeled glycoprotein by approximately the same percent (68–72%) in fetal and adult liver; (2) although the incorporation of radioactivity into cellular proteins was not especially changed by colchicine, incorporation of two of the sugars used (specifically fucose and *N*-acetylmannosamine) into total protein (cellular plus secreted) was reduced with the use of colchicine in both fetal and adult liver. This finding

Table 2. Effect of colchicine on ^{3}H-labeled sugar incorporation into cellular, secreted and total protein

Rats	^{3}H-Labeled sugar incorporated (pmol/mg tissue protein)		
	Cellular protein	Secreted protein	Total protein
		[^{3}H]Glucosamine	
Adult			
Control	253 ± 43	200 ± 28	453 ± 46
Colchicine	360 ± 14	62.7 ± 1.2 (69)	424 ± 15
Fetal			
Control	637 ± 42	783 ± 55	1363 ± 102
Colchicine (10 μM)	583 ± 67	248 ± 31 (68)	851 ± 134
		[^{3}H]Fucose	
Adult	28.4 ± 3.6	40.6 ± 3.6	69 ± 7.1
Colchicine (10 μM)	30.2 ± 4.4	13.1 ± 1.2 (68)	43.3 ± 4.1
Fetal			
Control	74.7 ± 3.2	164 ± 12	238 ± 13
Colchicine (10 μM)	52.2 ± 2.4	45.5 ± 4.3 (72)	48 ± 6
		N-Acetyl-[^{3}H]mannosamine	
Adult			
Control	209 ± 5	232 ± 23	432 ± 31
Colchicine (10 μM)	203 ± 14	66 ± 7 (72)	270 ± 26
Fetal			
Control	354 ± 28	678 ± 52	1032 ± 93
Colchicine (10 μM)	300 ± 30	214 ± 36 (68)	514 ± 42

Values are mean ± SE of four separate experiments. Incubations were carried out for 4 hr. The numbers in parentheses represent percent inhibition due to colchicine.

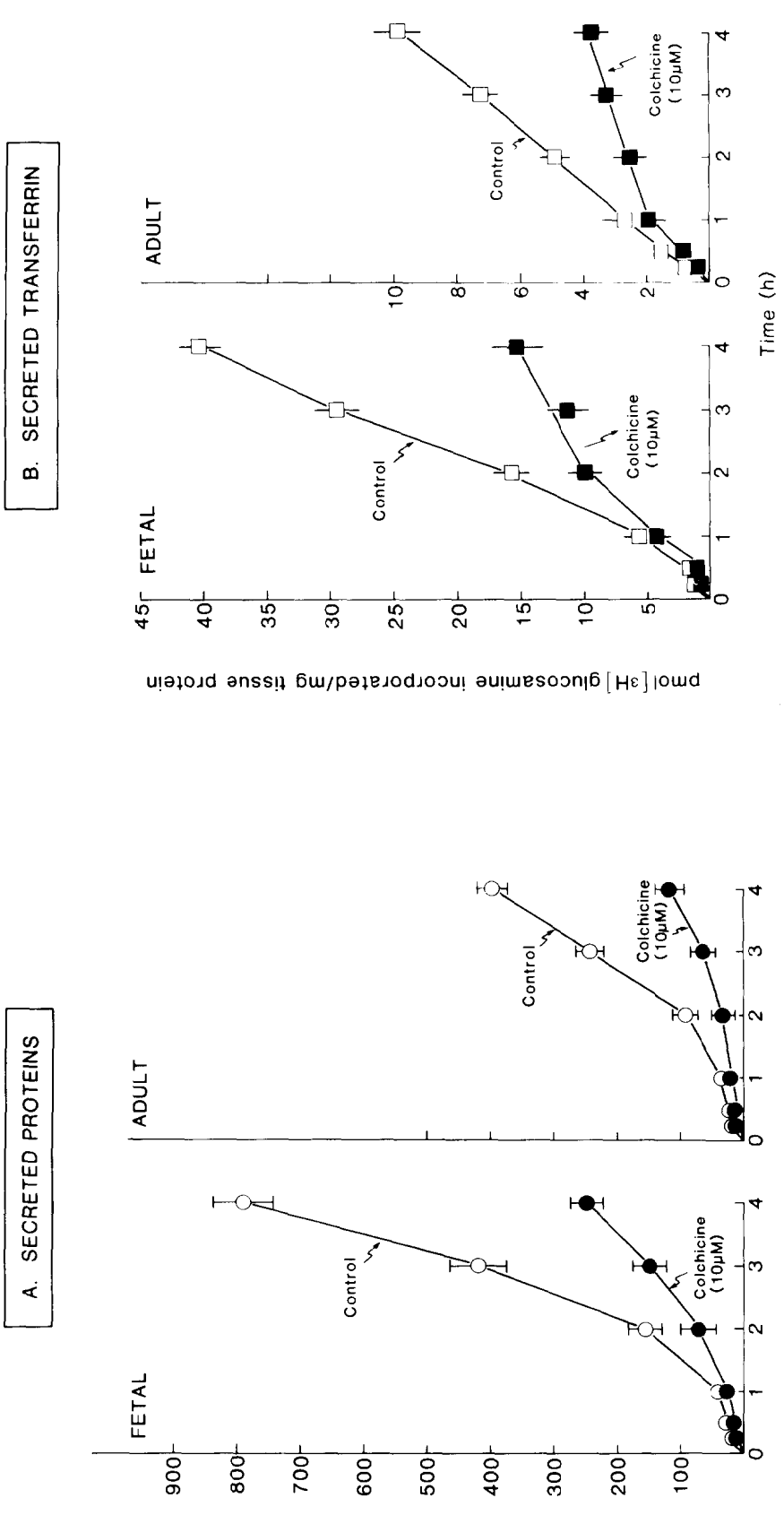

Fig. 5. Time-dependent incorporation of [^{3}H]glucosamine into secreted total proteins and transferrin in the presence and absence of colchicine. Liver slices were placed in 2.5 ml of modified medium without glucose, but containing 10 mM pyruvate with or without colchicine (10 μM). Following preincubation, 25 μCi [^{3}H]glucosamine (50 μM) was added. At indicated times, the medium and tissue samples were processed to determine radioactivity of secreted proteins (A) and transferrin (B). Transferrin was immunoprecipitated from medium as described under Materials and Methods. Results are mean ± SE of three experiments.

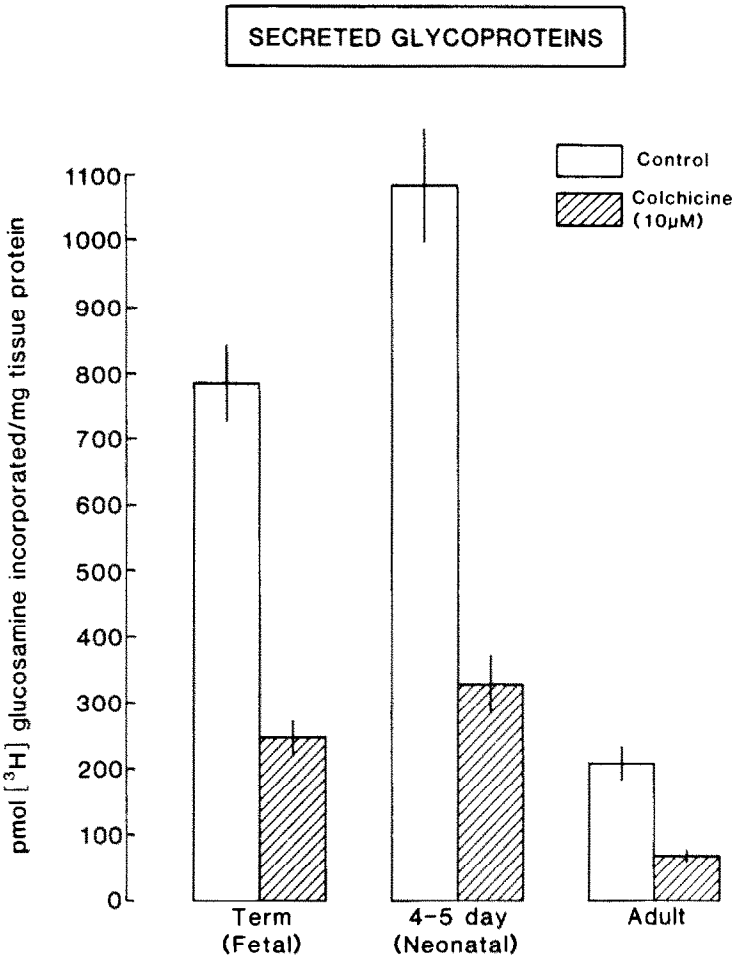

Fig. 6. Effect of colchicine on secretion of [^{3}H]glucosamine-labeled proteins from liver of fetal, neonatal and adult rats. Incubations were as described in the legend of Fig. 5 except that they were carried out for 4 hr only. Results include experiments from fetal, neonatal and adult rats (mean ± SE).

is consistent with previous information from this laboratory [6] showing that antimicrotubule drugs interfere with the activity of various Golgi-associated sugar transferases (N-linked terminal glycosyltransferases); (3) in contrast, colchicine did not interfere with the incorporation of a third sugar, glucosamine, into total protein of liver slices from adult rats. This finding is again consistent with our previous study [6] which shows that antimicrotubule drugs do not interfere with the activity of endoplasmic reticulum-associated sugar transferases (N-linked core glycosyltransferases). Insofar as glucosamine is used as a precursor for both N-linked oligosaccharide core and terminal sugar residues in liver tissue, it is not surprising that colchicine has less of an effect on total incorporation of this sugar compared to other sugars which are used exclusively in N-terminal glycosylation of proteins.

Table 3 summarizes all the data displayed earlier showing the differential action of colchicine on the secretion of total protein, albumin, and transferrin from slices of fetal and adult liver labeled with various amino acids and sugars. These data clearly show that the secretion of sugar-labeled proteins (total proteins or transferrin) was dramatically (and equally) inhibited when colchicine was used with liver samples of either fetal or adult liver. The secretion of amino acid-labeled proteins of adult tissues was similarly affected by colchicine, but amino acid-labeled proteins of fetal tissues (and neonatal tissues) were largely spared.

Lectin-agarose affinity chromatography of secreted transferrin

In these experiments we examined the possibility that there may be two pools of glycoprotein secretory proteins in the livers of immature rats. One form may be glycosylated and processed by the Golgi complex (and thereby sensitive to colchicine). The other form may not be processed through the Golgi complex and thus may not be a glycoprotein sensitive to colchicine. To test this idea experimentally, we made use of the known ability of plant lectins to bind glycoproteins with great specificity. Our expectation was that glycoproteins with incomplete terminal sugar groups would bind poorly to sugar specific lectins. For example, if fetal transferrin is a mixture of heterogeneous glycosylated forms, then only a

Table 3. Summary of effects of colchicine on total proteins, albumin and transferrin secretion by liver slices labeled with [^{3}H]leucine, [^{35}S]methionine or [^{3}H]sugars

Labeling agent used	Percent inhibition Fetal	Adult
Total proteins		
[^{3}H]Leucine	38	80
[^{35}S]Methionine	33	82
[^{3}H]Glucosamine	69	70
[^{3}H]Fucose	70	68
[^{3}H]Galactose	71	67
N-Acetyl-[^{3}H]mannosamine	69	70
Albumin		
[^{3}H]Leucine	37	76
[^{35}S]Methionine	35	79
Transferrin		
[^{3}H]Leucine	40	78
[^{35}S]Methionine	31	77
[^{3}H]Glucosamine	66	64
[^{3}H]Galactose	69	71
N-Acetyl-[^{3}H]mannosamine	67	69

Table 4. WGA-agarose column chromatography of transferrin

Labeling agents used	Percent not bound	Percent bound
[^{3}H]Leucine		
Fetal	58.7 ± 2.3	41.3 ± 2.3
Neonatal	58.0 ± 4.4	42.0 ± 4.4
Adult	16.0 ± 1.2	84.0 ± 1.2
N-Acetyl-[^{3}H]mannosamine		
Fetal	21.3 ± 2.0	78.7 ± 2.0
Neonatal	22.7 ± 1.8	77.3 ± 1.8
Adult	20.0 ± 1.2	80.0 ± 1.2

Preparations of secreted transferrin were cycled five times through WGA-agarose columns. The radioactivity in adsorbed (bound) and unadsorbed (not bound) fractions was determined by specific immunoprecipitation for transferrin. The results are mean ± SE of three experiments.

fraction (that which is terminally glycosylated) would bind to lectins specific for terminal sugars. We used two lectin columns (Con A-agarose specific for mannose residues [45–47] and WGA-agarose specific for glucosamine [45–48] and sialic acid [49, 50]) combined with immunoprecipitation to reveal a major molecular heterogeneity of the transferrin secreted from liver slices of fetal and neonatal rats. After 3-hr incubations with [^{3}H]leucine, the dialyzed incubation medium was cycled through ConA-agarose and WGA-agarose columns, and the resulting unbound and eluted (bound) fractions were immunoprecipitated with antibodies to transferrin. Since ConA-agarose bound 70–80% of the applied transferrin from all samples (data not shown), it was concluded that all preparations had comparable mannose residues (a constituent of core sugars catalyzed by mannosyltransferase present in endoplasmic reticulum). In contrast, WGA-agarose revealed significant differences in the ability of secreted transferrin from the different livers to interact with this lectin, which is specific for *N*-acetylglucosamine and sensitive to sialic acid residues (terminal sugars added during passage of proteins through Golgi complexes). As can be seen (Table 4), more transferrin of adult liver origin bound to the WGA-agarose column than that of fetal or neonatal origin; likewise, less of the transferrin from adult liver eluted without being adsorbed.

From these experiments we concluded that transferrin from adult liver contained mainly completed glycosylated molecules which bind to both lectin columns. On the other hand, transferrin from neonatal and fetal liver was either partially glycosylated with poor affinity for the WGA-lectin column, or existed as two molecular forms, one of which was completely glycosylated and the other not glycosylated. This observation was further corroborated by incorporation experiments with *N*-acetyl mannosamine, a precursor for sialic acid. When *N*-acetylmannosamine was used to label transferrin, a comparable percentage of label was retained by the WGA-agarose column irrespective of the source of transferrin (Table 4). Thus, fractions of fetal or neonatal transferrin processed through Golgi complexes do acquire all necessary terminal sugars and are, in turn, transformed into molecules effectively retained by WGA.

Endo-β-N-acetylglucosaminidase H (endoglycosidase H) treatment of immunoprecipitated intracellular transferrin

To assess differences in Golgi-mediated transferrin glycosylation, [^{35}S]methionine-labeled, immunoprecipitated transferrin from liver slices of neonatal and adult rats was subjected to endoglycosidase H digestion. This enzyme cleaves the GlcNAC-$\beta \rightarrow 4$ GlcNAC bond in Asn-linked oligosaccharides [15, 51], and is specific for glycoproteins of "high mannose" form (four or more mannose residues), i.e. the form present prior to terminal glycosylation in the Golgi complex [15]. These experiments revealed that radiolabeled transferrin from liver of both neonatal and adult animals was fully sensitive to endoglycosidase H action even after 120 min of incubation in the presence of excess, unlabeled methionine (data not shown). Thus, fully processed intracellular transferrin from both neonatal and adult rats probably retains high-mannose type oligosaccharide chains.

Studies to determine the function and availability of Golgi complexes for processing glycoproteins in liver of immature rats

The fact that transferrin secreted from liver slices of immature rats was only partially glycosylated suggested that hepatocytes of fetal or neonatal animals may not yet have sufficient or completely functional Golgi complexes to complete the glycosylation process of the synthesized glycoproteins.

To examine this question, liver homogenates and Golgi (GF_2) membranes were isolated from fetal and adult animals and examined for galactosyltransferase activity. The results of this experiment are shown in Table 5 and indicate that, when galactosyltransferase

Table 5. Terminal galactosyltransferase activity in liver fractions

Rats	Specific activity*	Relative specific activity†
Fetal		
Homogenate	339 ± 46	1
Golgi fraction (GF_2)	23700 ± 2100	70
Adult		
Homogenate	148 ± 24	1
Golgi fraction (GF_2)	8960 ± 623	61

* Specific activity is expressed as pmol [^{3}H]galactose transferred to ovomucoid·min^{-1}·(mg protein)$^{-1}$. Results are mean ± SE of four separate experiments.

† Relative specific activity is defined as the percentage of total recovered activity present in the individual fraction divided by the percentage of total recovered protein present in that fraction.

activity is expressed per mg tissue protein, fetal tissue has between 2- to 3-fold the galactosyltransferase activity of adult liver. The fact that this transferase activity was present in isolated Golgi (GF_2) membranes, as well as in tissue homogenate, suggests that the intracellular localization of the enzyme in liver from both fetal and adult animals is similar [6].

To obtain information on the content of Golgi complexes in livers of immature rats, we examined hepatocytes of perfusion-fixed livers of untreated neonatal rats and determined whether such cells had normal appearing and normal numbers of Golgi complexes. Livers from neonatal rather than fetal rats were chosen for this purpose since the neonatal liver more closely approximates the adult liver ultrastructurally, and the action of colchicine on secretion was similar in both groups of immature animals (Fig. 4). Panels A and B of Fig. 7 show representative electron micrographs of the neonatal and adult hepatocytes examined. Table 6 presents quantitative information from such micrographs and shows that the Golgi complexes were abundant in hepatocytes of neonatal rats, as was the number of microtubules associated with those complexes. It should be pointed out that not only were Golgi complexes plentiful in hepatocytes of neonatal animals, but they appeared highly developed (with prominently stacked cisternal membranes and a profusion of associated small vesicles). In other studies, liver slices of neonatal and adult rats were incubated with colchicine (10 μM) and subsequently examined to determine whether the microtubules of the tissues were equally sensitive to the action of colchicine. No differences were found in the colchicine-sensitivity of hepatocyte microtubules of neonatal and adult rats, i.e. essentially no microtubules remained in liver slices of either group of animals incubated for more than 1 hr at 37° with 10 μM colchicine (data not shown).

DISCUSSION

The goal of this study was to understand the differential effect of antimicrotubule agents, like colchicine, on hepatic protein and glycoprotein secretion in fetal, neonatal and adult rats, and to correlate this effect with possible changes in Golgi structure and function in hepatocytes from these respective animals. The results can be summarized as follows: (1) In experiments with adult liver, colchicine (and other antimicrotubule agents) inhibited the release (~70–80%) of newly synthesized proteins radiolabeled with amino acids. These results are similar to many previously published studies [1–3, 8, 9]. (2) In experiments with fetal or neonatal liver, the effect of colchicine was much less potent, and it inhibited the release of amino acid labeled proteins to an extent of only 30–40%. These results are identical to those published recently by Kaufman *et al.* [8, 9]. (3) However, this differential response of tissues to colchicine disappeared when the same hepatic proteins were labeled with sugars (rather than amino acids) during synthesis. In this case, secreted proteins from liver were equally inhibited (~70%) by colchicine, regardless of whether experiments were conducted with liver from adult, fetal or neonatal rats. This finding differs from that published before [9], but insofar as it occurs with total proteins, as well as a specific glycoprotein (transferrin), and holds true regardless of which sugar is used for labeling (glucosamine, fucose, galactose or *N*-acetyl mannosamine), it becomes difficult to ignore. (4) In characterizing the lectin specificity of secreted transferrin from liver slices of fetal and neonatal rats, we found a surprisingly high percentage (52–58%) of the protein lacked N-terminal sugars as compared to transferrin secreted by liver slices from adult animals (14–18%).

Table 6. Golgi complexes and number of associated microtubules in hepatocytes of neonatal and adult rats

Rats	Percent of hepatocyte cytoplasm occupied by Golgi complexes*	Number of microtubules counted per Golgi† (microtubules/μm^2 Golgi area)
Neonatal	3.7 ± 0.4	1.57 ± 0.2
Adult	3.4 ± 0.5	0.66 ± 0.8

* All visible Golgi complexes in randomly obtained electron micrographs of hepatocytes were outlined, and subsequently subjected to morphometric procedures as described in Materials and Methods (mean ± SE).

† Number of longitudinal or cross-sectional segments of microtubules associated with Golgi complexes as defined in Materials and Methods (mean ± SE).

Unexpectedly then, we found it was not the response to antimicrotubule agents, but rather the composition of the proteins themselves, which differed in liver from immature and adult animals. In this situation, the first question that comes to mind is whether Golgi development is incomplete in hepatocytes of the immature animals, resulting in the secretion of unfinished glycoproteins. In adult liver, it has been shown repeatedly that N-linked glucosamine residues are added to secretory proteins in the endoplasmic reticulum, as well as in Golgi complexes, but that N-linked terminal sugars (*N*-acetyl glucosamine, galactose, fucose and sialic acid) are added to proteins only as they progress through Golgi complexes [10–16]. Thus, if secreted transferrin from immature liver contains appropriate oligosaccharide core sugars, but lacks the N-linked terminal sugars found in mature transferrin, one must consider the possibility that the proteins are not being processed by Golgi-associated enzymes. It seems, however, that hepatocytes from neonatal rats have structurally well developed Golgi complexes; indeed, the percentage of hepatocyte cytoplasmic volume occupied by these organelles was equal to, or greater than, that found in adult liver. Moreover, Golgi-associated hepatic N-linked terminal galactosyltransferase activity of both fetal and neonatal rats was two to three times more active than in adult liver. These findings regarding the maturity of Golgi structure and function are supported by other data showing that large quantities of fully glycosylated proteins (i.e. labeled with N-linked terminal sugars) are, in fact, secreted by the livers of the immature rats, e.g. Table 2 indicates that, when considered on a tissue protein basis, liver slices from fetal rats secreted several-fold more newly synthesized proteins radiolabeled with N-linked galactose, fucose or *N*-acetyl mannosamine, than did liver samples from adult rats.

Given this evidence that Golgi function is probably

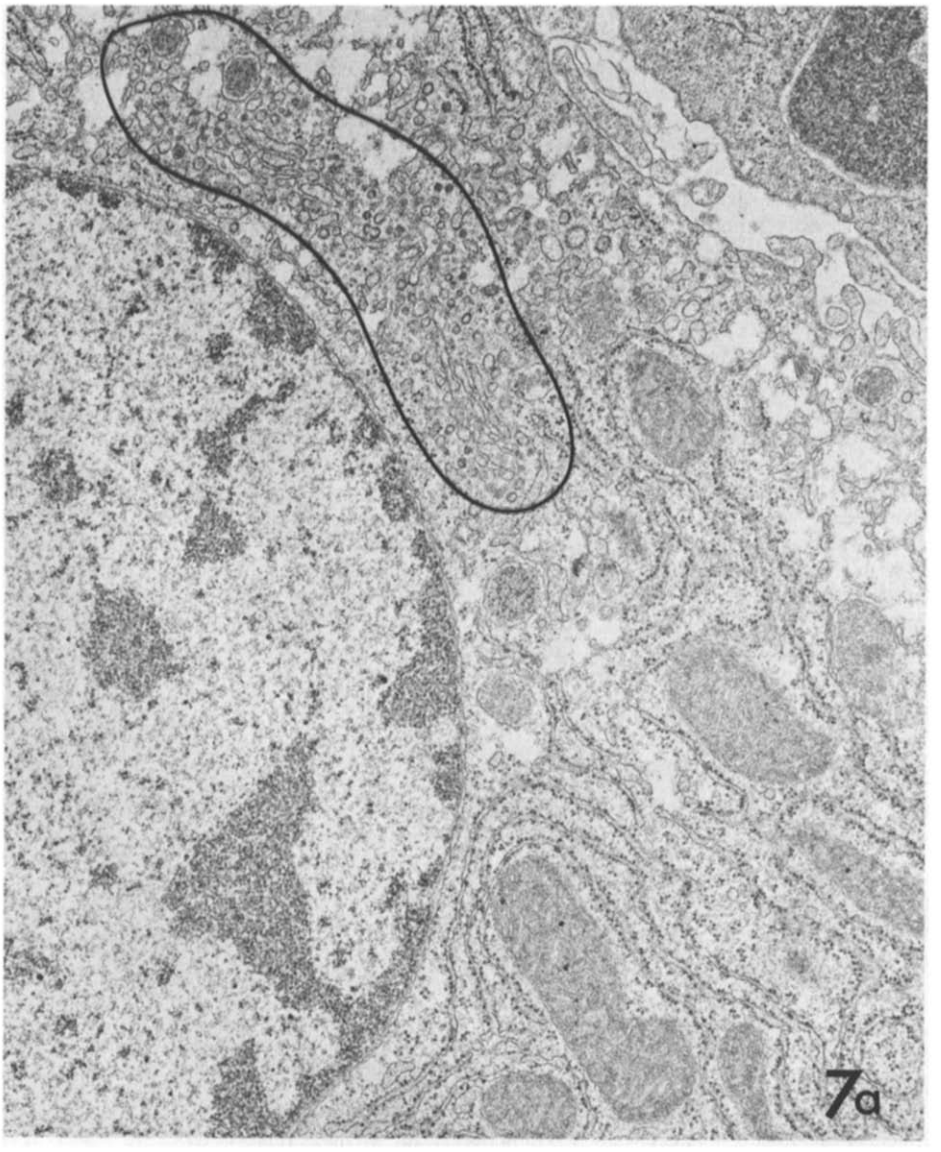

Fig. 7(a).

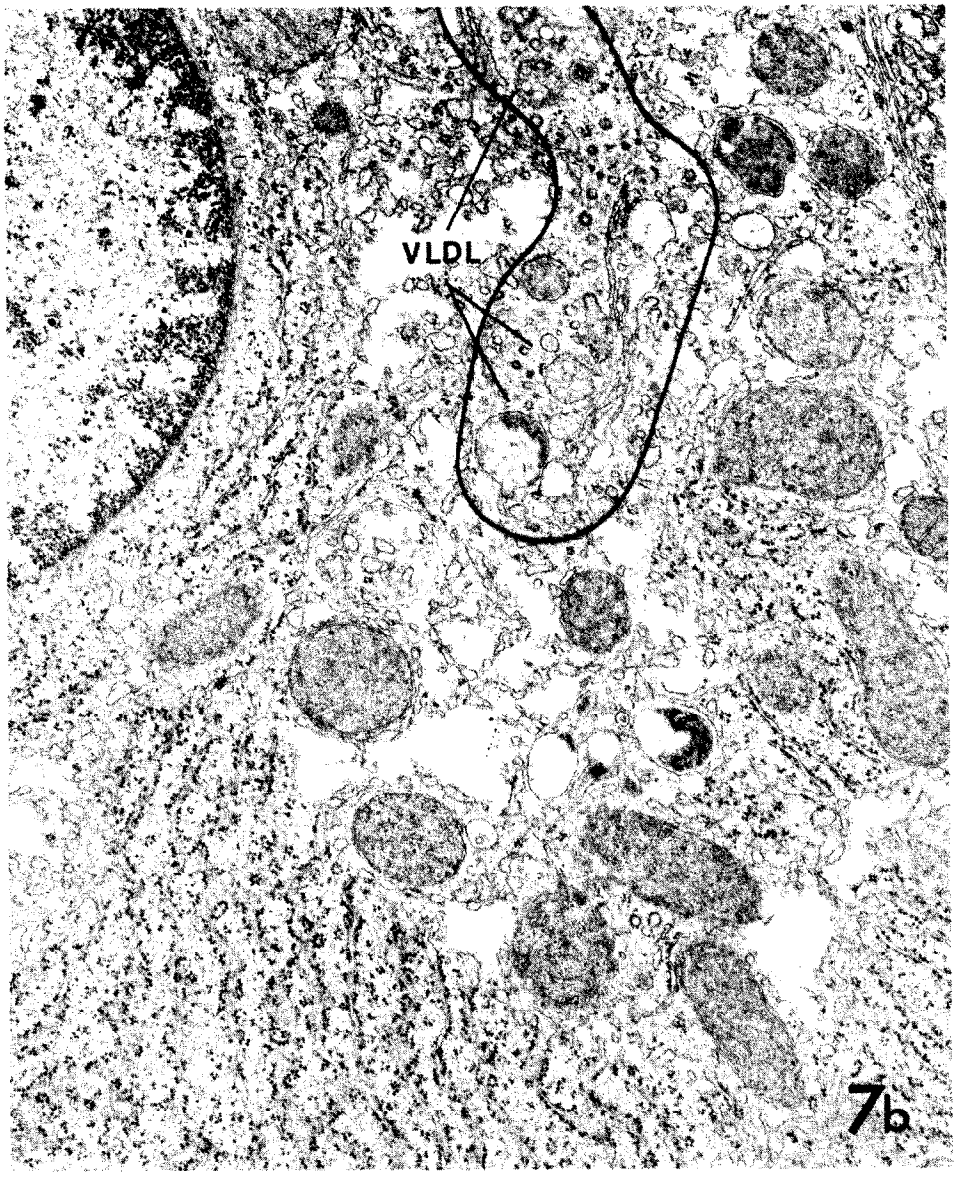

Fig. 7. Hepatocyte ultrastructure. Electron micrographs of hepatocytes from *in vivo* perfused livers of neonatal (A) and adult (B) rats showing representative Golgi complexes (encircled). Note that Golgi-associated vacuoles in cells from adult rat contain very low density lipoproteins (VLDL) not found in cells from neonatal animals. Magnification: 25,000×.

well developed in fetal and neonatal liver, and that liver tissue from these animals does secrete fully glycosylated proteins, how is it that such a relatively large percentage of a secreted glycoprotein, like transferrin, lacks terminal sugar residues? One explanation may be that the requirement for plasma proteins is so great in the developing organism that the amount of protein traffic flowing through the Golgi (or the speed of this flow) exceeds the capacity of the Golgi complexes to process the proteins at any given moment in time and the proteins escape without N-linked terminal sugars. (The fact that fetal and neonatal liver incorporated three and four times more labeled amino acids per weight tissue than did adult liver is consistent with this possibility.) Perhaps the Golgi transferases, or the cellular pool of sugar residues, are not sufficient, or correctly situated in the Golgi complex, to handle an excessive flow of protein [40]. Indeed, there is no guarantee that all aspects of the immature Golgi complex are operating at maximal capacity *in vivo* even if the appropriate biochemical and structural components are present. Another possibility is that some of the proteins leaving the endoplasmic reticulum are misdirected and somehow manage to bypass the Golgi membranes altogether. That hepatic secreted protein may bypass processing by Golgi membranes has been shown previously with albumin [27]. It is also clear that lack of terminal glycosylation does not, by itself, interfere with the secretion of cellular proteins [28, 52–54].

Were this latter situation to occur (i.e. that some proteins bypass Golgi processing), then the differential response by immature and adult liver to the effects of antimicrotubule drugs could be explained logically. As shown previously [1–3], colchicine and other antimicrotubule agents directly inhibit the secretion of Golgi-derived vesicles and, in addition, cause a number of changes in Golgi structure [4, 5] and function. These changes include a decline in the activity of sugar transferases involved in the N-linked terminal glycosylation of proteins [6]. On the other hand, the glycosylation of the core oligosaccharide chain of proteins is not affected by colchicine [6]. As such, proteins fully processed by Golgi complexes might be uniquely sensitive to the actions of the various antimicrotubule agents, and proteins and glycoproteins which bypass Golgi processing could well be protected. Therefore if, in fetal and neonatal rats, a proportionately larger share of total secreted protein is not transported through Golgi membranes, these proteins may avoid the inhibitory effects of colchicine. Due to the multiple sites where colchicine appears to act in Golgi complexes [3, 4, 6, 7], different proteins may be affected at different sites, e.g. nonglycosylated proteins may be affected at the level of Golgi-derived exit vesicles, whereas proteins undergoing glycosylation may be affected earlier in the sequence. Clearly, this explanation for the colchicine findings will have to be examined carefully by additional studies.

Apropos of this, our attempt to resolve the question with the use of endoglycosidase H was not practical since hepatocyte-processed transferrin from both adult and neonatal rats retains enough mannose residues to remain endoglycosidase H sensitive [55, 56]. Another concern has to do with the fact that this study used tissues from animals of diverse ages: it is always possible that structural changes in the tissues, unrelated to the question being examined, could explain the results. In this regard, it should be noted that the response of neonatal and fetal liver to the antimicrotubule agents was the same despite the fact that neonatal liver was structurally more similar to adult liver. Additionally, all aspects of the current study had built-in tissue controls, the most important being that the effect of colchicine on fetal and neonatal liver differs from adult depending on whether the labeling agent is an amino acid or a sugar. If colchicine were to have interfered with protein secretion because of some age-related tissue change (e.g. the presence of hemopoietic cells in the fetal liver), the nature of the precursor could not have resulted in different findings.

It is important to note that, since glucosamine is used both in N-linked core and terminal glycosylation of proteins in liver [14, 41, 42], results with the use of this sugar are not specific for the Golgi apparatus. We believe this may explain the differences between our results and those described by Kaufman *et al.* [9]. These authors used only glucosamine to label glycoproteins in their study, and it is possible that results obtained under the conditions of their particular experiments with glucosamine may have represented mainly oligosaccharide core glycosylation of proteins which appears to be insensitive to the action of antimicrotubule drugs.

Although the relationship of microtubules to the described Golgi-associated events is not necessarily clarified by the current experiments, it is difficult not to consider the role of microtubules when antimicrotubule agents are used. As in many previous studies [1, 4, 6–8], all the antimicrotubule agents used in this study produced the same results, despite the dissimilar structures [57, 58] of the drugs, themselves. It is worth noting also that the Golgi complexes in neonatal liver are associated with relatively more assembled microtubules than are Golgi complexes in adult liver, and that colchicine causes the disappearance of these visible microtubules under the experimental conditions used. We assume there is a link somewhere between these microtubules (or microtubule-protein) and the Golgi complex of cells, which is important to Golgi function—a link which is destroyed when antimicrotubule agents are used. The nature of this relationship has yet to be determined.

In all, we present evidence that fetal and neonatal rats are capable of synthesizing and fully glycosylating hepatic plasma proteins, and that the secretion of these proteins (believed to be processed by the Golgi apparatus of hepatocytes) is sensitive to the action of various antimicrotubule agents (as is the secretion of similar proteins from adult rats). However, some hepatic proteins and glycoproteins of fetal and neonatal rats appear to bypass critical processing sites in Golgi complexes. We suggest that these differently processed proteins are relatively insensitive to the action of antimicrotubule drugs and account for the differential effect that antimicrotubule agents have on hepatic protein secretion in immature and adult rats.

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