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Intralumenal pool and transport of CMP-*N*-acetylneuraminic acid, GDP-fucose and UDP-galactose

Study with plasma-membrane-permeabilized mouse thymocytes

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Treatment with NH_4Cl of mouse thymocytes renders their plasma membrane permeable to sugar nucleotides both inwards and outwards. Using this model, we studied the entry and utilization of CMP–NeuAc, GDP-Fuc and UDP-Gal into intracellular vesicles *in situ*. It is shown that CMP-NeuAc and GDP-Fuc enter the vesicles in a manner indicating a carrier-mediated transport (substrate saturation curve, inhibition by substrate analogues, temperature dependence) and are entrapped in their uncleaved form. This leads to the formation of an intralumenal pool of these precursors which can be further utilized by the sialyltransferases and fucosyltransferases. The occurrence of an endogenous pool of CMP-NeuAc and GDP-Fuc is demonstrated by the fact that, when the vesicles are disrupted by detergent, the release of the endogenous sugar nucleotides causes an isotopic dilution of the labelled precursors added to measure the glycosyltransferase activities. In contrast, no accumulation of UDP-Gal has been detected, suggesting that transport and transfer reaction are simultaneous events. However, experiments with UDP 2′,3′-dialdehyde indicate that UDP-Gal is not transported through the membrane by galactosyltransferase action but by a distinct carrier molecule.

Sugar nucleotides are donors for the biosynthesis of glycoconjugates and are used routinely to assay glycosyltransferases. In cells, most glycosyltransferase activities occur within the lumen of intracellular vesicles (Fleischer, 1981), whereas the sugar nucleotides are present in the cytoplasm on the other side of the membrane barrier, raising the question of how sugar nucleotides cross the membranes (Snider *et al.*, 1980; Hanover & Lennarz, 1982). Studies *in vitro* by Carey *et al.* (1980) have indicated that mouse liver microsomes can transfer CMP-NeuAc from the incubation medium into the lumenal compartment in a manner suggesting carrier-mediated transport. Subsequent to transport, a portion of the NeuAc moiety becomes covalently linked to the glycoconjugates facing the lumen of Golgi vesicles (Carey & Hirschberg, 1981). More recently, Sommers & Hirschberg (1982) have demonstrated that Golgi vesicles can transport GDP-fucose from the incubation medium into their lumen.

For UDP-Gal, Kuhn & White (1976, 1977), Kuhn *et al.* (1980), and Brandan & Fleischer (1982) presented indirect evidence for transport of UDP-Gal by rat Golgi membrane. Using rat-spleen lymphocytes, we had observed the occurrence of an intracellular pool of CMP-NeuAc (Hoflack *et al.*, 1979) and GDP-Fuc (Hoflack *et al.*, 1978) leading to isotopic dilution when sialyltransferases and fucosyltransferases are measured in cell homogenates with labelled sugar nucleotides. In contrast no isotopic dilution was observed when UDP-[^{14}C]Gal was used to measure the galactosyltransferase activity in rat spleen lymphocyte homogenates (Cacan *et al.*, 1977).

In the present paper, we demonstrate that thymocytes whose plasma membrane was rendered permeable to sugar nucleotides by NH_4Cl treatment (Cecchelli *et al.*, 1983) can be suitably used as a model for studying sugar nucleotide transport through intracellular vesicles and their intralumenal fate. This has been examined for CMP-NeuAc, GDP-Fuc and UDP-Gal.

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; Dol, dolichol.

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Materials and methods

Chemicals

The following radioactive sugars and sugar nucleotides from Amersham were used: CMP-[^{14}C]NeuAc (247mCi/mmol), GDP-[^{14}C]Fuc (180mCi/mmol), GDP-[^{14}C]Man (307mCi/mmol), UDP-[^{14}C]Gal (309mCi/mmol) and [^{14}C]NeuAc (247mCi/mmol). CMP, GDP, UDP, UDP 2',3'-dialdehyde and UDP-Gal were from Sigma. CMP-NeuAc was generously given by Dr. J. C. Michalski (Villeneuve d'Ascq, France).

Preparation of low-M_r acceptors

p-Nitrophenyl-β-D-galactoside and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were from Sigma; *p*-nitrophenyl-β-D-[^{3}H]galactoside was labelled by oxidation with galactose oxidase followed by reduction with KB^3H_4 (C.E.A., Saclay, France; 50Ci/mmol) as described by Bauvois *et al.* (1982). The final specific radioactivity of *p*-nitrophenyl-β-D-[^{3}H]galactoside was 1Ci/mg.

Preparation of NH_4Cl-treated thymocytes

Thymocytes were prepared from 4-week-old Swiss mice. Briefly, a suspension of thymocytes was prepared by pressing the thymus through a fine stainless steel gauze (0.5mm mesh) and subsequent washing with the medium described by Cox & Peters (1979). NH_4Cl treatment of cells was achieved by incubation in a medium containing 0.154M-NH_4Cl, 0.1mM-EDTA and 10mM-$KHCO_3$, pH7.4, at 37°C for 30min. After treatment, the cells were layered over a 66% Lymphoprep (Nyegaard Co., Norway) solution in the medium described by Cox & Peters (1979), and centrifuged at 1000*g* for 10min. As described by Cecchelli *et al.* (1983), this treatment gave a cell population which was permeable at 90% to Trypan Blue as judged by the exclusion test achieved with 0.2% (final concn.) Trypan Blue solution.

Incubation medium for the determination of transport and transfer activities

For transport and glycosyltransferase assays, the NH_4Cl-treated thymocytes were resuspended in the medium described by Cox & Peters (1979) at a concentration of 10^9cells/ml and all the assays were performed in this medium in a final volume of 100μl.

CMP-N-acetylneuraminic acid. When CMP-[^{14}C]NeuAc was used, standard assays were performed using 2μM-CMP-[^{14}C]NeuAc in a medium containing 5mM-$MnCl_2$. For the study of the concentration-dependence of CMP-NeuAc transport and transfer, the specific radioactivity of the precursor was brought to 57mCi/mmol with unlabelled CMP-NeuAc. When low-M_r exogenous acceptor was used, the incubation medium contained either 2μM-CMP-[^{14}C]NeuAc and 5mg of *p*-nitrophenyl-β-D-galactoside/ml or 1mM unlabelled CMP-NeuAc and 0.5mg of *p*-nitrophenyl-[^{3}H]galactoside/ml.

GDP-fucose. The incubation medium contained 2.5μM-GDP-[^{14}C]Fuc in the presence of 2mM-$MgCl_2$. As it has been reported that UMP is a potent inhibitor of GDP-Fuc hydrolysis (Hoflack *et al.*, 1978), 5mM-UMP was added to the incubation medium. When *p*-nitrophenyl-β-D-galactoside was used as exogenous acceptor, the concentration was 5mg/ml.

UDP-galactose. UDP-[^{14}C]Gal used in the incubation was brought to a specific radioactivity of 200mCi/mmol by the addition of unlabelled UDP-Gal. The incubation medium contained 5μM-UDP-[^{14}C]Gal, 5mM-$MnCl_2$ and 1mM-UMP to protect the precursor from the degradation (Verbert *et al.*, 1976). When *p*-nitrophenyl-β-D-glucosaminide was used as exogenous acceptor, the concentration was 0.5mg/ml.

GDP-mannose. The incubation medium contained 5μM-GDP-[^{14}C]Man in the presence of 5mM-$MgCl_2$ and 2mM-$MnCl_2$. In the incubation conditions (10min incubation) most of the covalently bound [^{14}C]mannose residues correspond to Dol-*P*-[^{14}C]Man when analysed after sequential extraction, as already described (Cacan *et al.*, 1982).

Determination of the total cell-associated radioactivity

After incubation with labelled sugar nucleotides, the total cell-associated radioactivity was a measure of the radioactivity entrapped within intracellular vesicles of NH_4Cl-treated thymocytes, i.e. the transport capacity. To separate cells from the radioactive incubation medium at the end of each incubation period the incubation mixture was transferred to a polypropylene microtube in which 200μM of Merck microscope immersion oil (density 1.01g/cm^3) had been layered on 225μl of Cox & Peters (1979) medium containing 30% (v/v) Lymphoprep (Lernmark *et al.*, 1975). The tube was centrifuged at 3000*g* for 30s and the cell pellet was collected after discarding the supernatant. Further washings in 0.154M-NaCl/0.1M-sodium cacodylate, pH7.4, lead to removal of the non-entrapped radioactive material, as demonstrated in the Results and discussion section (see Fig. 1). After these washings, the cells were dissolved in 200μl of formic acid (100°C for 10min) and the radioactivity was determined by counting in scintillation fluid with the external-ratio standardization method.

Determination of the trichloroacetic acid-precipitable radioactivity

The trichloroacetic acid-precipitable radioactivity was a measure of the transfer activity of the NH_4Cl-treated thymocytes on to the endogenous acceptors. For the determination of the covalently bound radioactivity, the cells were treated as described above, but after the final washing the pellet was precipitated with 5ml of 5% (w/v) trichloroacetic acid. Then, the precipitate was washed free of soluble radioactivity by repeated low-speed centrifugation in 5% (w/v) trichloroacetic acid. Determination of the radioactivity was made by dissolving the precipitate in 200 μl of formic acid (100°C for 10min). Counting was carried out in scintillation medium as described above.

Determination of the radioactivity incorporated in low-M_r acceptors

At the end of each incubation period in the presence of low-M_r exogenous acceptors, the radioactive products of the incubation mixture were analysed by descending paper chromatography (Whatman no. 3). Separation of [^{14}C]sialyl-α2-3-*p*-nitrophenyl-β-D-galactoside from [^{14}C]sialyl-α2-6-*p*-nitrophenyl-galactoside and from CMP-[^{14}C]NeuAc, [^{14}C]NeuAc and *p*-nitrophenyl-[^{3}H]galactoside was achieved in solvent system A [ethyl acetate/pyridine/water (10:4:3, by vol.)] as described by Bauvois *et al.* (1982). This same solvent system was used for the separation of [^{14}C]fucosyl-*p*-nitrophenyl-galactoside from GDP-[^{14}C]Fuc, [^{14}C]Fuc-1-*P* and [^{14}C]Fuc, and for the separation of the [^{14}C]galactosyl-*p*-nitrophenyl-*N*-acetylglucosaminide from UDP-[^{14}C]Gal, [^{14}C]Gal-1-*P* and [^{14}C]Gal.

Determination of CMP-NeuAc acid-soluble incubation products

For the determination of acid-soluble incubation products derived from CMP-NeuAc, the cells were treated as for the determination of the total cell-associated radioactivity but, after the final washing, the cell pellet was extracted with ethanol/water (2:1, v/v) and aliquots were analysed by descending paper chromatography in solvent systems B [ethanol/1M-ammonium acetate (7:3, v/v)] and C [pyridine/ethyl acetate/acetic acid/water (5:5:1:3, by vol.)]. It was checked that the radioactivity which was not released by this treatment was totally recovered in the acid-precipitated material.

Results and discussion

Permeable cells

We have previously shown that NH_4Cl treatment of thymocytes renders the plasma membranes permeable to sugar nucleotides (Cecchelli *et al.*, 1983). This effect is not reversible, since after several washings (0.154M-NaCl/0.1M-sodium cacodylate, pH7.4) NH_4Cl-treated cells still exhibit the same property, namely, the entry and utilization of sugar nucleotides as donors for the glycosylation of intracellular endogenous acceptors. To determine whether the NH_4Cl-induced permeability of the plasma membrane for sugar nucleotides is unidirectional or both inwards and outwards, we investigated if, after uptake of labelled sugar nucleotides by the leaky cells, these sugar donors could be eliminated by washings. In Fig. 1 is reported the effect of successive washings on the total cell-associated radioactivity and on the covalently bound radioactivity after incubation of NH_4Cl-treated cells with GDP-[^{14}C]Man (Fig. 1*a*) and CMP-[^{14}C]NeuAc (Fig. 1*b*). The results clearly indicated that with GDP-[^{14}C]Man, the cell-associated acid-soluble radioactivity can be entirely eliminated by the washings, although with CMP-[^{14}C]NeuAc a part of the acid-soluble radioactivity remains associated with the cells whatever the number of washings. In addition, in the case of GDP-Man, the acid-soluble radioactivity released from the cells by the first washing was identified as GDP-Man by paper chromatography. In the case of CMP-NeuAc, the acid-soluble radioactivity remaining associated with the cells, after the washings, could only be released after membrane disruption (see the Material and methods section), suggesting that it has been entrapped within vesicles. Identification by paper chromatography (solvent systems B and C) revealed that 95% of this acid-soluble radioactive material was CMP-[^{14}C]NeuAc and 5% was [^{14}C]NeuAc, presumably originating from the action of CMP-NeuAc hydrolase as already mentioned by Carey *et al.* (1980). These results demonstrate that these sugar nucleotides may enter the NH_4Cl-treated cells in their uncleaved form. Since they can be washed off, this indicates that the plasma membrane has been rendered permeable to sugar nucleotides both inwards and outwards. However, in the case of CMP-NeuAc, a proportion of this sugar donor remains entrapped in the permeabilized cells. How CMP-NeuAc can be accumulated is investigated below; using the same biological model, similar studies were performed with GDP-Fuc and UDP-Gal.

Transport of CMP-NeuAc

To determine whether the trapping of CMP-NeuAc in NH_4Cl-treated cells occurs within intracellular vesicles by simple diffusion or by a specific carrier-mediated transport, such as the one described as a Golgi marker activity by Sommers & Hirschberg (1982), we investigated the depend-

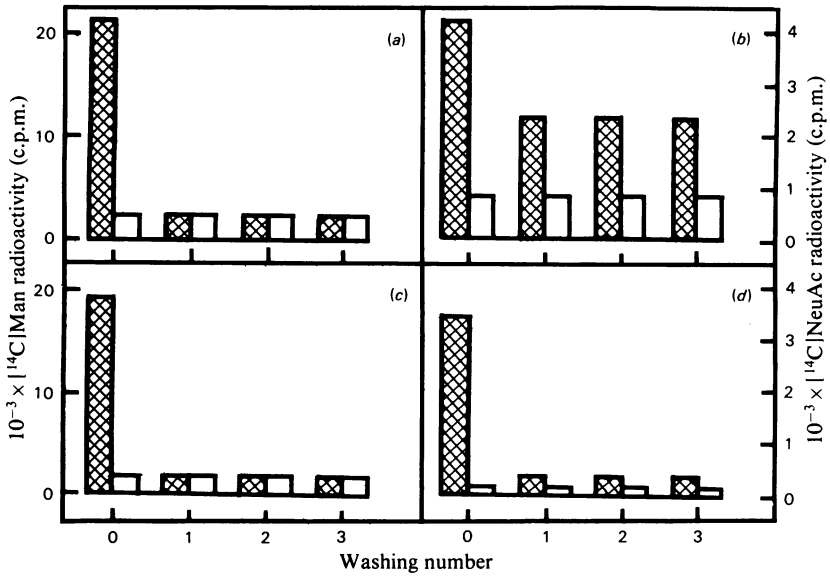

Fig. 1. *Effect of sequential washings after loading NH_4Cl-treated thymocytes with GDP-[^{14}C]Man or CMP-[^{14}C]NeuAc*
NH_4Cl-treated cells at a concentration of 10^9 cells/ml were incubated either with 5 μM-GDP-[^{14}C]Man (*a* and *c*) or with 2 μM-[^{14}C]NeuAc (*b* and *d*) in incubation medium for 10 min, either at 37°C (*a* and *b*) or at 0°C (*c* and *d*). After incubation, the cells were washed through a microscope oil immersion layer as indicated in the Materials and methods section. The total cell-associated radioactivity (hatched bars) and the covalently bound radioactivity (open bars) were measured on the cell pellet (washing number 0) or after each sequential washing. Most of the covalently bound [^{14}C]Man residues correspond to Dol-*P*-[^{14}C]Man, whose synthesis is known to be only slightly affected by low temperature.

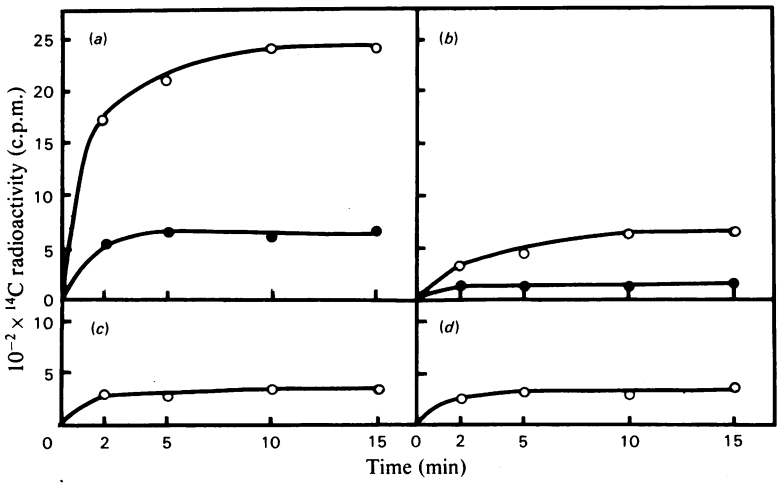

Fig. 2. *Time course of CMP-NeuAc and NeuAc penetration and transfer in NH_4Cl-treated thymocytes*
After incubation of 10^9 cells/ml at 37°C for the time indicated in the Figure, the total cell-associated radioactivity (○) and the trichloroacetic acid-precipitable radioactivity (●) were measured. (*a*) Incubation with 2 μM-CMP-[^{14}C]NeuAc; (*b*), incubation with 2 μM-CMP-[^{14}C]NeuAc in the presence of 5 mM-CMP; incubation with 2 μM-[^{14}C]NeuAc; (*d*) incubation with 2 μM-[^{14}C]NeuAc in the presence of 5 mM-CMP.

ence of penetration on time, temperature and sugar nucleotide concentration and the susceptibility to inhibition by nucleotides. Fig. 2(*a*) shows the accumulation of CMP-NeuAc as a function of time of incubation. The entry of CMP-NeuAc is very rapid for the first 2 min, after which the rate decreases. More interesting is the fact that at each time, as shown by the difference between total and

acid-precipitable radioactivity, free CMP-NeuAc remains trapped in the intracellular vesicles. CMP was a potent inhibitor of both entry and transfer reactions, with 80% inhibition at 5mM (Fig. 2*b*). When the same experiment was performed with 2 μM-[^{14}C]NeuAc, the accumulation was very low (Fig. 2*c*) and was not inhibited by 5mM-CMP (Fig. 2*d*), indicating a specific carrier-mediated transport for CMP-NeuAc. Fig. 3 shows the relationship between CMP-NeuAc concentration and the entry measured after 2min of incubation; the penetration of CMP-NeuAc was saturable with increasing concentration. The temperature-dependence of CMP-NeuAc accumulation is shown in Fig. 1(*d*); it has to be noted that the acid-soluble radioactivity associated with the cells can also be washed off at 0°C (Fig. 1*c*), thus indicating that the NH_4Cl-induced leakage was maintained at lower temperature. In contrast, the accumulation of CMP-[^{14}C]NeuAc depends on temperature and at 0°C it was less than 10% of that observed at 37°C.

The observations that transport of CMP-NeuAc is saturable, temperature-dependent and inhibited by CMP strongly suggest that this sugar nucleotide is translocated via a transport mechanism into vesicles. Part of this CMP-NeuAc is used as donor for the sialylation of endogenous acceptors but, as demonstrated by the analysis of the acid-soluble incubation products, the major proportion of this sugar nucleotide remains trapped in its uncleaved form. Previous observations (Cecchelli *et al.*, 1983) with rat liver and hen oviduct microsomal vesicles showed that NH_4Cl treatment does not affect the latency of the lumenal glucose-6-phosphatase marker enzyme; thus is can be assumed that accumulation of CMP-NeuAc occurs within sealed intracellular vesicles.

Additional proof for the validity of permeabilized thymocytes for studying sugar nucleotide transport: study with GDP-Fuc

It has been shown that GDP-Fuc, like CMP-NeuAc, enters Golgi vesicles via a transport mechanism (Sommers & Hirschberg, 1982). To demonstrate the validity of our model, similar studies have been performed with GDP-Fuc, and we report here that GDF-Fuc is transported via a carrier which is saturable and can be inhibited by substrate analogues.

Fig. 4 shows that when NH_4Cl-treated thymocytes are incubated in the presence of increasing concentrations of GDP-[^{14}C]Fuc, a concentration-dependent incorporation of radioactivity is observed. As described above for CMP-NeuAc, at each concentration a difference is found between the total and the acid-precipitable radioactivity, suggesting an accumulation of GDP-[^{14}C]Fuc into intracellular vesicles. In addition, 50% inhibition of the transport of GDP-Fuc is obtained in the

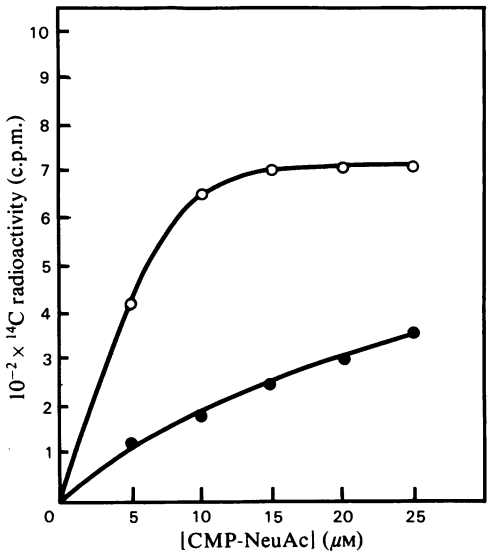

Fig. 3. *Concentration-dependence of CMP-[^{14}C]NeuAc transport and transfer activity*

NH_4Cl-treated cells at the concentration of 10^9 cells/ml were incubated for 2min at 37°C, with various concentrations of CMP-[^{14}C]NeuAc (57mCi/mmol). After incubation, the total cell-associated radioactivity (○) and the trichloroacetic acid-precipitable radioactivity (●) were measured.

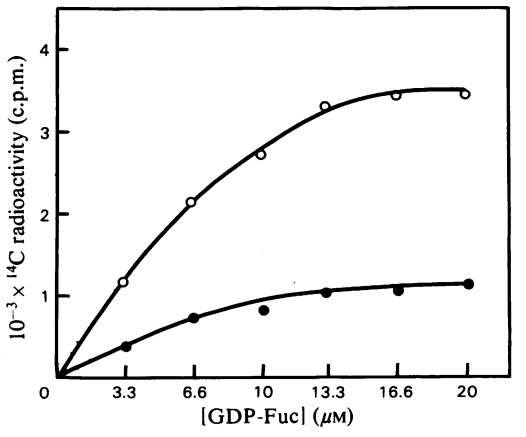

Fig. 4. *Concentration-dependence of GDP-[^{14}C]Fuc transport and transfer activity*

NH_4Cl-treated cells at the concentration of 10^9 cells/ml were incubated for 5min at 37°C with various concentration of GDP-[^{14}C]Fuc. After incubation, the total cell-associated radioactivity (○) and the trichloroacetic acid-precipitable radioactivity (●) were measured.

presence of 5mM-GDP, indicating the specificity of the transport of this sugar nucleotide.

The results obtained above with CMP-NeuAc and GDP-Fuc indicate that cells whose plasma membrane has been rendered leaky by NH_4Cl treatment provide a simple model to study the transport of sugar nucleotides into intracellular vesicles.

Transport of UDP-Gal

It has been demonstrated that galactosyltransferase activity was localized within the lumen of rat liver microsomes (Andersson & Eriksson, 1980; Fleischer, 1981) raising the question of how does UDP-galactose cross the membrane. Although the fate of the nucleotidyl moiety released by the galactosylation process has been reported (Kuhn & White, 1976, 1977; Brandan & Fleischer, 1982) the existence of an intralumenal pool of UDP-Gal is still speculative. However, studies from Fleischer & Brandan (1983) and Yusuf *et al.* (1983) indicated that a portion of the transported UDP-[^{14}C]Gal is recovered as unidentified acid-soluble radioactivity. Using our model we investigated whether a transport mechanism for UDP-[^{14}C]Gal leads to the accumulation of uncleaved UDP-[^{14}C]Gal. Fig. 5 shows that when permeabilized cells were incubated in the presence of 3 μM-UDP-[^{14}C]Gal, the acid-soluble radioactivity is low compared with covalently bound radioactivity and, in contrast to CMP-NeuAc and GDP-Fuc, no more acid-soluble radioactivity can be detected after 15min incubation. Presumably because of high galactosyltransferase activity, UDP-Gal does not accumulate. In this condition, the characterization of the nature of the acid-soluble labelled material was uncertain, and the occurrence of a specific carrier for UDP-Gal had to be assayed in another way. We have used UDP 2',3'-dialdehyde, which has been shown to cause a progressive inactivation of galactosyltransferase (Powell & Brew, 1976). This compound binds covalently to the enzyme by Schiff base formation and has been used to study the sidedness and role of galactosyltransferase (Cummings *et al.*, 1979; Shur & Hall, 1982). In our case, permeabilized cells were treated for 30min with 5mM-UDP dialdehyde and the unreacted UDP dialdehyde was washed off by low-speed centrifugation through an immersion oil layer as described in the Materials and methods section. After this treatment, the [^{14}C]Gal incorporation from UDP-[^{14}C]Gal to endogenous acceptors shows a 75% inhibition. However, the addition of 2% (v/v) Triton X-100 restores most (75% of the control) of the galactosyltransferase activity toward ovomucoid used as exogenous acceptor. This result indicates that UDP-Gal is transported by a carrier molecule

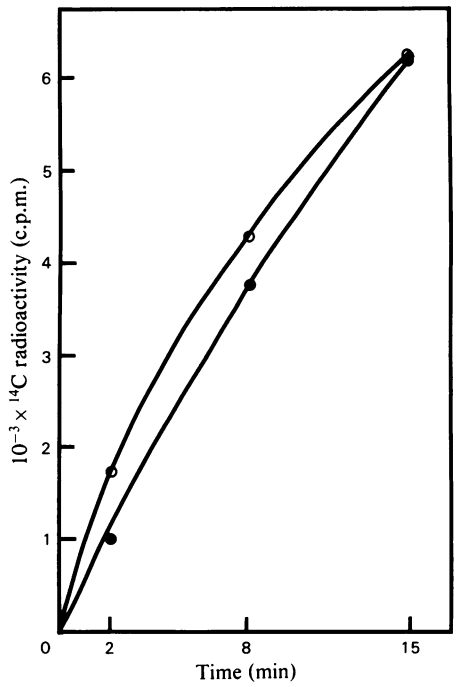

Fig. 5. *UDP-Gal penetration and galactosyltransferase activity in NH_4Cl-treated thymocytes*

NH_4Cl-treated cells at the concentration of 10^9 cells/ml were incubated with 3 μM-UDP-[^{14}C]Gal at 37°C for the time indicated in the Figure. After incubation, the total cell-associated radioactivity (○) and the trichloroacetic acid-precipitable radioactivity (●) were measured.

which can be blocked without affecting the catalytic activity of the galactosyltransferase.

Occurrence of intravesicular pools of sugar nucleotides

The results obtained above establish the existence of a carrier mechanism for sugar nucleotides leading, *in vitro*, to the transport of CMP-NeuAc, GDP-Fuc and UDP-Gal and to the accumulation of the two former within intracellular vesicles. If these transport mechanisms occur *in vivo*, endogenous pools of the respective sugar nucleotides must exist, as already suggested for CMP-NeuAc by Hoflack *et al.* (1979). As reported in Fig. 6, when sialyltransferase (Fig. 6*a*) and fucosyltransferase (Fig. 6*b*) were assayed with increasing concentrations of Triton X-100 using labelled sugar nucleotides and unlabelled exogenous acceptor [*p*-nitrophenyl-galactoside, Bauvois *et al.* (1982); Chester *et al.* (1976)] a decreased incorporation is observed. In fact, when *p*-nitrophenyl-[^{3}H]galactoside is used as exogenous acceptor, with unlabelled CMP-NeuAc, the transfer activity is stimulated

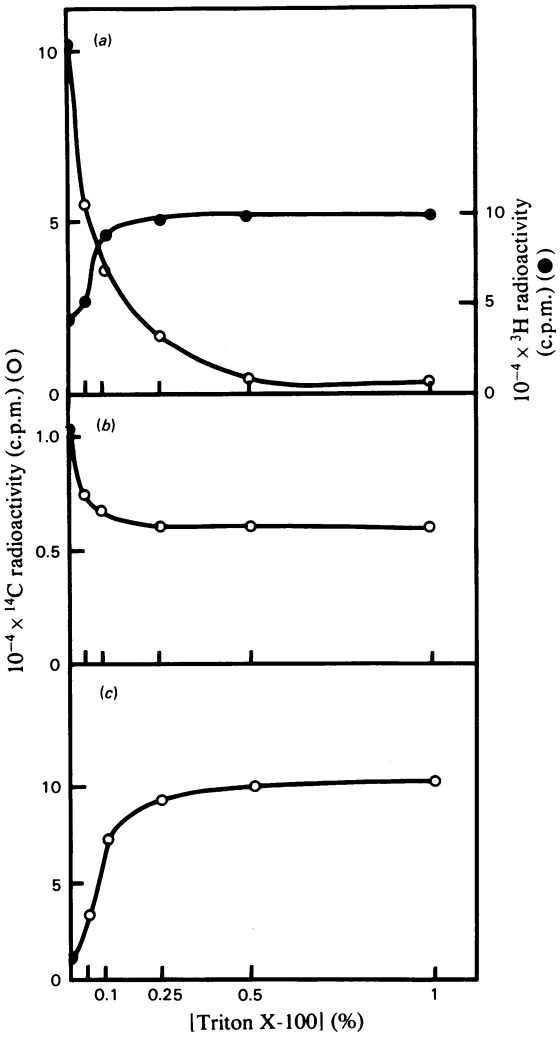

Fig. 6. *Occurrence of intravesicular pools of sugar nucleotides*

NH_4Cl-treated cells at the concentration of 10^9 cells/ml were incubated at 37°C for 60min in the presence of sugar nucleotide and of *p*-nitrophenyl-glycoside as exogenous acceptor with various concentrations of Triton X-100. After incubation, the glycosylated acceptors were isolated by paper chromatography in solvent system A. (*a*) Formation of sialyl-α2-3-*p*-nitrophenyl-galactoside: ○, incubations with 2μM-CMP-[^{14}C]NeuAc and 5mg of *p*-nitrophenyl-galactoside/ml; ●, incubations with 1mM-CMP-NeuAc and 0.5mg of *p*-nitrophenyl-[^{3}H]galactoside/ml. (*b*), Formation of fucosyl *p*-nitrophenyl galactoside when incubations were performed with 2.5μM-GDP-[^{14}C]Fuc and 5mg of *p*-nitrophenyl-galactoside/ml. (*c*), Formation of galactosyl *p*-nitrophenyl-*N*-acetylglucosaminide when incubations were performed with 5μM-UDP-[^{14}C]Gal and 0.5mg of *p*-nitrophenyl-*N*-acetyl-glucosaminide/ml.

about 2.5-fold by the addition of Triton X-100 (Fig. 6*a*), indicating that the previously observed decreased incorporation is not due to an inactivation of the sialyltransferase by the detergent but is due to an isotopic dilution of the CMP-[^{14}C]NeuAc by endogenous CMP-NeuAc. This result shows the existence of an intravesicular pool formed *in situ* which is maintained during the NH_4Cl treatment, proving that the intracellular vesicles are sealed and that the accumulation due to the transport mechanism of these sugar nucleotides occurs *in vivo*. Conversely, then the galactosyltransferase is assayed with increasing concentration of Triton X-100, using UDP-[^{14}C]Gal and unlabelled *p*-nitrophenyl-*N*-acetylglucosaminide, we observed a 4.5-fold increase of the formation of [^{14}C]galactosyl-*p*-nitrophenyl-*N*-acetylglucosaminide. This result demonstrates that no intralumenal pool of UDP-Gal can be detected *in vivo*.

Conclusions

Cell permeabilization is a technique which allows the utilization of radiolabelled sugar nucleotides as direct donors to examine intracellular glycosylation process (Cecchelli *et al.*, 1983; Rudick *et al.*, 1983). The results presented in the present paper demonstrate that in thymocytes whose plasma membrane has been rendered leaky by NH_4Cl treatment the sugar nucleotides can enter the cell and, as has been demonstrated using labelled GDP-Man, they can be washed off. With this sugar nucleotide, the cell-associated radioactivity is entirely covalently bound, contrary to what is observed with CMP-NeuAc and GDP-Fuc, which can be entrapped in their uncleaved form in intracellular vesicles. As already described by Sommers & Hirschberg (1982) with Golgi vesicles, we demonstrated with plasma membrane-permeabilized cells that the accumulation of these two sugar nucleotides is saturable, can be inhibited by substrate analogues, and is temperature dependent. These NH_4Cl-treated cells offer a model to study the translocation of sugar nucleotides through intracellular membranes. In contrast, no accumulation of UDP-Gal has been observed, although a carrier-mediated transport of this sugar nucleotide is shown by using inhibition experiments with UDP dialdehyde. In agreement with these results, we demonstrate the occurrence of an intralumenal pool of CMP-NeuAc and GDP-Fuc, although no such a pool can be detected for UDP-Gal.

The question raised by this study is the regulation of the glycosylation process. For galactosylation, a suitable acceptor would be the only signal for the transfer reaction if donor is present on the other side of the membrane. In contrast, the regulation of sialylation and fucosylation would depend

not only on the availability of acceptors but also on a sufficient intravesicular concentration of donors. The carrier and the transferase might be two keys for the regulation of the transfer of these two terminal sugars.

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