Effect of antimicrotubule agents on terminal glycosyltransferases and other enzymes associated with rat liver subcellular fractions

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Previous studies have shown that anti-microtubule agents disrupt Golgi complexes in hepatocytes and other cells, causing breakdown or vesiculation of Golgi cisternal membranes. Whether this change in the structure of the Golgi membranes is associated with changes in Golgi membrane function is not known. The present study was initiated to investigate this issue; i.e., to determine whether anti-microtubule agents that cause structural changes in Golgi membranes *in vivo* would, at the same time, affect characteristic enzyme functions of Golgi membranes. To this end, colchicine was given to young rats *in vivo* and various hepatic subcellular membranes were subsequently isolated and utilized for enzyme assays. Initially it was shown that colchicine (2.5 mg/kg body wt.) given for 5 h significantly decreased the activities of the Golgi-membrane-associated enzymes galactosyl-, sialyl- and *N*-acetylglucosaminyl-transferases. More detailed experiments indicated that low doses of colchicine (0.8 mg/kg body wt.), although less effective than higher doses, decreased the activities of the terminal glycosylating enzymes maximally at 5 h, with partial and complete recovery at 12 and 24 h respectively. Treatment *in vivo* of rats with vinblastine (20 mg/kg body wt.) for 5 h mimicked the action of colchicine. Two microsomal glycosylating enzymes (mannosyl- and *N*-acetylglucosaminyl-transferases) were unaffected by the treatment with colchicine, as were various hepatic 'marker' enzymes such as 5'-nucleotidase, glucose 6-phosphatase and succinate: 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase (succinate dehydrogenase; EC 1.3.99.1), which were found to be enriched in plasma-membrane, endoplasmic-reticulum and mitochondrial-membrane fractions respectively. These results show that anti-microtubule agents specifically suppress the activity of Golgi-associated glycosyltransferases in liver. Although it seems likely that these changes are related to the previously observed structural changes in hepatocyte Golgi complexes after colchicine treatment, to what extent the results are linked to the interaction of colchicine with microtubule protein remains to be clarified.

It is well known that antimicrotubule agents block the release of secretory products in many different cell types (Obberghen *et al.*, 1974; Stein *et al.*, 1974; Chertow *et al.*, 1975; Kemper *et al.*, 1975; LeMarchand *et al.*, 1975; Poisner & Cooke, 1975; Redman *et al.*, 1975; Banerjee *et al.*, 1976; Williams, 1976; Reaven & Reaven, 1980) and that these products accumulate in the cells within vesicles derived from

Golgi complexes. The prevailing view is that the block occurs at some site distal to the processing of the secretory product by the Golgi membranes (Banerjee *et al.*, 1976). It is noteworthy, however, that the structure of the Golgi complexes of many cells is itself extremely sensitive to the action of anti-microtubule drugs (Moskalewski *et al.*, 1975; Seybold *et al.*, 1975; Hindelang-Gertner *et al.*, 1976; Hoffstein *et al.*, 1977; Patzelt *et al.*, 1977; Reaven & Reaven, 1977, 1980; DeBrabander *et al.*, 1978; Knudson *et al.*, 1978; Stock *et al.*, 1978). Two types of morphological changes have been described. The

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first type of change involves the drug-induced topographical redistribution of Golgi complexes within the cytoplasm of cells (Moskalewski *et al.*, 1975, 1976; Hoffstein *et al.*, 1977; Reaven & Reaven, 1977; DeBrabander *et al.*, 1978; Knudson *et al.*, 1978; Stock *et al.*, 1978). The second observed change involves the alteration of the most characteristic structural feature of Golgi complexes, their stacked cisternal membranes. After treatment of cells with colchicine, vinblastine or $^2\text{H}_2\text{O}$, the number of Golgi cisternal membranes per Golgi complex is found to be markedly reduced (Moskalewski *et al.*, 1975, 1976; Seybold *et al.*, 1975; Hindelang-Gertner *et al.*, 1976; Reaven & Reaven, 1980); indeed, our recent quantification of this phenomenon in hepatocytes suggests that approx. 80% of Golgi cisternal membranes are disrupted after treatment of animals with colchicine (Reaven & Reaven, 1980).

Despite the frequency of these observations, it is not yet clear whether the drug-induced relocation of Golgi complexes or the 'disruption' or 'vesiculation' of Golgi cisternal membranes in any way alters the function of the Golgi complexes in cells. The Golgi apparatus is known to be a plastic changeable organelle with no uniform shape, and it is not certain what is its most active form; there are, in fact, well-documented instances where the stacked Golgi membranes are reduced in number (used up?) when the cells are in their most active stage of processing secretory products (Friedman & Cardell, 1977). Also, vesiculation of membranes (as may occur after treatment of tissues with anti-microtubule drugs) does not by itself imply loss of function, as is clear from the many enzyme studies that are routinely carried out on homogenized and sonicated preparations of membranes. On the other hand, one cannot be certain in any given instance that vesiculation of membranes is a benign event, and it is possible that certain Golgi-associated functions may be altered after the disruption of Golgi complexes with anti-microtubule drugs. Were this to be the case, one might reasonably question whether or not the anti-microtubule-drug-induced block in secretion was a function of improper processing of the secretory proteins (or their limiting membranes) by the affected Golgi structures.

The current study represents the first of several studies to examine this possibility; in particular, it seeks to determine if anti-microtubule agents, which seem to alter specifically the organization of Golgi membranes, also affect a specific function of these membranes. The primary objective of the study has been to determine the effect of colchicine and vinblastine administered *in vivo* on the activity *in vitro* of terminal glycosyltransferases (galactosyl-, *N*-acetylglucosaminyl- and sialyl-transferases) thought to be located exclusively in Golgi mem-

branes (Bretz *et al.*, 1980). The decision to use an '*in vitro*' approach was based on our desire to distinguish between drug-related glycosylation and secretory events, i.e., to exclude whatever effects a primary or secondary block in secretion might impose on the interpretation of the glycosylation data.

The results show that the concentration of anti-microtubule agents that induces ultrastructural changes in hepatocyte Golgi complexes also affects hepatic Golgi-associated terminal glycosyltransferases. In contrast, core glycosyltransferases and hepatic 'marker' enzymes associated with other non-Golgi membranes of liver cells are not affected.

Experimental procedures

Materials

UDP-D-[U- ^{14}C]galactose (sp. radioactivity 305 Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-*N*-acetyl-D-[U- ^{14}C]glucosamine (sp. radioactivity 207 Ci/mol), GDP-[U- ^{14}C]mannose (sp. radioactivity 269 Ci/mol), UDP-*N*-acetyl-D-[6- ^3H]glucosamine (sp. radioactivity 6.6 Ci/mol), CMP-[4,5,6,7,8,9- ^{14}C]sialic acid (sp. radioactivity 302 Ci/mol) and [*ring C* methoxy- ^3H]colchicine (sp. radioactivity 19.6 Ci/mol) were obtained from New England Nuclear, Boston, MA, U.S.A.

Treatment of rats and isolation of various membrane subcellular fractions

Groups of three male Sprague-Dawley rats (250–280 g; Simonsen Laboratories, Gilroy, CA, U.S.A.) were injected intravenously under light diethyl ether anaesthesia with freshly prepared colchicine (usually 2.5 mg/kg body wt.) in normal saline (0.9% NaCl). Control groups of rats received an equal volume of normal saline. The animals had free access to water and, unless otherwise stated, were killed 5 h after colchicine injection.

The livers were quickly removed, placed in 0.25 M-sucrose at 4°C and immediately processed for the isolation of Golgi and smooth- and rough-endoplasmic reticulum fractions by a slightly modified procedure of Ehrenreich *et al.* (1973) and Bergeron *et al.* (1978). The 105 000 g pellet (referred to as 'starting microsomal fraction') was used as the source of the various Golgi fractions. The starting microsomal fraction was suspended in 6.5 ml of 0.25 M-sucrose (equivalent to 10 g of liver) and 8 ml of 2 M-sucrose was added to achieve a final sucrose concentration of 1.22 M. A portion (14 ml) of this fraction was subsequently overlaid with 8 ml of 1.05 M-, 14 ml of 0.86 M-, 14 ml of 0.6 M- and 9.5 ml of 0.25 M-sucrose solutions and the tubes were centrifuged in a Beckman SW 25.2 rotor at 76 900 g_{av} for 3 h. Three Golgi fractions, referred to as 'GF1' (light), 'GF2' (intermediate) and 'GF3' (heavy), were

collected, diluted to 0.25 M-sucrose and resedimented at 105 000 g for 1 h. All the fractions were resuspended in 1 ml of 0.25 M-sucrose and immediately assayed for enzyme activities or processed for electron microscopy. Similarly, the load fractions from the above gradients, which consist of microsomes, were diluted to 0.25 M-sucrose and further processed for the isolation of smooth and rough endoplasmic reticulum as described previously (Reaven & Azhar, 1981).

Discontinuous-sucrose-density-gradient centrifugation of galactosyltransferase and other non-glycosylating enzymes

Starting microsomal fraction (8 ml; sucrose concentration 1.22 M) was subsequently overlaid with 5 ml of 1.05 M-, 8 ml of 0.86 M-, 8 ml of 0.6 M- and 6 ml of 0.25 M-sucrose solutions. Centrifugation was carried out at 104 000 g_{av} in a Beckman SW 28.2 rotor for 3 h. At the end of centrifugation, 1 ml fractions were collected from the top of the tube (total 35 fractions) and each fraction was then assayed immediately for various marker enzymes.

Isolation of other liver subcellular fractions

Rat liver plasma membranes and mitochondria were isolated as described previously (Reaven & Azhar, 1981).

Enzyme assays

All assays were carried out under experimental conditions in which enzyme activity was linear with respect to enzyme protein concentration and incubation time.

Measurement of Golgi glycosyltransferases. Sialyltransferase. Activity was measured by the procedure of Bretz *et al.* (1980). The samples were processed for radioactivity determination as described by Kim *et al.* (1971).

Galactosyltransferase. Activity was assayed by the procedures of Bretz & Staubli (1977) and Bretz *et al.* (1980). The incubation mixture in a final volume of 50 μ l contained 100 mM-sodium cacodylate, pH 6.8, 50 mM-MnCl₂, 40 mM-2-mercaptoethanol, 2 mM-ATP, 0.4% Triton X-100, 17.5 mg of ovomucoid/ml and 2.1 mM-UDP-[¹⁴C]galactose (0.95 μ Ci/ml) and appropriate amounts of membrane fraction. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 3 ml of 1% (w/v) phosphotungstic acid in 0.5 M-HCl. The samples were processed for radioactivity determination as described by Kim *et al.* (1971). In some instances *N*-acetylneuraminic acid- and galactose-free fetuin or *N*-acetylneuraminic acid-free mucin (17.5 mg/ml) instead of ovomucoid was used as acceptor protein. When *N*-acetyl-D-glucosamine (40 mM) was used as acceptor, the product *N*-[¹⁴C]acetyl-lactosamine was separated by Dowex column chromatography (Morre, 1971).

N-Acetylglucosaminyltransferase. The incubation conditions of this enzyme were the same as those described by Bretz *et al.* (1980). Transfer of radioactivity from UDP-*N*-[¹⁴C]acetyl-D-glucosamine to Smith-degraded ovomucoid (25 mg/ml; Bretz *et al.*, 1980) or ovalbumin (28 mg/ml; Amar-Costesec, 1981) was determined as described by Kim *et al.* (1971).

Measurement of transfer of core sugars to microsomes. *N*-Acetylglucosamine transfer. The incorporation of *N*-[³H]acetyl-D-glucosamine from UDP-*N*-[³H]acetyl-D-glucosamine into total microsomal and smooth- and rough-endoplasmic-reticulum fractions was measured by a modification of the procedures of Heifetz & Elbein (1977) and Waechter & Harford (1977).

Mannose transfer. Transfer of [¹⁴C]mannose from GDP-[¹⁴C]mannose into total microsomal and smooth- and rough-endoplasmic-reticulum fractions was measured by a modification of previously published procedures (Chambers & Elbein, 1975; Heifetz & Elbein, 1977; Waechter & Harford, 1977).

Miscellaneous enzyme assays. 5'-Nucleotidase and glucose 6-phosphatase activities were measured by the procedure of Aronson & Touster (1974). Microchondrial succinate:2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase activity was measured by the procedure of Pennington (1961).

Analytical methods

The protein concentration of each membrane fraction was measured by the technique of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978). P_i was quantified colorimetrically (Fiske & SubbaRow, 1925).

Electron-microscopic procedures

Processing of membrane fractions. Immediately after isolation, portions of the various membrane fractions were fixed for 10 min at 4°C in 1.5% glutaraldehyde prepared in 0.1 M-cacodylate buffer (pH 7.2; 420 mosM), and subsequently spun for 2 min at 100 000 g in a microcentrifuge (Airfuge; Beckman Instruments, Palo Alto, CA, U.S.A.). The resulting pellets were left overnight in cold fixative, then processed by standard techniques (Reaven & Reaven, 1980; Reaven & Azhar, 1981) the next day. All preparations were examined electron microscopically to check for membrane purity.

Quantification of contaminating membranes within Golgi-membrane fractions. When suitably embedded, the pelleted Golgi membranes could be thin-sectioned through their entire thickness. Six electron micrographs (16 000 $\times$) were taken at random from one embedded block of each of such

Fig. 1. Effect of a low and high dose of colchicine *in vivo* on terminal glycosyltransferase activity associated with hepatic Golgi light (GF1), intermediate (GF2) and heavy (GF3) fractions

The animals were injected with colchicine 5 h before isolation of Golgi fractions. Results are means $\pm$ S.E.M. (indicated by the bars) of three experiments.

Golgi-membrane specimens, and subjected to quantification as follows. Photographic prints (enlarged 3 $\times$) were prepared from each micrograph and all vesicular profiles bounded by ribosomes were identified on each print. The percentage frequency of rough-surfaced profiles (containing very-low-density lipoprotein), among other smooth-membraned structures (containing very-low-density lipoproteins), was estimated by using stereological volume density techniques (Weibel, 1973).

Results

Effect of colchicine on glycosyltransferase activity in Golgi fractions

Colchicine dose and treatment time on galactosyl-, sialyl- and N-acetylglucosaminyl-transferase activities. In these studies two doses of colchicine were used; one (2.5 mg/kg body wt.) was known to be maximally effective after 5 h in decreasing very-low-density-lipoprotein secretion in liver, disrupting Golgi complexes and eliminating microtubules in hepatocytes (Reaven & Reaven, 1980); approx. 50% of the rats given this dose of colchicine died between 12 and 24 h. The second dose of colchicine (0.8 mg/kg body wt.) was equally effective in altering liver structure and function at 5 h, but partial recovery of these hepatocyte features was seen at 12 h and all processes, in all the animals studied, were normal by 24 h after treatment with the drug (Azhar & Reaven, 1982). Given these results, it was of interest to see whether both doses of colchicine supplied *in vivo* had the same effect on

terminal glycosyltransferases located in membranes of hepatic Golgi complexes. The results presented in Fig. 1 indicate that 0.8 mg of colchicine/kg body wt. produced a 40–50% decrease in galactosyl-, sialyl-, and N-acetylglucosaminyl-transferase activities in GF1, GF2 and GF3 fractions. The effect was more marked in the case of sialyltransferase, with 50–70% decrease in all three Golgi fractions tested. Increasing the dose of colchicine to 2.5 mg/kg body wt. further decreased the activities of all three terminal glycosyltransferases, with an average decrease of 70–80% in Golgi fractions.

Administration of colchicine at a dose of 2.5 mg/kg body wt. produced a linear decrease in activity of the transferases in total (pooled) Golgi fractions. The drug effect was evident as early as 1 h and the extent of inhibition increased with time up to 5 h, the maximum time point studied (results not shown).

Insofar as the effect of colchicine on all three Golgi-associated glycosyltransferases was identical, subsequent experiments were simplified and results are provided for only one enzyme, galactosyltransferase.

Effect of colchicine on galactosyltransferase kinetics and acceptor specificity. To determine whether colchicine given *in vivo* (2.5 mg/kg body wt.) altered the catalytic properties of galactosyltransferase, Golgi membranes from livers of saline- or colchicine-treated rats were incubated with various concentrations of UDP-[¹⁴C]galactose, MnCl₂ and the acceptor (ovomucoid). Our experiments indicated that colchicine treatment did not alter the K_m of galactosyltransferase for UDP-

EXPLANATION OF PLATE 1

Electron micrographs of liver GF1 fraction from saline- and colchicine-treated rats ($\times 60\,000$)

(a) Shows a representative sample of GF1 from saline-treated animals. This fraction shows a variety of smooth-surfaced vacuoles, many of which are filled with very-low-density lipoproteins embedded in electron-dense matrix (arrowheads); (b) shows a sample of GF1 from colchicine-treated (2.5 mg/kg body wt. 5 h before death) rats. In addition to the structures seen in (a), a certain number of lipoprotein-filled rough-endoplasmic-reticulum vacuoles are also seen (arrowheads); such vacuoles have also been found in intact liver after colchicine treatment (see Reaven & Reaven, 1980).

galactose, MnCl_2 and ovomucoid in any Golgi fraction. However, the $V_{\max}$ of the enzyme was greatly decreased regardless of the acceptor used (see Table 1).

Purity of isolated Golgi-membrane fractions. Golgi-membrane fractions from all experiments conducted with 2.5 mg of colchicine/kg body wt. were examined by electron microscopy. The various fractions from saline-treated rats were identical with those previously described from this laboratory (Reaven & Azhar, 1981) and many others (Ehrenreich *et al.*, 1973; Bergeron *et al.*, 1978); Plate 1(a) is representative of the GF1 fraction from control rats. In contrast, GF1, GF2 and, to a much lesser extent GF3 fractions from colchicine-treated

animals showed some contamination with endoplasmic reticulum; a micrograph showing ribosome-studded membranes (rough endoplasmic reticulum) with luminal lipoproteins from a GF1 fraction of colchicine-treated animals is shown in Plate 1(b) (see also Reaven & Reaven, 1980). The level of such rough-endoplasmic-reticulum contamination was estimated on the GF1 fractions of all experiments by using quantitative microscopic techniques, and was found to vary from 7 to 15% of the fractions obtained from colchicine-treated rats.

Although this finding suggests that the GF1 and GF2 fractions of colchicine-treated rats may be contaminated to some extent with elements of the rough endoplasmic reticulum (by virtue of their luminal lipoproteins and lighter density), the extent of this 7–15% contamination cannot account for the extent of the decrease (70–80%) found in the activities of the various Golgi glycosyltransferases.

Reversal of inhibitory action of colchicine on galactosyltransferase activity. To determine if the decrease in hepatic Golgi glycosyltransferase activity observed after colchicine treatment was reversible with time, rats were injected with colchicine (0.8 mg/kg body wt.), killed 5, 12 and 24 hours later and hepatic Golgi membranes were isolated for enzyme assays. As shown in Fig. 2, this dose of colchicine, though less effective than a higher (2.5 mg/kg body wt.) dose (see also Fig. 1), decreased galactosyltransferase activity maximally at 5 h. Partial recovery was apparent at 12 h, and complete recovery occurred by 24 h.

Effect of colchicine on galactosyltransferase activity in various subcellular fractions of liver

Initially we examined the total homogenate of livers from control and colchicine-treated (2.5 mg/kg

Table 1. Effect of colchicine treatment on galactosyltransferase activity of GF2 fraction using various acceptors

For simplicity, data are limited to the GF2 fraction only, but similar results were obtained with GF1 and GF3 fractions also. Incubation conditions were as described in the Experimental procedures section. Results are means of three experiments. Colchicine dose was 2.5 mg/kg body wt. 5 h before death.

Acceptors	^{14}C Galactose transferred (nmol/min per mg of protein)	
	Saline	Colchicine
Ovomucoid	9.44 ± 0.69	1.78 ± 0.15
N-Acetylneuraminic acid-free mucin	11.08 ± 0.85	2.67 ± 0.55
N-Acetylneuraminic acid- and galactose-free fetuin	7.51 ± 0.71	1.64 ± 0.31
GlcNAc	7.98 ± 0.47	1.59 ± 0.83

Fig. 2. Time-dependent recovery of galactosyltransferase activity after injection of rats with 0.8 mg of colchicine/kg body wt. Samples of hepatic Golgi fractions (GF1, GF2 and GF3) were taken 5, 12 and 24 h after injection of colchicine or saline. Results are means $\pm$ S.E.M. (indicated by the bars) of three separate experiments.

Table 2. *Effect of colchicine administration on subcellular distribution of galactosyltransferase and protein in rat liver* Galactosyltransferase activity is measured as pmol of [^{14}C]galactose transferred to DS-mucin/min per g of liver, except for the serum fraction, where it is measured as pmol of [^{14}C]galactose transferred to DS-mucin/min per ml of serum. 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.

	Galactosyltransferase		Protein		Specific activity (pmol/min per mg of protein)	Relative specific activity
	(pmol/min per g of liver)	Recovery (%)	($\mu\text{g/g}$ of liver)	Recovery (%)		
Saline treatment						
Homogenate	53 991 $\pm$ 741	100.0	242 833 $\pm$ 5020	100.0	223 $\pm$ 8	1
Nuclear/mitochondrial fraction	24 724 $\pm$ 962	45.8 $\pm$ 1.8	110 417 $\pm$ 1828	45.6 $\pm$ 0.8	224 $\pm$ 12	1
Soluble fraction	653 $\pm$ 134	1.2 $\pm$ 0.3	91 500 $\pm$ 6172	37.7 $\pm$ 2.5	7 $\pm$ 1	<1
Starting microsomal fraction	26 161 $\pm$ 2802	48.5 $\pm$ 5.2	40 280 $\pm$ 1989	16.6 $\pm$ 0.8	646 $\pm$ 41	2.9
GF1	704 $\pm$ 159	1.3 $\pm$ 0.3	54 $\pm$ 9	0.02 $\pm$ 0	13 482 $\pm$ 532	60.5
GF2	2285 $\pm$ 89	4.2 $\pm$ 0.2	160 $\pm$ 1	0.07 $\pm$ 0	14 274 $\pm$ 532	64.0
GF3	5519 $\pm$ 271	10.2 $\pm$ 0.5	699 $\pm$ 65	0.28 $\pm$ 0.03	8331 $\pm$ 453	37.4
Microsomes	13 353 $\pm$ 1819	24.7 $\pm$ 3.4	30 171 $\pm$ 535	12.4 $\pm$ 0.2	442 $\pm$ 58	2.0
Serum	3067 $\pm$ 178	—	—	—	—	—
Colchicine treatment						
Homogenate	55 089 $\pm$ 3489	100.0	239 167 $\pm$ 4475	100.0	230 $\pm$ 17	1
Nuclear/mitochondrial fraction	28 018 $\pm$ 3887	50.9 $\pm$ 7.1	117 167 $\pm$ 4068	49.0 $\pm$ 1.7	240 $\pm$ 35	1
Soluble fraction	1053 $\pm$ 237	1.9 $\pm$ 0.4	94 950 $\pm$ 2029	39.7 $\pm$ 0.9	11 $\pm$ 2	<1
Starting microsomal fraction	19 310 $\pm$ 937	35.1 $\pm$ 1.7	41 610 $\pm$ 1140	17.4 $\pm$ 0.5	464 $\pm$ 14	2.0
GF1	387 $\pm$ 47	0.7 $\pm$ 0.1	120 $\pm$ 10	0.05 $\pm$ 0	3222 $\pm$ 322	14.0
GF2	758 $\pm$ 56	1.4 $\pm$ 0.1	225 $\pm$ 10	0.09 $\pm$ 0.01	3371 $\pm$ 200	14.7
GF3	2460 $\pm$ 230	4.5 $\pm$ 0.4	639 $\pm$ 16	0.27 $\pm$ 0.01	3838 $\pm$ 298	16.7
Microsomes	11 560 $\pm$ 111	21.0 $\pm$ 0.2	28 513 $\pm$ 955	11.92 $\pm$ 0.4	407 $\pm$ 18	1.8
Serum	3140 $\pm$ 247	—	—	—	—	—

body wt.) animals. Twelve separate experiments were carried out using two proteins as [^{14}C]galactose acceptors (*N*-acetylneuraminic acid-free mucin and ovomucoid). The results showed no effect of colchicine on galactosyltransferase activity in the total homogenate. To explore this issue further we examined galactosyltransferase activity in various subcellular fractions of liver. In Table 2, the effect of colchicine on galactosyltransferase activity is shown for total homogenate, serum and every subfraction of liver. For each fraction, enzyme activity is expressed per g of liver and the percentage recovery in each specific fraction (compared with its content in total homogenate) is indicated. Likewise, the protein content of the identical fraction and its percentage recovery (compared with that in total homogenate) is given. As can be seen from the relative specific activity, there is no enrichment of enzyme activity in the nuclear/mitochondrial fraction of either saline- or colchicine-treated animals. On the other hand, enzyme enrichment does occur in the starting microsomal fraction of both groups of animals, and the percentage increase is less, after colchicine treatment. In both situations, the amount

of enzyme activity in the cytosolic (soluble) fraction is negligible. Details of Golgi and microsomal fractions (which are derived from the starting microsomal material) are discussed below.

For fine-resolution of the starting microsomal material, identical amounts of these fractions were placed in discontinuous sucrose gradients, centrifuged for 3 h (104 000 g_{av}) and 1 ml fractions were collected from the top for enzyme assays. Fig. 3 shows the distribution of galactosyltransferase activity from saline- and colchicine-treated rats. Data from GF1 (fractions 4–8), GF2 (fractions 12–16), GF3 (fractions 20–24) and microsomes (25–35) were pooled for additional calculations in Table 2. In Fig. 3(a), three peaks of enzyme activity can be identified, with GF1 containing the lowest and GF3 the highest level of activity in both groups of animals, though colchicine-treated animals show less activity at every point. The load zone (which represents the microsomal fraction) also contains significant amounts of activity. Fig. 3(b) shows that the protein obtained from the GF1 and GF2 peaks of colchicine-treated rats is more than that of the saline-treated controls. Thus, when the enzyme data

Fig. 3. Sucrose-density-gradient distribution pattern of hepatic galactosyltransferase activity in starting microsomal material from saline- (○) and colchicine-treated (●) rats

Sequential 1 ml fractions from the discontinuous sucrose gradient were analysed separately for enzyme activity (a) and protein (b). (c) shows the specific activities of enzymes in these fractions. Gradient concentrations are indicated by the horizontal arrows at the top of each Figure. GF1 was assumed to be fractions 4–8, GF2 fractions 12–16, GF3 fractions 20–24 and microsomes fractions 25–35. Results are means $\pm$ S.E.M. (indicated by the bars); $n = 3$. Colchicine dose was 2.5 mg/kg body wt. 5 h before death.

are corrected for protein content, a new picture emerges, showing large differences in the specific activity (Fig. 3c) of Golgi-associated galactosyltransferase activity between the saline- and colchicine-treated animals, but showing almost no differences among the various Golgi fractions within a given animal group. The pooled data for these fractions are shown in Table 2. From Table 2 and Fig. 2, one can make the following points. (1) Galactosyltransferase activity is present in identical fractions in liver of both saline- and colchicine-treated rats. (2) Most of the activity is found in Golgi fractions. (3) Colchicine has a profound effect on Golgi-associated galactosyltransferase, reducing its activity in GF1, GF2 and GF3 fractions by 77, 77 and 55% respectively.

Effect of colchicine on the activity of hepatic marker enzymes in various subcellular fractions of liver

Up to this point, all experiments were conducted to assess the effect of colchicine treatment on the activity of terminal glycosyltransferases known to be located in liver Golgi fractions. To determine whether other (non-glycosylating) enzymes were similarly affected, separate assays were carried out for the marker enzymes 5'-nucleotidase (plasma membrane), glucose 6-phosphatase (rough and smooth endoplasmic reticulum) and succinate: 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium reductase (mitochondria). For this purpose,

we used portions of the identical sucrose-density-gradient fractions obtained for the assessment of galactosyltransferase as in Fig. 3. Marker enzymes were also assayed in purified plasma membranes, mitochondria, total microsomes and rough- and smooth-endoplasmic-reticulum fractions from the same liver homogenates of saline- and colchicine-injected rats used for quantification of galactosyltransferase activity. The results of these measurements are seen in Fig. 4 and indicate that the appropriate enzymes are enriched in the appropriate fractions, but, more importantly, that none of these enzymes are enriched in (thus contaminating) the Golgi fractions.

Effect of colchicine on transfer of core sugars to microsomes and non-glycosylating enzymes

The studies conducted with microsomal enzymes were meant to answer the question of whether all glycosylating enzymes were affected by colchicine treatment (regardless of their location in the cell) or whether only the terminal glycosylating enzymes of the Golgi membranes were affected. For this purpose, transfer of two core sugars (*N*-acetylglucosamine and mannose) to microsomes was studied (Parodi & Leloir, 1979).

***N*-Acetylglucosamine transfer.** The incorporation of *N*-[3 H]acetyl-D-glucosamine from UDP-*N*-[3 H]acetyl-D-glucosamine into three endogenous products is presented in Table 3. The major portion of the incorporated radioactivity was extracted in a

Fig. 4. Comparison of marker enzymes for homogenate and various hepatic subcellular fractions obtained from saline- (a) and colchicine-treated (b) animals

H, Homogenate; PM, plasma membrane; M, mitochondria; GF1-3, Golgi fractions; MC, microsomes; SER, smooth endoplasmic reticulum; RER, rough endoplasmic reticulum. Colchicine dose was 2.5 mg/kg body wt. Units for galactosyltransferase are pmol/min per mg of protein and those for 5'-nucleotidase, glucose 6-phosphatase and succinate: 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium (INT) reductase are μ mol/min per mg of protein.

mixture of chloroform/methanol (2:1, v/v). This product probably represents a mixture of dolichyl diphosphate *N*-acetylglucosamine and dolichyl diphosphate *NN'*-diacetylchitobiose. A smaller amount of radioactivity was also incorporated into oligosaccharide lipid [extracted with chloroform/methanol/water (10:10:3, by vol.)] and glycoproteins. Addition of dolichylphosphate further enhanced the incorporation of *N*-[3 H]acetylglucosamine into all three products, indicating that endogenous dolichol was in short supply. Colchicine administered *in vivo* did not change the level of incorporated radioactivity or the distribution of the radioactivity in any of these products.

Mannose transfer. The effect of colchicine on mannose transfer to liver microsomes was also investigated and, as with *N*-acetylglucosamine transfer, no changes were observed in dolichyl phosphate mannose, oligosaccharide lipid or the glycoprotein fraction (results not shown).

Effect of vinblastine sulphate on galactosyltransferase activity in various subcellular fractions of liver

To determine whether the drug colchicine had a unique effect on Golgi enzymes, we injected animals with a second anti-microtubule agent (vinblastine; 20 mg/kg body wt.) (Wilson *et al.*, 1975; Na & Timasheff, 1980) and assayed for galactosyltransferase activity in various hepatic functions. Vinblastine, like colchicine, markedly reduced the activity of this enzyme in all Golgi fractions (results not shown).

Discussion

These various data show that anti-microtubule agents have a specific inhibitory effect on the activities of various Golgi-associated sugar transferases in liver. This conclusion follows from the information that colchicine and vinblastine (to the extent that it was tested) (1) affect only hepatic Golgi terminal glycosyltransferases and not endoplasmic-reticulum-associated transferases, (2) affect only the level of terminal glycosyltransferase activities, and not the distribution of the enzymes in various subcellular fractions of liver homogenate and (3) do not affect the distribution, or the activities, of a number of other (non-Golgi) hepatic enzymes.

The first of the above points is straightforward and requires little explanation. Hepatic Golgi fractions show substantial decreases in galactosyl-, sialyl- and *N*-acetylglucosaminyl-transferase activity 5 h after treatment with either a low or a high dose of colchicine, or vinblastine sulphate. In contrast, transfer of mannose or *N*-acetylglucosamine to microsomes (assayed from the microsomal fractions

Table 3. *Effect of colchicine treatment on transfer of core sugar (N-acetylglucosamine) to microsomes*

Incubation mixtures (final volume, 0.4 ml) contained 30 mM-Tris/HCl buffer (pH 7.4), 10 mM-MnCl₂, 2.5 mM-EDTA, 0.5% Triton X-100, 2×10^5 c.p.m. of UDP-[³H]GlcNAc, microsomal fractions (2–3 mg of protein) and, where required, 20 µg of dolichyl phosphate (suspended in Triton X-100 as described by Chen & Lennarz, 1977). After incubation at 37°C for 15 min, the radioactivity incorporated into various fractions was determined as described in the Experimental procedures section. Results are means $\pm$ S.E.M. of three experiments. Abbreviation used: GlcNAc-*P*₂-Dol, dolichyl diphosphate *N*-acetylglucosamine.

	[³ H]GlcNAc incorporation into fractions (d.p.m./mg of protein)		
	GlcNAc- <i>P</i> ₂ -Dol	Oligosaccharide lipid	Glycoprotein
Saline			
Starting microsomal fraction	1532 $\pm$ 598	398 $\pm$ 145	896 $\pm$ 116
Smooth endoplasmic reticulum	532 $\pm$ 86	204 $\pm$ 24	268 $\pm$ 5
Rough endoplasmic reticulum	3064 $\pm$ 1308	403 $\pm$ 159	408 $\pm$ 106
Smooth endoplasmic reticulum + dolichylphosphate (20 µg)	1676 $\pm$ 486	273 $\pm$ 36	272 $\pm$ 23
Rough endoplasmic reticulum + dolichylphosphate (20 µg)	5559 $\pm$ 999	496 $\pm$ 150	433 $\pm$ 103
Colchicine (2.5 mg/kg body wt. 5 h before death)			
Starting microsomal fraction	1832 $\pm$ 581	336 $\pm$ 72	788 $\pm$ 62
Smooth endoplasmic reticulum	461 $\pm$ 124	212 $\pm$ 8	224 $\pm$ 5
Rough endoplasmic reticulum	2189 $\pm$ 294	266 $\pm$ 30	330 $\pm$ 10
Smooth endoplasmic reticulum + dolichyl phosphate (20 µg)	1561 $\pm$ 319	214 $\pm$ 32	204 $\pm$ 4
Rough endoplasmic reticulum + dolichyl phosphate (20 µg)	5798 $\pm$ 1755	408 $\pm$ 89	378 $\pm$ 42

of the very same livers) is not affected. The colchicine effect does not alter the kinetic properties of the transferases tested, and is not specific for any particular acceptor: only the $V_{\max}$ values of the enzymes are reduced. Colchicine affects the terminal glycosylating enzymes in a dose- and time-dependent manner, and it is noteworthy that when the drug is given in low doses (0.8 g/kg body wt.), the effect seen at 5 h is totally reversed by 24 h.

As straightforward as these statements are, there is always some concern when working with sub-cellular fractions that the action of a drug may be to alter the physical make-up of the affected cells, resulting in a shift of either membranes or enzymes into different subcellular fractions during the isolation procedure. This concern is particularly felt in the present study, since previous morphological studies indicated that the organization of the hepatocyte Golgi complex is altered by the use of anti-microtubule drugs. Thus, to determine if the loss of Golgi glycosyltransferase activity is due to a change in the cellular distribution of the enzyme after colchicine treatment, we monitored and kept track of the activity of these transferases in all cell fractions derived from the initial homogenate and, in addition, we examined 35 sequential (1 ml) fractions of the starting microsomal/Golgi material run in discontinuous sucrose gradients. The results unequivocally show that the colchicine-induced loss of Golgi-associated glycosyltransferase activity cannot

be explained by a shift in the distribution of the enzyme. The following points can be made from Table 2 and Fig. 3, which display the complete 'book-keeping' done for one enzyme, galactosyltransferase. When considering the homogenate as the starting material, one sees no colchicine-induced loss of galactosyltransferase activity to either the cytosol or the nuclear/mitochondrial fraction, and only a marginal decrease in activity is apparent in the starting microsomal fraction. This latter is comprised of the Golgi fractions plus the endoplasmic reticulum and only the enzyme that is present in the Golgi fractions appears to be sensitive to colchicine (Table 2). Since an endoplasmic-reticulum-based galactosyltransferase has recently been shown to have characteristics different from that of the Golgi enzyme (Andersson & Eriksson, 1981), it perhaps is not too surprising that a difference in colchicine sensitivity exists in the two fractions. Moreover, since the colchicine-sensitive Golgi-associated enzyme comprises such a small proportion of the enzyme found in the whole homogenate (<15%; Table 2), one would not expect a change to be apparent in the activity of the homogenate itself. This expectation is borne out by the results of Table 2. In addition, when one considers a detailed accounting of the enzyme within the starting microsomal fraction (as in Fig. 3), it can be seen that colchicine affects only the maximal activity and not the distribution pattern of galacto-

syltransferase; i.e., enzyme activity is located in three peaks identified as Golgi fractions GF1, GF2 and GF3 in saline-treated animals and smaller enzyme peaks, in identical locations, are obtained from the samples of colchicine-treated rats, suggesting that the enzyme is decreased in activity in treated rats but sediments in the same location in the gradient.

That the Golgi fractions are not substantially contaminated with other membranes (in either group of animals) is shown by the fact that marker enzymes for plasma membranes, endoplasmic reticulum and mitochondria assayed along with galactosyltransferase (Fig. 4) are not enriched in the Golgi regions. In this regard, the fact that we can discern a 7–15% contamination of rough-endoplasmic-reticulum vesicles by electron microscopy (Plate 1) in the GF1 fractions in the colchicine-treated rats, and not in the controls, gives cause for some concern. We have no reasonable explanation for our inability to measure equivalent changes in glucose 6-phosphatase in the membranes derived from the colchicine-treated animals, but insofar as these data have been confirmed by us many times, we must accept them. Perhaps those particular rough-endoplasmic-reticulum vesicles that are associated with secretory product no longer contain glucose 6-phosphatase. In any event, contamination of the magnitude displayed in the electron-microscopy samples cannot account for the colchicine-induced enzyme changes observed in the Golgi fractions.

At this point, the mechanism responsible for these Golgi-associated changes in galactosyltransferase activity is not understood, i.e., we do not know if colchicine reduces the enzyme content of the Golgi membranes or if enzyme activity that is present cannot be normally expressed. We are quite certain, however, that the specific decrease in galactosyltransferase activity is not due to drug inactivation of the enzyme during the prolonged isolation of the membranes, i.e., the homogenate and all subcellular fractions were assayed at the same time and were therefore equally exposed to colchicine, yet only the enzyme activity of the Golgi fractions was affected.

In all, the findings from the present study are consistent with morphological data that show that membranes of the Golgi samples are altered *in vivo* after the treatment of rats with colchicine or vinblastine under precisely the conditions used in the present study (Reaven & Reaven, 1980). We believe these events are linked somehow, i.e., that anti-microtubule agents specifically interfere with membrane reconstruction in the Golgi region and that this, in turn, affects Golgi-associated enzymes such as the terminal glycotransferases. To what extent these results are related to the interaction of the

anti-microtubule drugs with hepatic microtubule protein remains to be clarified.

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References

- Amar-Costesec, A. (1981) *J. Cell Biol.* **89**, 62–69
- Andersson, G. N. & Erikson, L. C. (1981) *J. Biol. Chem.* **256**, 9633–9639
- Aronson, N. J., Jr. & Touster, O. (1974) *Methods Enzymol.* **31**, 90–102
- Azhar, S. & Reaven, E. (1982) *J. Cell Biol.* **95**, 396a
- Banerjee, D., Manning, C. P. & Redman, C. M. (1976) *J. Biol. Chem.* **251**, 3887–3892
- Bergeron, J. J. M., Posner, B. I., Josefsberg, Z. & Sikstrom, R. (1978) *J. Biol. Chem.* **253**, 4058–4066
- Bretz, R. & Staubli, W. (1977) *Eur. J. Biochem.* **77**, 181–192
- Bretz, R., Bretz, H. & Palade, G. E. (1980) *J. Cell Biol.* **84**, 87–101
- Chambers, J. & Elbein, A. D. (1975) *J. Biol. Chem.* **250**, 6904–6915
- Chen, W. W. & Lennarz, W. J. (1977) *J. Biol. Chem.* **252**, 3473–3479
- Chertow, B. W., Williams, G. A., Barker, G. R., Surbaugh, R. D. & Hargis, G. K. (1975) *Exp. Cell Res.* **93**, 388–394
- Chertow, B. W., Williams, G. A., Barker, G. R., Surbaugh, R. D. & Hargis, G. K. (1975) *Exp. Cell Res.* **93**, 388–394
- DeBrabander, M., Wanson, J., Mossellmans, T., Geuens, G. & Drohmans, P. (1978) *Bio. Cell.* **31**, 127–140
- Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. & Palade, G. E. (1973) *J. Cell Biol.* **59**, 45–72
- Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Friedman, H. I. & Cardell, R. R., Jr. (1977) *Anat. Rec.* **188**, 77–102
- Heifetz, A. & Elbein, A. D. (1977) *J. Biol. Chem.* **252**, 3047–3063
- Hindelang-Gertner, C., Stoeckel, M., Porte, A. & Stutinsky, F. (1976) *Cell Tiss. Res.* **170**, 17–14
- Hoffstein, S., Goldstein, I. M. & Weissman, G. (1977) *J. Cell Biol.* **73**, 242–256
- Kemper, B., Habener, J. F., Rich, A., Potts, J. T., Jr. (1975) *Endocrinology* **96**, 903–912
- Kim, Y. S., Perdomo, J. & Nordberg, J. (1971) *J. Biol. Chem.* **246**, 5466–5476
- Knudson, C. M., Stemberger, B. H. & Patton, S. (1978) *Cell Tiss. Res.* **195**, 169–181
- LeMarchand, Y., Singh, A., Patzelt, C., Orci, L. & Jeanrenaud, B. (1975) in *Microtubules and Microtubule Inhibitors* (Borgers, M. & DeBrabander, M., eds.), p. 153, North Holland Publishing Co., Amsterdam and Oxford
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

- Markwell, M. A. K., Hass, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210
- Morre, D. J. (1971) *Methods Enzymol.* **22**, 130–148
- Moskalewski, S., Thyberg, J., Lohmander, S. & Friberg, U. (1975) *Cell Res.* **95**, 440–454
- Moskalewski, S., Thyberg, S. J. & Friberg, U. (1976) *J. Ultrastruct. Res.* **54**, 304–317
- Na, G. C. & Timasheff, S. M. (1980) *Biochemistry* **19**, 1347–1354
- Obberghen, E., Devis, G., Somers, G., Ravazzola, M., Malaisse-Lagae, F. & Malaisse, W. J. (1974) *Eur. J. Clin. Invest.* **4**, 307–312
- Parodi, A. J. & Leloir, L. F. (1979) *Biochim. Biophys. Acta* **559**, 1–37
- Patzelt, C., Brown, D. & Jeanrenaud, B. (1977) *J. Cell Biol.* **73**, 578–593
- Pennington, R. J. (1961) *Biochem. J.* **80**, 649–654
- Poisner, A. M. & Cooke, P. (1975) *Ann. N.Y. Acad. Sci.* **253**, 653–669
- Reaven, E. P. & Azhar, S. (1981) *J. Cell Biol.* **89**, 300–308
- Reaven, E. P. & Reaven, G. M. (1977) *J. Cell Biol.* **75**, 559–572
- Reaven, E. P. & Reaven, G. M. (1980) *J. Cell Biol.* **84**, 28–39
- Redman, C. M., Banerjee, D., Howell, K. & Palade, G. E. (1975) *J. Cell Biol.* **66**, 42–59
- Seybold, J., Bieger, W. & Kern, H. F. (1975) *Virchows Arch. Pathol. Anat. Physiol.* **368**, 309–327
- Stein, O., Sanger, L. & Stein, Y. (1974) *J. Cell Biol.* **62**, 90–98
- Stock, C., Launay, J. F., Grenier, J. F. & Bauduin, J. (1978) *Lab. Invest.* **38**, 157–163
- Waechter, C. J. & Harford, J. B. (1977) *Arch. Biochem. Biophys.* **181**, 185–198
- Weibel, E. R. (1973) in *Principles and Techniques of Electron Microscopy* (Hayat, M. A., ed.), vol. 3, pp. 237–291, Van Nostrand Reinhold Co., New York
- Williams, J. (1976) *Endocrinology* **98**, 1351–1358
- Wilson, L., Creswell, K. M. & Chin, D. (1975) *Biochemistry* **14**, 5586–5592