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Biochemical and Ultrastructural Studies of Ectoglycosyltransferase Systems of Murine L1210 Leukemic Cells

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Intact murine L1210 leukemic cells incorporated significant quantities of [^{3}H]-N-acetylneuraminic acid directly from CMP-N-acetylneuraminic acid. When pretreated with Vibrio cholerae neuraminidase, incorporation increased sixfold to tenfold. Biochemical studies comparing incorporation of N-acetylneuraminic acid from the nucleotide sugar with that from free sugar demonstrated that the relatively high levels of incorporation from CMP-N-acetylneuraminic acid could not be due to the incorporation of free sugar generated by extracellular degradation of the nucleotide sugar. Very little N-acetylneuraminic acid was taken up or incorporated by L1210 cells from free sugar and this incorporation was not increased by neuraminidase pretreatment. Moreover, extracellular breakdown of CMP-N-acetylneuraminic acid during incubations with L1210 cells was rather insignificant.

Electron microscope autoradiography of cells incubated with CMP-N-acetylneuraminic acid demonstrated that greater than 84% of the incorporated radioactivity was associated with the plasma membrane and less than 1% with the Golgi apparatus. These findings are consistent with the conclusion that incorporation of N-acetylneuraminic acid from CMP-N-acetylneuraminic acid is the consequence of a cell surface sialyltransferase system. Pretreatment of cells with the nonpenetrating reagent, diazonium salt of sulfonilic acid, significantly inhibited this ectoenzyme system while only marginally affecting galactose uptake and incorporation at the Golgi apparatus. Interestingly, incorporation from CMP-N-acetylneuraminic acid declined as the viability of the cell population declined. When taken together, the above evidence develops a rigorous argument for the presence of a sialyltransferase enzyme system at the cell surface of L1210 cells.

Studies directed towards the detection of a similar ectogalactosyltransferase system were also undertaken. Cells incubated in the presence of UDP-[^{3}H]-galactose incorporated radioactivity into a macromolecular fraction. The presence of excess unlabeled galactose in the incubation medium significantly reduced this incorporation. Electron microscope autoradiographs of cells incubated with UDP-[^{3}H]-galactose, demonstrated that incorporation occurred primarily at the Golgi apparatus. The grain distribution in these

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autoradiographs was similar to that for free galactose. Thus, the incorporation observed for L-1210 cells incubated in UDP-[^{3}H]-galactose was due primarily to the intracellular utilization of free galactose generated by extracellular degradation of the nucleotide sugar. Inability to demonstrate an ectogalactosyltransferase system on L1210 cells does not rule out the possibility that the enzyme is present but undetectable due to the absence of appropriate cell surface acceptor molecules.

Key words: **sialyltransferase, galactosyltransferase, electron microscope autoradiography, plasma membrane, Golgi apparatus**

Among the ectoenzymes which have been detected on the surface of various normal and neoplastic cell types are glycosyltransferases – enzymes which catalyze the transfer of sugar moieties from nucleotide sugar donors to glycoconjugate acceptor molecules. Characteristically, glycosyltransferases have been associated with the membranes of the Golgi apparatus [1], where they function to glycosylate proteins and lipids destined for plasma membrane and/or secretion.

Following Roseman's proposal that cell-to-cell adhesion and communication might be mediated by cell surface glycosyltransferases [2], a number of investigators detected these enzymes on the plasma membrane of a variety of cell types [4, 11–14]. Among the more convincing evidence of their presence at this location was the ability of whole intact cells to glycosylate exogenous acceptors bound to glass beads [3]; alterations in the lectin agglutinability of lymphocytes following whole-cell incubations with nucleotide sugars; and electron microscope (EM) autoradiographic localization of labeled acceptor molecules at the cell surface (but not at the Golgi region) following incubation of cells with CMP-[^{3}H]-N-acetylneuraminic acid (CMP-NANA) [5]. A great deal of the remaining evidence originally put forth, however, was based on the premise that if whole viable cells can incorporate labeled sugar from a nucleotide sugar, the reaction must be due to gylcosyltransferases at the cell surface. This assumes that the intact nucleotide sugar does not enter the cell and that it is not broken down extracellularly.

Recent studies have shown, however, the extracellular breakdown of nucleotide sugars does occur, especially for those containing a diphosphate linkage [6, 7]. Hirschberg, Goodman, and Green [7] have detected free NANA in the extracellular medium following incubations of fibroblasts with CMP-NANA and found that free NANA, albeit in very small quantities, could enter intact cells and become incorporated intracellularly [20]. This finding raised the possibility that at least some of the incorporated NANA in our studies with L1210 cells [5, 8] could be due to intracellular incorporation of free sugar. This, in turn, could be directly related to the integrity of the cell and, hence, to its permeability to NANA or the intact nucleotide sugar. Based on the EM autoradiographic evidence for ectosialyltransferase discussed above, these possibilities seem unlikely. Nonetheless, we have undertaken in the present study to demonstrate, by several lines of evidence, that incorporation of NANA from CMP-NANA is the consequence of glycosyltransferase activity associated with the cell surface and not within the Golgi apparatus. In addition, we have applied biochemical and EM autoradiographic techniques in an attempt to detect an ectogalactosyltransferase system associated with these same L1210 cells.

MATERIALS AND METHODS

L1210 Leukemic Cells

Female DBA/2J mice, weighing 20–25 g (Jackson Laboratories, Bar Harbor, Maine) were inoculated IP with 10^5 L1210 murine leukemic cells suspended in saline. The animals were sacrificed five to seven days later by cervical dislocation and the ascites fluid containing the tumor cells was withdrawn and diluted with ice-cold Dulbecco's phosphate-buffered saline (PBS). The cells were washed twice with PBS and counted with an electronic particle counter (Coulter Electronics, Hialeah, Florida) and the cell density was adjusted in RPMI 1640 (Gibco, Buffalo, New York) containing 20 mM HEPES (Sigma, St Louis, Missouri) and 10 mM MOPS (Sigma), pH 7.0 (RPMI 1640 HM).

Detection of Breakdown of Nucleotide Sugars

For 30 and 60 min 0.15 μCi of CMP-[^{14}C]-NANA (227 mCi/mmole, Amersham, Arlington Heights, Illinois) or UDP-[^{14}C]-galactose (274 mCi/mmole, New England Nuclear, Boston) were incubated in 0.2 ml RPMI 1640 HM containing 10% fetal calf serum (FCS) or 10% heat-inactivated fetal calf serum (56°, 1 h, HI-FCS) in the presence and absence of 10^7 Vibrio cholerae neuraminidase (VCN)-treated L1210 cells. Assays were terminated by placing the incubation tubes in an ice-water bath. The cells were removed by centrifugation and aliquots of the supernatant were chromatographed on S and S orange ribbon paper (Schleicher and Schuell, Keene, New Hampshire) using ethanol:1M ammonium acetate (7:3, v/v) as the solvent system. Descending chromatography was performed for 16 h and the dried paper strips were monitored for radioactivity using a Nuclear Chicago strip scanner. The radioactive areas were then cut into small strips and counted with a scintillation counter.

Preparation of [^{3}H]-NANA

Commercial CMP-[^{3}H]-NANA (2.33 Ci/mmole, New England Nuclear) was dried in a Pyrex tube under nitrogen and then redissolved in 0.03 ml of distilled water. Then 0.03 ml of 0.2 N sulfuric acid was added and the tube was heated to 80°. After 1 h, 0.06 ml of 0.1 N sodium hydroxide was added. The hydrolysate was examined by descending paper chromatography and the purity of the prepared [^{3}H]-NANA preparation was verified.

Uptake and Incorporation of Sialic Acid

Washed L1210 cells (5×10^7) were treated with 10 units of VCN (Behring Diagnostic Company, Somerville, New Jersey, EC 3.2.1.18) in 0.5 ml RPMI 1640 HM for 15 min at 37°. VCN-treated and untreated cells were washed three times in 5 ml RPMI 1640 HM, diluted in 1.0 ml of media, and dispensed in 0.2 ml volumes (10^7 cells) in glass tubes. VCN-treated or untreated L1210 cells were incubated for 30 min with 1 μM CMP-[^{3}H]-NANA or 1 μM or 10 μM [^{3}H]-NANA prepared as described above. The incubation was terminated by the addition of 2 ml cold RPMI 1640 HM, and the cells were centrifuged

and washed twice with cold RPMI 1640 HM, followed by the addition of 0.5 ml ethanol (EtOH). The mixture was centrifuged and an aliquot of the supernatant was transferred to glass filters, dried, and counted. The pellet was washed once with 0.5 ml 1% phosphotungstic acid (PTA) and twice with 5% trichloroacetic acid (TCA). An aliquot of each supernatant was removed for scintillation counting and the final pellet was dissolved in 0.2 ml of 1 N NaOH and neutralized, and the incorporated radioactivity was determined.

Viability Studies

VCN-pretreated and control L1210 cells were suspended in RPMI 1640 HM and kept at either 23° or 4° for 21 h. At this time viability was assessed by trypan blue dye exclusion, and ectosialyltransferase activity was measured as described above.

Effect of Diazonium Salt of Sulfonilic Acid on Sugar Incorporation

Intact cells were treated with the diazonium salt of sulfonilic acid, a nonpenetrating reagent inhibitory to ectoenzymes [9, 18]. The reagent was prepared as described by DePierre and Karnovsky [9] from sodium nitrite and sulfonilic acid. Untreated and VCN-treated cells were exposed for 15 min at 37° to either 8.5 mM or 17 mM diazonium salt of sulfonilic acid in RPMI 1640 HM (pH 7.0) prior to a 30-min incubation in the presence of 1 μM CMP-[^{3}H]-NANA or 1 μM [^{3}H]-galactose (0.6 Ci/mole, New England Nuclear). Incorporation was measured as described above.

Assay for Ectogalactosyltransferase

The ability of intact L1210 cells to incorporate galactose from the nucleotide sugar UDP-galactose was examined. For these studies, 2 or 20 μM UDP-[^{3}H]-galactose (1.15 Ci/mole, New England Nuclear) was added to 0.2 ml RPMI 1640 HM containing 1×10^7 L1210 cells. Cells were incubated for 30 min at 37° in a shaking water bath. Cellular incorporation was quantitated in the acid-insoluble fraction as described for NANA. The effect of an excess of unlabeled galactose (1 mM) was assessed with respect to galactose incorporation from UDP-galactose.

Electron Microscope Autoradiography

L1210 cells were pretreated with VCN and incubated in RPMI 1640 HM with either 10 μM CMP-[^{3}H]-NANA, 60 μM UDP-[^{3}H]-galactose (4.53 Ci/mole, New England Nuclear), or 30 μM [^{3}H]-galactose for 30 min at 37°. The cells were thoroughly washed with 5 ml of cold RPMI 1640 HM, centrifuged, fixed 2 h in 3% phosphate buffered glutaraldehyde (pH 7.0), postfixed 2 h in 1% buffered osmium tetroxide, dehydrated in a graded alcohol series, and embedded in Epon-araldite. Ultramicrotome sections (100 nm thick) were processed for EM autoradiography [5]. The sections were mounted on collodionized slides, stained with 2% uranyl acetate, carbon-coated, and overlaid with a monolayer of Ilford L-4 emulsion. The preparations were exposed 25–55 days, developed in D-19 (Kodak) for 2 min at 24°, and photographed with a Siemens Elmiskop 101.

Electron micrographs were examined and the cellular location of more than 400 grains were scored for each experiment. The percentage of the total number of grains in each cellular compartment (plasma membrane, lipid vacuole, Golgi apparatus, nuclear membrane, and nuclei) was then calculated.

RESULTS

Breakdown of Nucleotide-Sugars

Incubation with VCN-treated cells did not significantly increase the breakdown of CMP-[^{14}C]-NANA over that detectable after incubation in RPMI 1640 HM alone (Table I). In both cases breakdown amounted to about 5% of the added CMP-NANA after 60 min. However, breakdown of CMP-[^{14}C]-NANA readily took place in the presence of fetal calf serum. After 30 min incubation, 18.8% of the radioactivity was associated with free NANA and by 60 min this increased to 33%. Heat inactivation of the sera substantially reduced the hydrolysis of CMP-[^{14}C]-NANA but did not completely abolish it.

UDP-[^{14}C]-galactose as obtained commercially was found to be 98% pure, containing 2% galactose-1-phosphate prior to incubations with cells. Incubating UDP-[^{14}C]-galactose with RPMI 1640 HM at 37° for 30 or 60 min did not alter the radioisotope purity. In the presence of 10% FCS or VCN-treated L1210 cells, however, small amounts (0.6–1.6%) of free [^{14}C]-galactose were generated within 30 min and 1.6–2.4% after 60 min. After 1 h, the quantity of galactose-1-phosphate present decreased from 2.4% to 1.9% in the presence of FCS and decreased from 3.7% to 2.9% with intact cells.

Subcellular Distribution of Radioactivity Following Incubation With CMP-[^{3}H]-NANA

Intact L1210 cells were capable of taking up and incorporating small quantities of free NANA into a macromolecular fraction (Table II). Cells incubated in 1 μM [^{3}H]-NANA and extensively washed with RPMI 1640 HM contained some radioactivity in the ethanol-extractable soluble fraction and a small amount incorporated into the acid-precipitable fraction. Increasing the concentration of [^{3}H]-NANA from 1 to 10 μM essentially increased both uptake and incorporation tenfold. Cells pretreated with VCN exhibited similar levels of uptake and incorporation of [^{3}H]-NANA. By contrast, and as found previously, cells pretreated with VCN and incubated with 1 μM CMP-[^{3}H]-NANA incorporated 6.5 times more [^{3}H]-NANA than untreated cells. Uptake of [^{3}H]-NANA was also somewhat higher in VCN-treated cells (203 vs 150 DPM) but the total radioactivity in the soluble fraction was only a small fraction of the total amount incorporated (203 vs 6421 DPM).

In all cases, cells incorporated much more radioactivity from CMP-[^{3}H]-NANA than from [^{3}H]-NANA, both having the same specific activity. Cells not treated with VCN incorporated 3.8 times more radioactivity from 1 μM CMP-[^{3}H]-NANA than from 1 μM [^{3}H]-NANA while cells pretreated with VCN incorporated 17 times more radioactivity.

Effect of Cell Viability on Ectosialyltransferase Activity

Maintenance of cells at room temperature (23°) for 21 h decreased cell viability to 71% (Table III). L1210 cells treated with VCN experienced an initial decrease in viability to 92% immediately following VCN treatment and a further decline to 70% after 21 h at 23°. Cells maintained at 4° retained higher cell viabilities. Sialyltransferase activity did not increase with decreasing cell viability over time as might be expected as the cells become more permeable to the nucleotide sugar. Instead incorporation by the enzyme-treated cells decreased along with, but not proportionally to, the decreasing viability. Following a 21-h incubation at 4° cell viability fell to 87% and sialyltransferase

TABLE I. Extracellular Degradation of Nucleotide-Sugars (% total radioactivity)*

	30 min					60 min				
	UDP-Gal	Gal-P	Gal	CMP-NANA	NANA	UDP-Gal	Gal-P	Gal	CMP-NANA	NANA
Standard[a]	98	2.0	0	97.9	2.1	–	–	–	–	–
RPMI 1640 HM	98.8	1.2	0	98.1	2.9	97.6	2.4	0	95.1	4.9
" + 10% FCS	97.0	2.4	0.6	81.2	18.8	96.5	1.9	1.6	67	33
" + 10% HI-FCS	97.0	3.0	0	92.8	7.2	96.7	3.3	0	88.8	11.2
" + VCN-L1210 Cells	94.7	3.7	1.6	96.4	3.6	94.7	2.9	2.4	94.2	5.8

*Either UDP-[^{14}C]-galactose or CMP-[^{14}C]-NANA (0.5 μCi) were added to 200 μl of RPMI 1640 HM containing the additions listed above. These mixtures were incubated at 37° for 30 min or 60 min, at which time 20-μl cell-free aliquots were removed and analyzed using paper chromatography. Compounds were separated and identified using authentic standards and these areas on the paper strips were cut out and counted with a scintillation counter. Total CPM were summated and percentage total radioactivity was calculated for each peak. R_f values were as follows: UDP-galactose 0.33, galactose phosphate 0.57, galactose 0.80; CMP-NANA 0.32, NANA 0.63. Solvent system, ethanol:1 M ammonium acetate (7:3).

[a] 0.1 μCi of UDP-[^{14}C]-galactose or CMP-[^{14}C]-NANA of authentic standards were analyzed chromatographically as described above.

TABLE II. Distribution of Radioactivity in L1210 Leukemic Cells Incubated With CMP-[^{3}H]-NANA or Free [^{3}H]-NANA

VCN[a] pretreatment	Radioisotope[b] present (final concentration)	Total DPM in soluble fraction from 10^7 cells[c]				Incorporation[c] (DPM/10^7 cells/ 30 min)
		EtOH extract	PTA extract	TCA extract	TCA extract	
–	1 μM CMP-[^{3}H]-NANA	150	120	50	0	991
–	1 μM [^{3}H]-NANA	381	95	22	0	255
–	10 μM [^{3}H]-NANA	3,365	860	143	8	2,202
+	1 μM CMP-[^{3}H]-NANA	203	89	52	24	6,421
+	1 μM [^{3}H]-NANA	513	92	40	0	373
+	10 μM [^{3}H]-NANA	3,814	664	145	0	1,845

[a] Cells were pretreated with Vibrio cholerae neuraminidase.

[b] These isotopes were used at identical specific activities (2.33 Ci/mmole).

[c] 10^7-VCN treated or untreated cells were incubated for 30 min at 37° in either CMP-[^{3}H]-NANA or [^{3}H]-NANA in a final volume of 0.2 ml RPMI 1640 HM. Following the incubation, the cells were washed three times in 2 ml of RPMI 1640 HM and extracted with 0.5 ml ethanol (EtOH). The EtOH-soluble material was transferred to glass filters, dried, and counted; the EtOH-insoluble material was washed once with 0.5 ml 1% phosphotungstic acid (PTA) in 0.5 N HCl and twice with 0.5 ml 10% trichloroacetic acid (TCA). The soluble material was counted directly in 10-ml ACS, and the final pellet was dissolved in 0.2 ml 1 N NaOH, neutralized, and counted with a Packard scintillation counter. Experiments were performed in duplicate on two separate occasions and the data are the means of those experiments.

TABLE III. Dependence of Incorporation on Cell Viability*

	Incorporation of NANA, DPM/10^7 cells/30 min (% viability)		
	0 h	4°, 21 h	23°, 21 h
L1210	986 (99%)	841 (93%)	815 (71%)
VCN-L1210	6,749 (92%)	5,542 (87%)	2,210 (70%)

* Untreated and VCN-treated cells were suspended in RPMI 1640 HM (10^6 cells/ml) and maintained for 21 h at room temperature (23°C) or on ice (4°). Cell viabilities were determined (% in brackets) by trypan blue dye exclusion. Cells were incubated for 30 min in 1 μM CMP-[^{3}H]-NANA at 37°. The cells were washed once with 2 ml 1% PTA and twice with 5% TCA. Insoluble radioactivity was quantitated. Incorporation levels represent the average of duplicate enzyme assays performed on two separate occasions.

activity to 86% of the zero-time control value. Similarly, following a 21-h incubation at 23°, cell viability fell to 70% and enzyme activity plummeted to 34%.

Effect of Diazonium Salt of Sulfonilic Acid Pretreatment on Sugar Incorporation

Preincubation of VCN-treated cells with the diazonium salt of sulfonilic acid decreased incorporation of label from CMP-[^{3}H]-NANA by about 80%, while reducing that for [^{3}H]-galactose by about 32% (Table IV). The effect was dependent on the concentration of the diazonium salt. Interestingly, VCN treatment increased incorporation from CMP-NANA about sixfold while having no effect on galactose incorporation.

TABLE IV. Effects of Pretreatment With the Diazonium Salt of Sulfonilic Acid on the Incorporation of Sugars by L1210 Cells

VCN[a] treatment 15′, 37°C	DSA[b] treatment 15′, 37°C	Radioisotope 30′, 37°C	Incorporation[c] (DPM/10^7 cells/30 min)
–	–	1 μM CMP-[^{3}H]-NANA	1,290
+	–	1 μM CMP-[^{3}H]-NANA	9,800
+	8.5 mM	1 μM CMP-[^{3}H]-NANA	3,136
+	17 mM	1 μM CMP-[^{3}H]-NANA	1,960
–	–	1 μM [^{3}H]-Galactose	63,844
+	–	1 μM [^{3}H]-Galactose	65,060
+	8.5 mM	1 μM [^{3}H]-Galactose	49,380
+	17 mM	1 μM [^{3}H]-Galactose	44,553

[a] Pretreatment with Vibrio cholerae neuraminidase (VCN).

[b] DSA, diazonium salt of sulfonilic acid.

[c] Untreated or VCN-treated L1210 cells (5 × 10^7 cells/ml) were incubated 15 min at 37°C in the presence or absence of 8.5 mM or 17 mM diazonium salt of sulfonilic acid in RPMI 1640 HM, pH 7.0. Aliquots of cells (0.2 ml) were then placed in separate tubes and incubated in either 1 μM CMP-[^{3}H]-NANA or 1 μM [^{3}H]-galactose for 30 min at 37°C. Incorporated radioactivity expressed as DPM/10^7 cells/30 min was determined as described in the text. Cell viability at the end of the incubations with label was greater than 88% for all samples. The data represent the means of two experiments performed in duplicate.

Studies With UDP-[^{3}H]-Galactose

L1210 cells incubated with UDP-[^{3}H]-galactose incorporate radioactivity in a concentration-dependent manner (Table V). The addition of 1 mM cold galactose to an assay system containing 20 μM UDP-[^{3}H]-galactose substantially lowered the amount of incorporated galactose by 62%. A 51% decline in incorporation was noted at 2 μM UDP-galactose, and both studies suggest competition for uptake and incorporation between unlabeled galactose and [^{3}H]-galactose generated by the extracellular breakdown of the nucleotide sugar.

Electron Microscope Autoradiography

EM autoradiographs of L1210 cells incubated in 60 μM UDP-[^{3}H]-galactose or 30 μM [^{3}H]-galactose for 30 min, or of VCN-treated cells incubated in 10 μM CMP-[^{3}H]-NANA for 30 min, were prepared and analyzed. Only sugar incorporated into glycoconjugate and not that present in the cell as free sugar or nucleotide-sugar are retained after tissue processing. The radioactivity incorporated from CMP-[^{3}H]-NANA was clearly confined to the plasma membrane of L1210 cells (Fig 1). All cells were quantitatively similar in the amount of label at their surface. An analysis of grain distribution was performed (Table VI) and 84% of the total number of grains were found to be associated with this structure. Occasional grains were associated with other cellular compartments and were attributed to background or possibly to the incorporation of free [^{3}H]-NANA subsequent to extracellular breakdown of CMP-NANA to NANA (Table I).

In EM autoradiographs of cells incubated with UDP-[^{3}H]-galactose (Fig 2a, 2b), the majority (43%) of the grains were located over the Golgi apparatus, indicative of intracellular glycosylation. A similar grain distribution was found with cells incubated with [^{3}H]-galactose, where 40% of the grains were associated with the Golgi apparatus. Cellular grain distributions were calculated without regard to radiation scatter, which would not appreciably alter the grain distribution frequencies calculated in these studies. By paper chromatography, the purity of the commercial preparation of UDP-[^{3}H]-galactose was found to be nearly identical to that for UDP-[^{14}C]-galactose (Table I). Unlike sialic acid, free galactose generated extracellularly rapidly enters the cell and is incorporated by Golgi enzymes.

TABLE V. Effects of Unlabeled Galactose on the Incorporation (DPM/10^7 cells/30 min) of [^{3}H]-Galactose From UDP-[^{3}H]-Galactose*

	UDP-[^{3}H]-Galactose	
Additions	2 μM	20 μM
None	99	1,278
1 mM galactose	48	483

*1×10^7 cells were incubated with various concentrations of UDP-[^{3}H]-galactose in the presence or absence of 1 mM unlabeled galactose in a final volume of 200 μl for 30 min at 37°. Incorporation was measured as described earlier. Data expressed as the means of three experiments.

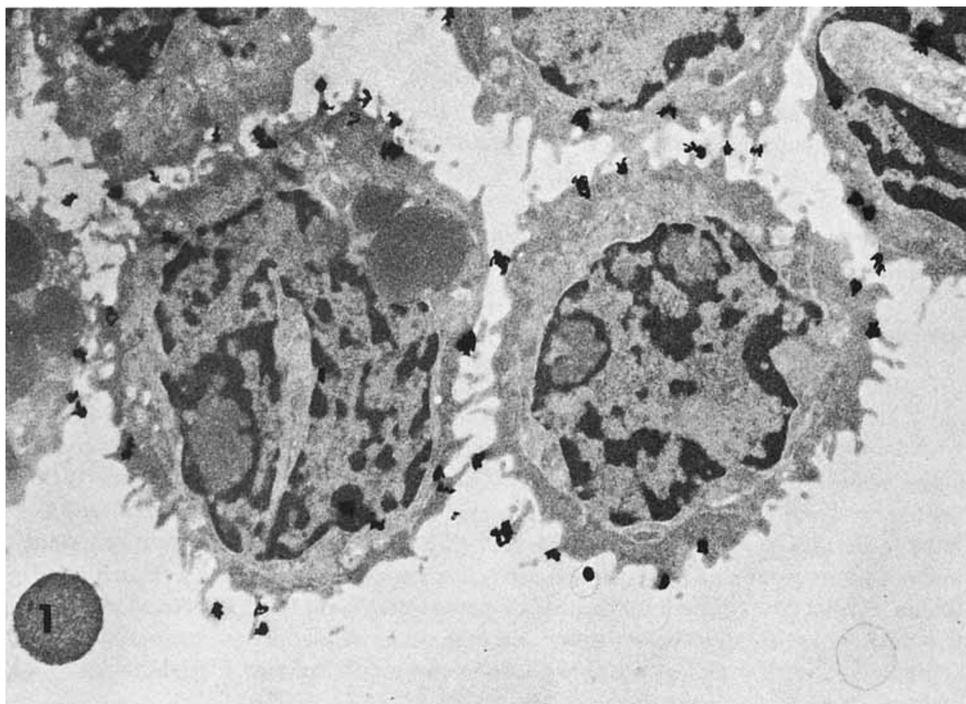

Fig 1. Electron microscope autoradiograph of L1210 cells incubated 30 min in the presence of 10 μM CMP-[^{3}H]-NANA. The majority of the silver grains, and hence the radioactivity, is associated with the plasma membrane (× 7,500).

TABLE VI. Distribution of Incorporated [^{3}H]-Sugars in L1210 Leukemic Cells as Determined by Electron Microscope Autoradiography*

Compartment	Silver grain distribution (% total)		
	CMP-[^{3}H]-NANA[a]	UDP-[^{3}H]-Galactose[b]	[^{3}H]-Galactose[c]
Plasma membrane	84	11	19
Cytoplasm	8	21	24
Lipid vacuole	1	8	5
Golgi apparatus	1	43	40
Nuclear membrane	0	1	7
Nuclei	6	6	4

* Exposure times varied between 1 and 3 months depending on the amount of radioisotope incorporated per cell.

[a] Cells were incubated in 10 μM CMP-[^{3}H]-NANA (2.33 Ci/mole) for 30 min at 37°. Grain distribution is based on 780 grains and 231 cells.

[b] Cells were incubated in 60 μM UDP-[^{3}H]-galactose (4.53 Ci/mmole) for 30 min at 37°. Grain distribution is based on 405 grains and 90 cells.

[c] Cells were incubated in 30 μM [^{3}H]-galactose (0.61 Ci/mmole) for 30 min at 37°. Grain distribution is based on 1,254 grains and 277 cells.

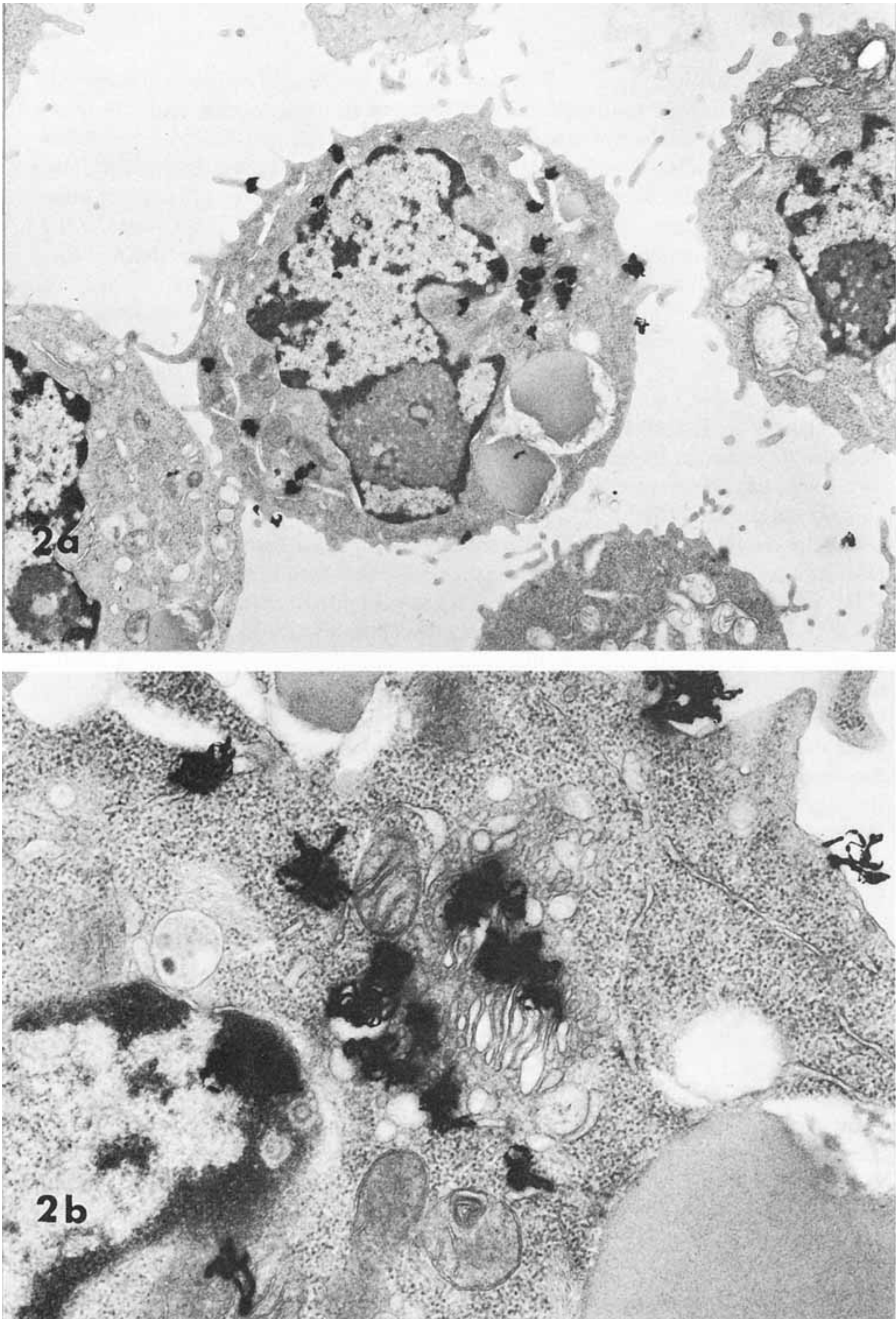

Fig 2. a) Radioactivity distribution of L1210 cells labeled for 30 min in the presence of 60 μM UDP-[^{3}H]-galactose. The grains are located over the cell interior, and in particular over the Golgi region. The grain distribution for cells with [^{3}H]-galactose appeared identical to this (× 9,000). b) Enlargement of portion of cell interior from Figure 2a, showing the membrane lamellae of the Golgi region and the associated silver grains (× 32,000).

DISCUSSION

Over the past few years, we have developed several lines of evidence in support of the contention that glycosyltransferases, in addition to being located at the Golgi apparatus, exist at the surface of certain mammalian cell types. Specifically, these studies involve the sialyltransferase of L-1210 cells and were initiated by the finding that intact cells, incubated in the presence of CMP-[^{3}H]-NANA, incorporate radioactivity into a macromolecular fraction [8]. This ectosialyltransferase system [5] is composed of the enzyme together with endogenous cell surface acceptor molecules. The activity of the enzyme system is limited by the availability of suitable acceptor molecules for sialylation. Thus pretreatment of whole cells with neuraminidase [8] increases enzyme activity 6–10 times presumably by generating new acceptor molecules at the surface. A number of desialylated glycoproteins, including desialylated fetuin, α_1-acid glycoprotein, and bovine submaxillary mucin, have been tested and found to be unsuitable as exogenous acceptor molecules for the L-1210 ectosialyltransferase (unpublished results). In fact, additional evidence suggests that the enzyme may be incapable of reacting with exogenous acceptors, since enzyme on one cell surface apparently does not sialylate acceptor molecules on another cell surface [10].

The enzyme assays described herein rely on the measurement of incorporated NANA onto endogenous acceptors by intact cells incubated in the presence of CMP-[^{3}H]-NANA. Fluctuations in enzyme activity may be due to changes in enzyme activity per se or to changes in endogenous membrane acceptor availability, since the latter is present in nonsaturating amounts. Increasing the concentration of CMP-NANA results in a nonlinear increase in incorporation approaching saturation in the millimolar range. At high concentrations of CMP-NANA more intracellular incorporation would be taking place due to the higher extracellular concentrations of free NANA. Awareness of these limitations are important in interpreting the results with this enzyme system. Recent findings in other laboratories [6, 7, 15] also suggest that the use of whole-cell enzyme systems, such as those we have employed, have similar limitations. Nucleotide-sugar substrates are degraded extracellularly and the free labeled sugars can be taken up and incorporated intracellularly by Golgi glycosyltransferases.

The possibility that whole cells may enzymatically degrade CMP-NANA to CMP and NANA was first noted by Kean and Bighouse [16], who detected such hydrolase activity on the surface of liver cells. In subsequent studies Deppert, Werchau, and Walter [6] and Hirschberg, Goodman, and Green [7] noted that nucleotide-sugars containing a diphosphate linkage were readily broken down by cells, while CMP-NANA was less rapidly degraded at pH 7.0. The present findings show that although a small amount of breakdown of both UDP-galactose and CMP-NANA occurs in media alone, there is no significant increase in degradation in the presence of L1210 cells alone. Though not relevant to our enzyme assay system, considerable hydrolytic activity towards CMP-NANA was observed with fetal calf serum, where 19% of the nucleotide-sugar was degraded within 30 min. This activity could not be completely eliminated by heat inactivation of the serum. We have noted previously [17] that the breakdown of CMP-NANA by serum hydrolases can be diminished by the addition of millimolar amounts of UTP.

Hirschberg, Goodman, and Green [7] and Hirschberg and Yeh [20] have recently found that cells are capable of transporting and incorporating small amounts of free NANA when incubated in high concentrations of the sugar. The present data confirmed this finding but clearly showed that incorporation from free NANA cannot account for

the levels of incorporation observed when VCN-pretreated cells were incubated with CMP-NANA. When incorporation levels from NANA and CMP-NANA were compared, VCN-treated cells incorporated over 17 times more sugar from CMP-NANA. These comparisons were made at equimolar concentrations but, as discussed above, less than 4% of the CMP-NANA was degraded in 30 min with cells. The total contribution of NANA in the whole-cell enzyme assays therefore becomes negligible. Consistent with the cell surface incorporation of CMP-NANA was the finding that uptake of sugar into the soluble cytosol (ethanol extract) was much higher for free NANA than CMP-NANA. Moreover, neuraminidase had no effect on the incorporation of free NANA, while increasing that from CMP-NANA by a factor of six to seven. As further evidence for the insignificance of free [^{3}H]-NANA incorporation in our ectoenzyme assays we have already found that the presence of 100-fold molar excess of free unlabeled NANA (1 mM) in the enzyme assay medium does not diminish the level of incorporation of radioactivity [5]. Inclusion of cytidine (1.25 mM) similarly does not effect enzyme activity, but CMP does decrease enzyme activity to 82% at 125 μM and to 28% at 1.25 mM [21]. No effect of CMP would be anticipated at 10 μM, the maximal concentration reached during the [^{3}H]-NANA incorporation studies reported in Table II.

Our EM autoradiographs of L1210 cells incubated with CMP-[^{3}H]-NANA (Fig 1) show that over 84% of the grains, and hence the incorporated sialic acid, was associated with the cell's plasma membrane. This percentage would probably have been higher had radiation scatter been taken into account when localizing each grain. The paucity of grains over the Golgi apparatus (< 1%) argues that the overall grain distribution which we have observed was not simply a reflection of the cellular distribution of sialic acid but an indication of a plasma membrane site for sialic acid incorporation. Even if all cellular sialic acid were associated with the plasma membrane, which in fact is not the case [19], a higher percentage of the radioactivity would be expected to fall over the Golgi apparatus if any of the incorporation was due to the uptake of free sialic acid. With the free sugar galactose, the majority of the grains were associated with the Golgi apparatus. This was expected, since the primary site for incorporation of a free sugar is the Golgi apparatus. The data in this report differ from those presented in an earlier study [5] in that the incubation periods were slightly longer and the grain distribution data are expressed as the percentage of the total cellular radioactivity (or grains) rather than as a normalized grain density. When expressed in this manner, the data are more relevant to biochemical data such as those regarding subcellular distribution of radioactivity.

In contrast to the evidence for sialyltransferase at the surface of L1210 cells, we were unable to obtain similar support for a galactosyltransferase at that location. When intact L1210 cells were incubated with UDP-[^{3}H]-galactose, they incorporated substantial amounts of sugar into a macromolecular fraction. The inclusion of a 40-fold molar excess of free galactose in the medium during these incubations significantly reduced the levels of incorporation by over 50%, suggesting competition with [^{3}H]-galactose for uptake and incorporation. A small but significant amount of UDP-galactose was found to be degraded during short incubations with cells. The free galactose thus generated could easily account for all of the cellular incorporation observed.

EM autoradiographs of these cells demonstrate a grain distribution nearly identical to that for cells incubated in [^{3}H]-galactose. After a 30-min incubation with either compound, approximately 40% of the grains were associated with the Golgi apparatus, 20% with the plasma membrane, and 20–25% with the remaining cytoplasm. There was no increase in grains over plasma membrane for cells incubated with UDP-[^{3}H]-galactose as

might be expected if an ectogalactosyltransferase were present there. The internal labeling of these cells is attributed to extracellular breakdown of UDP-[^{3}H]-galactose and the subsequent entry of the labeled sugar into the cell. Though low (1–3% of the added UDP-galactose), the amount of free [^{3}H]-galactose generated extracellularly would be sufficient to account for the total radioactivity incorporated by these cells. Thus, incorporation of [^{3}H]-galactose by L1210 cells incubated with UDP-[^{3}H]-galactose is due to glycosyltransferase located at the Golgi apparatus and not at the plasma membrane. This does not exclude the possibility that such enzymes are present at the plasma membrane but undetectable in the absence of appropriate acceptor molecules.

It is our experience, then, that whole-cell incubations with nucleotide-sugars in which endogenous acceptors are used vary in their validity as assays for ectoglycosyltransferases. In the case of sialyltransferase, no evidence was obtained to indicate that free sialic acid accounts partially or wholly for incorporation levels. This is due mainly to the relative impermeability of cells to this particular sugar. However, with UDP-galactose and possibly most other nucleotide-sugars, the free sugar released during whole-cell incubations probably accounts for most if not all of the observed incorporation, making such incubations unreliable as ectoglycosyltransferase assays. These findings are not relevant to ectoglycosyltransferase assays in which exogenous acceptors have been used, especially if they are linked to a nonphagocytizable carrier [3].

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