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Glycosylation of proteins from sugar nucleotides by whole cells

Effect of ammonium chloride treatment on mouse thymocytes

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When thymocytes are treated with iso-osmotic NH_4Cl, the sugar incorporation into endogenous acceptors from labelled sugar nucleotides is largely increased compared with that in control thymocytes. This effect was obtained with labelled GDP-mannose, UDP-galactose and CMP-*N*-acetylneuraminic acid. The stimulation observed with NH_4Cl-treated thymocytes does not involve the glycosylation of exogenous acceptors, and it was proved that the NH_4Cl treatment (1) does not stimulate glycosyltransferase activities themselves, (2) does not lead to the release of soluble glycosyltransferases as the result of an extensive lysis of the thymocytes and (3) does not cause the emergence of glycosyltransferases at the cell surface. In fact, electron-microscopy observations showed that, although marked changes had occurred in the cytoplasm, the plasma membrane is sufficiently maintained to allow the cell to keep roughly its original shape and to retain the intracellular vesicles. We thus demonstrate that this stimulation is due to an enhancement of the entry of sugar nucleotides into the cell. As demonstrated by the inclusion of Trypan Blue within the cells, and the non-stimulation of glycosylation of exogenous large-molecular-mass acceptors, the effect of NH_4Cl seems to be limited to the penetration of small-molecular-sized compounds through the plasma membrane. Thus NH_4Cl treatment allows the labelled sugar nucleotides to penetrate the cell and to behave as the cellular pool to be utilized for glycosylation by intracellular vesicles.

Assembly of carbohydrate chains of glycoproteins proceeds by sequential attachment of each sugar unit to the growing glycan. The glycosyltransferases involved in this process are bound to intracellular membranes and mainly to the rough endoplasmic reticulum and Golgi apparatus. As demonstrated by Coates *et al.* (1980), Carey *et al.* (1980) and Sommers & Hirschberg (1982), sugar nucleotides, which are the initial donors in each transfer reaction, are synthesized in the cytoplasm for most of them and in the nucleus for CMP-*N*-acetylneuraminic acid. Thus study of glycoprotein biosynthesis can be performed in two ways: either with whole cells with labelled monosaccharides, which can penetrate the cells, being further activated into sugar nucleotides and incorporated into nascent glycoproteins, or with isolated rough endoplasmic reticulum or Golgi-derived vesicles, which require labelled sugar nucleotides as precursors.

In the present paper we describe a different approach, in which NH_4Cl treatment of whole cells leads to the intracellular utilization of labelled sugar nucleotides to be incorporated into lipid intermediates and nascent cellular glycoproteins.

Materials and methods

Chemicals

All reagents were of analytical grade. GDP-[^{14}C]mannose, UDP-[^{14}C]galactose and CMP-*N*-acetyl[^{14}C]neuraminic acid were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. UDP-*N*-acetylglucosamine, AMP and UMP were from Sigma Chemical Co., St. Louis, MO, U.S.A. RPMI-1640 medium and 0.5% Trypan Blue solution were from GIBCO Laboratories, Grand Island, NY, U.S.A.

Cell preparation

Thymocytes were prepared from 4-week-old Swiss mice. Briefly, suspensions of thymocytes were prepared by pressing the thymus through a fine stainless-steel gauze (0.5 mm mesh) and subsequent washings with RPMI-1640 medium. NH_4Cl treatment of cells was performed by resuspension in the

following medium: 0.154M-NH_4Cl/0.1mM-EDTA/10mM-$KHCO_3$, pH 7.4. Unless otherwise stated, the total duration (including centrifugation period) of NH_4Cl treatment was 15min at room temperature. The cells were further resuspended in the medium described by Cox & Peters (1979). The Trypan Blue-exclusion test was performed with 0.2% (final concn.) Trypan Blue solution in the medium described by Cox & Peters (1979).

Glycosyltransferase assays

For glycosyltransferase assays the cells were resuspended in the medium described by Cox & Peters (1979) at a concentration of 10^9cells/ml and the assays were performed in a final volume of 100μl. With regard to the glycosyltransferases involved in the dolichol cycle, the assays were performed with unlabelled 20μM-UDP-*N*-acetylglucosamine and 6μM-GDP-[^{14}C]mannose (180c.p.m./pmol) and the sequential lipid extraction was as described by Cacan *et al.* (1982).

Galactosyltransferase assays were performed with 6μM-UDP-[^{14}C]galactose (200c.p.m./pmol) in medium containing 10mM-$MnCl_2$, 5mM-UMP and 5mg of ovomucoid/ml as exogenous acceptor (Verbert *et al.*, 1976). Sialyltransferases were assayed with 2.5μM-CMP-*N*-acetyl[^{14}C]neuraminic acid (250c.p.m./pmol) in medium containing 5mM-$MnCl_2$ and 5mg of asialofetuin/ml as exogenous acceptor (Cacan *et al.*, 1977). Asialofetuin was prepared by mild acid hydrolysis (0.1M-trifluoroacetic acid, 80°C, 30min) of fetuin. Radioactivity incorporated into the exogenous acceptors was measured by difference between two assays, one with and one without exogenous acceptor. Recovery of acid-precipitable material, analysis of precursor degradation and counting of radioactivity were performed as previously described (Verbert *et al.*, 1976).

Marker enzymes

Lactate dehydrogenase activity was measured (Wroblewski & La Due, 1955) as a control of cell leakage. Glucose-6-phosphatase activity was measured as described by Beaufay *et al.* (1974) and the liberated inorganic phosphate was assayed by the method of Fiske & SubbaRow (1925).

Electron microscopy

Thymocytes were fixed in 2.5% (v/v) glutaraldehyde in 0.1M-cacodylate buffer, then post-fixed for 2h in 1% osmium tetroxyde in the same buffer. Embedding was in Epon. Sections were examined with a Siemens Elmiskop I or JEOL 120 CX electron microscope.

Results and discussion

Effect of NH_4Cl treatment of thymocytes on lipid intermediate formation and glycosylation

It has been shown previously that whole splenocytes are able to utilize exogenous GDP-mannose to synthesize dolichyl phosphate mannose and to form dolichyl diphosphate oligosaccharides on the addition of UDP-*N*-acetylglucosamine, and further transfer on to proteins was observed (Cacan *et al.*, 1980). When similar experiments were performed with thymocytes, low activities of these glycosyltransferases were recovered (Fig. 1), indicating poor cell-surface activities. These differences could be

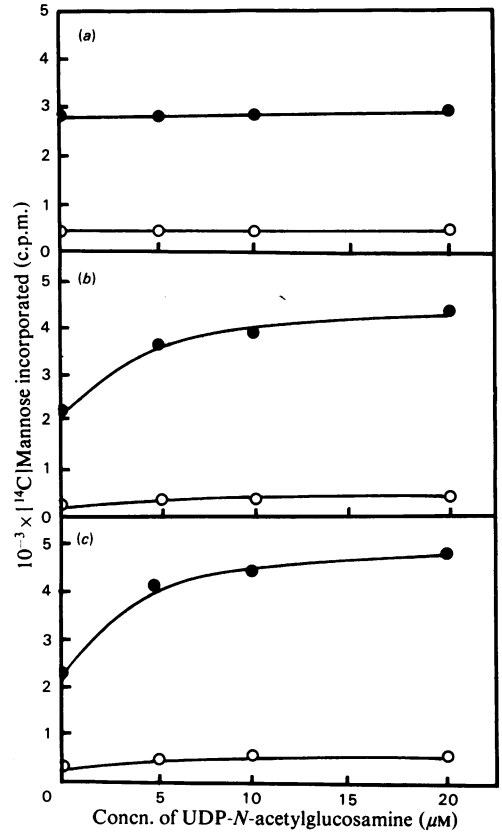


Fig. 1. *Effect of UDP-N-acetylglucosamine concentration on the incorporation of* [^{14}C]*mannose from GDP-*[^{14}C]*mannose into lipid intermediates and proteins by thymocytes and NH_4Cl-treated thymocytes*

Incubations were performed as described in the Materials and methods section with GDP-[^{14}C]-mannose at 30°C for 30min with thymocytes (○) and NH_4Cl-treated thymocytes (●). After sequential lipid extraction, the radioactivity was measured in the chloroform/methanol extract (*a*), in the chloroform/methanol/water extract (*b*) and in the protein pellet (*c*).

due to the origin of the cells, but, as splenocyte preparations require erythrocyte lysis by NH_4Cl (Agostoni & Ideo, 1965), it was checked whether this treatment was responsible for the observed phenomenon. In fact, when thymocytes were treated for 15 min with iso-osmotic NH_4Cl (0.154 M) and then washed, the incorporation into dolichyl phosphate mannose, dolichyl diphosphate oligosaccharides and proteins was considerably enhanced, about 10-fold (see Fig. 1).

This effect was not restricted to enzymes dealing with lipid intermediates, but was also observed with other glycosyltransferases such as galactosyltransferase and sialyltransferase (Figs. 2*a* and 2*c*). It has to be noted that for both transferases an increase is observed of transfer to endogenous acceptors but transfer activity towards exogenous acceptors is not changed at all (Figs. 2*b* and 2*d*). Thus NH_4Cl treatment of thymocytes stimulates utilization of sugar nucleotides for the glycosylation of endogenous acceptors, but does not affect the glycosylation of non-permeant macromolecular exogenous acceptors.

This effect of NH_4Cl treatment could be explained in four ways: (1) a direct stimulation effect on glycosyltransferase activities themselves; (2) an increased amount of broken cells and subsequent release of intracellular enzymes; (3) a remodelling of plasma membrane with the emergence of intracellular glycosyltransferases, as NH_4Cl is known to perturb the intracellular membrane traffic (Tietze *et al.*, 1980); (4) a permeation of plasma membrane to sugar nucleotides promoted by NH_4Cl, thus allowing their intracellular utilization for glycosylation.

Does NH_4Cl directly stimulate glycosyltransferase activities?

Sialyltransferase and galactosyltransferase activities were solubilized from thymocytes in 0.05% and 2% Triton X-100 respectively and assayed under optimal conditions, in the presence of increasing concentrations of NH_4Cl. Fig. 3 shows that NH_4Cl leads to inhibition of galactosyltransferase and

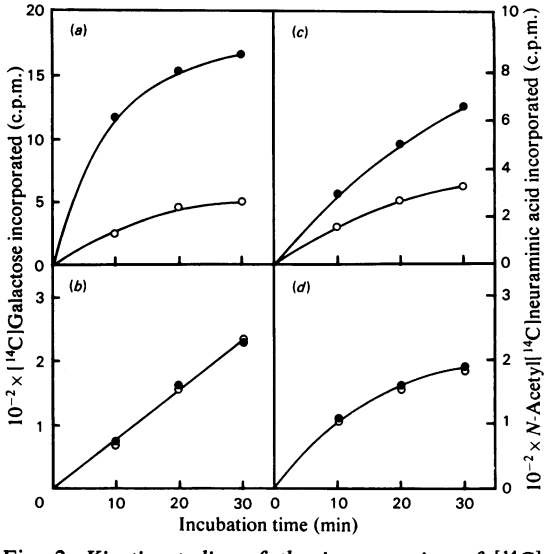


Fig. 2. *Kinetic studies of the incorporation of* [^{14}C]*galactose and N-acetyl*[^{14}C]*neuraminic acid from UDP-*[^{14}C]*galactose and CMP-N-acetyl*[^{14}C]*neuraminic acid into endogenous and exogenous acceptors by thymocytes and NH_4Cl-treated thymocytes*

Incubations were performed as described in the Materials and methods section with UDP-[^{14}C]-galactose (*a* and *b*) or CMP-*N*-acetyl[^{14}C]neuraminic acid (*c* and *d*) at 30°C with thymocytes (○) and NH_4Cl-treated thymocytes (●). The incorporation was measured, after acid precipitation, into endogenous acceptors (*a* and *c*) and into exogenous acceptors: ovomucoid (*b*) and asialofetuin (*d*).

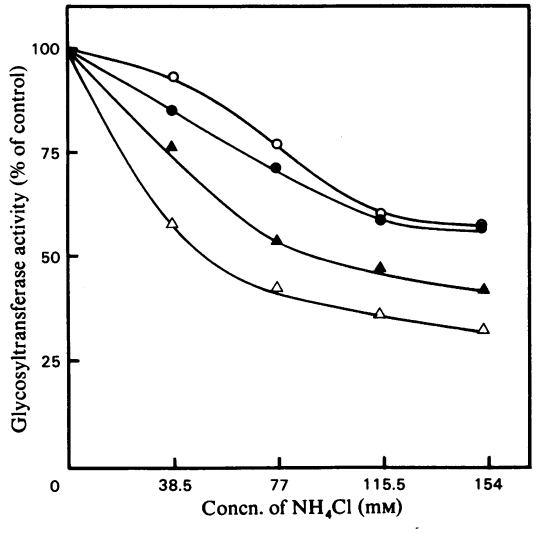


Fig. 3. *Effect of NH_4Cl concentration on galactosyltransferase and sialyltransferase activities.*

Incubations were performed as described in the Materials and methods section with UDP-[^{14}C]-galactose in the presence of 2% Triton X-100 (○ and ●) or with CMP-*N*-acetyl[^{14}C]neuraminic acid (△ and ▲) in the presence of 0.5% Triton X-100 at 30°C for 30 min with increasing concentrations of NH_4Cl, the ionic strength being kept constant by the addition of NaCl. The incorporation of [^{14}C]-galactose was measured into endogenous acceptors (○) and into ovomucoid (●). The incorporation of *N*-acetyl[^{14}C]neuraminic acid was measured into endogenous acceptors (△) and into asialofetuin (▲). The results are expressed as percentages of the activity measured without the NH_4Cl treatment.

sialyltransferase, and a 50% inhibition is obtained at 0.154M whether the activities were measured on endogenous or on exogenous acceptors. A similar effect was observed on the formation of lipid intermediates (results not shown). Thus the stimulating effect observed with NH_4Cl-treated cells cannot be due to contamination of NH_4Cl remaining after washing of cells, as this salt would have led, in contrast, to an inhibition.

Is the NH_4Cl stimulation effect due to broken cells?

To estimate the extent of cell lysis after NH_4Cl treatment the lactate dehydrogenase activity was measured in the cell suspension. The total lactate dehydrogenase activity was obtained by complete cell lysis with 0.5% Triton X-100. Under these conditions the percentage of broken cells in the control was about 0.6%, and never higher than 0.7% when cells were treated with NH_4Cl, showing that the percentage of broken cells in the initial cell preparation was similar to that in the NH_4Cl-treated cells. More importantly, no glycosyltransferase activity was recovered in the supernatant after low-speed centrifugation of the NH_4Cl-treated cells.

Does NH_4Cl allow the emergence of intracellular enzymes at the cell surface?

It is now well known that addition of 5–10mM-NH_4Cl for 24h in cell-culture medium induces modification of intracellular traffic, such as lysosomal secretion (Tietze *et al.*, 1980). Such events could be accompanied by the arrival at the cell surface of intracellular membrane-bound glycosyltransferases. If that were the case, intracellular membrane-bound enzymes such as glucose-6-phosphatase (marker of the luminal side of endoplasmic reticulum) could be detected on the cell outer surface. In our case, NH_4Cl treatment of thymocytes did not enhance the glucose-6-phosphatase activity expressed by thymocyte suspensions. This result is confirmed by the fact that enhancement of glycosyltransferase activity after NH_4Cl treatment is restricted to endogenous acceptors. The fact that glycosylation of exogenous macromolecular acceptors, accessible only from the outside of the cell, is not affected indicates that no glycosyltransferases have emerged to the cell surface.

Does NH_4Cl render plasma membrane permeable to sugar nucleotides?

After treatment with 0.154M-NH_4Cl, the thymocytes were recovered by low-speed centrifugation and assayed for sialyltransferase activity, a sample also being submitted to the Trypan Blue-exclusion text. As shown in Fig. 4, the proportion of cells including Trypan Blue increased with the duration of the treatment, and it is noteworthy that the sialyltransferase activity towards endogenous acceptors is

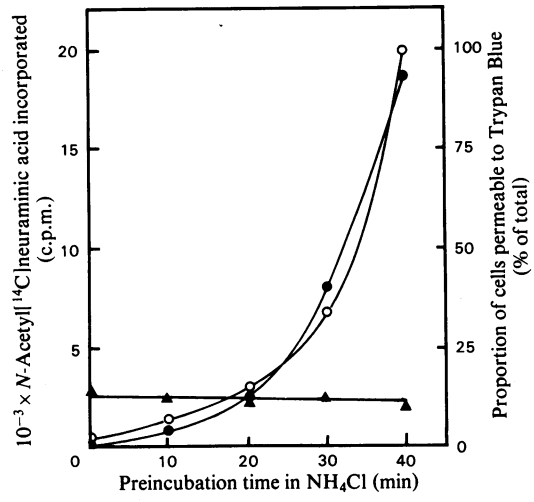


Fig. 4. *Correlation between the incorporation of N-acetyl[^{14}C]neuraminic acid from CMP-N-acetyl[^{14}C]-neuraminic acid into endogenous acceptors and the permeability of cells to Trypan Blue*

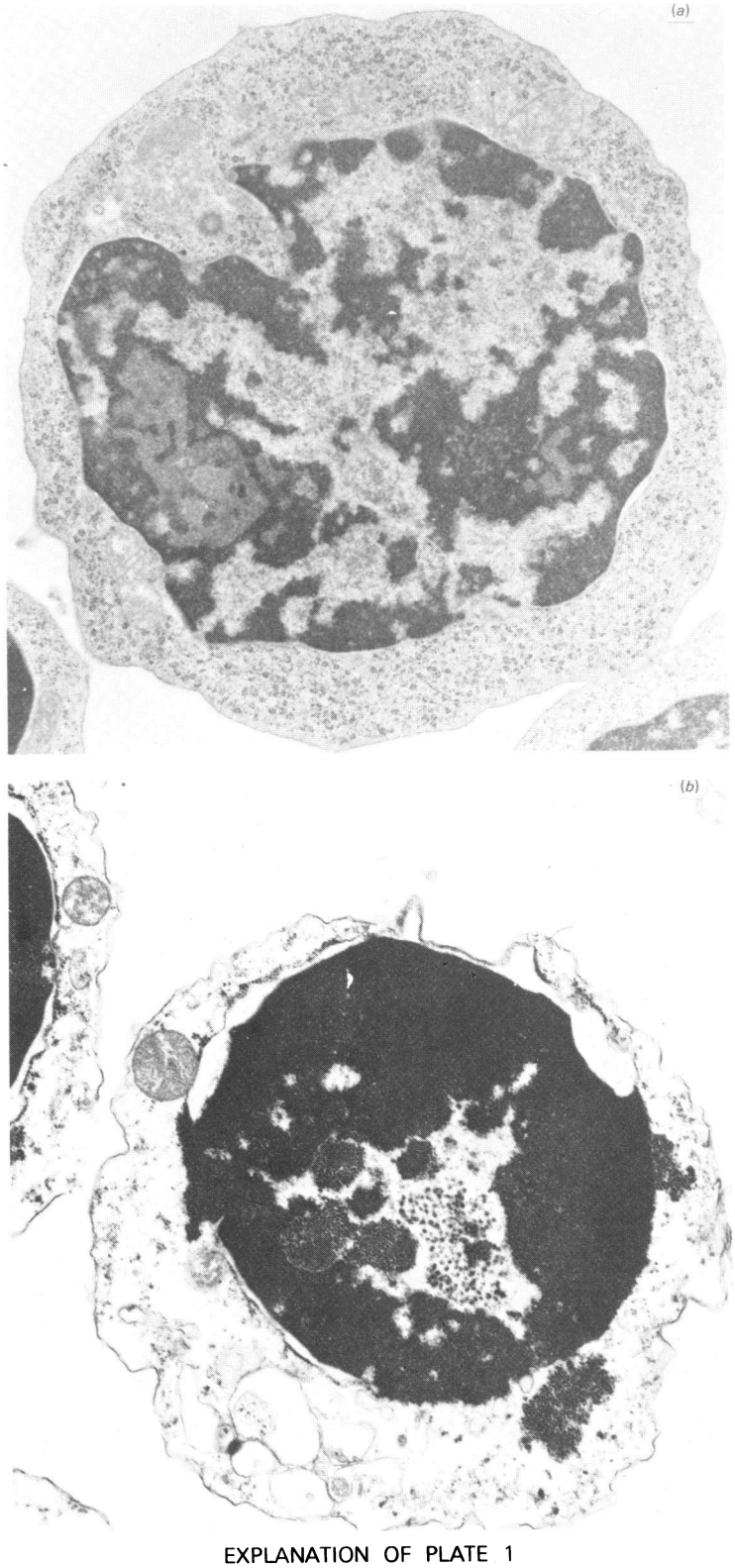
Thymocytes were treated with NH_4Cl for times ranging from 0 to 40min. After this preincubation cells were washed and the incorporation of *N*-acetyl[^{14}C]neuraminic acid from CMP-*N*-acetyl-[^{14}C]neuraminic acid at 30°C during 30min was allowed to proceed as described in the Materials and methods section. The incorporated radioactivity in endogenous acceptors (○) and in asialofetuin (▲) was measured. Before each incubation the percentage of cells that are permeable to Trypan Blue (●) was determined as described in the Materials and methods section.

strictly proportional to the number of Trypan Blue-permeable cells, although the sialyltransferase activity measured on asialofetuin remains constant.

These results suggest that sialyltransferase activity occurs in two different cellular locations: one exhibited by intact cells and unaffected by NH_4Cl treatment and being active on exogenous macromolecular acceptors mainly (Fig. 4), the other being active on intracellular endogenous acceptors when NH_4Cl treatment has rendered the cells permeable to CMP-*N*-acetylneuraminic acid. Similar results were obtained for galactosyltransferase with ovomucoid as exogenous acceptor (results not shown), indicating that permeation is also observed for UDP-galactose.

Additional proof for the effect of NH_4Cl treatment

After incubation of thymocytes with sugar nucleotide (either UDP-galactose or CMP-*N*-acetylneuraminic acid) for 40min in order to saturate cell-surface sites of glycosylation, the thymocytes were washed in the absence or in the presence of NH_4Cl,



EXPLANATION OF PLATE 1

Electron micrographs of intact and NH_4Cl-treated thymocytes

(*a*) Untreated thymocytes; (*b*) thymocytes that were treated with 0.154 M-NH_4Cl during 40 min. Magnification ×18000.

(*facing p.* 684)

and they were then further incubated again with the same sugar nucleotide. Fig. 5 shows that, although cell-surface sites of glycosylation were saturated (no increase in the control), an increase of the glycosylation was observed only when cells were treated with NH_4Cl, this being due to intracellular utilization of the labelled sugar nucleotides.

These experiments reinforce the observation of two cellular sites of utilization of sugar nucleotides: the first one, exhibited by whole cells, and presumably corresponding to cell-surface glycosyltransferase activities (for review see Pierce *et al.*, 1980), and the second one, representing the major proportion of the activity, being located inside the cell but accessible to sugar nucleotides owing to cell leakage after NH_4Cl treatment. The fact that the first site is saturable without affecting the second one indicates that the two sites are cytologically separated.

Electron-microscopy observations

The effect of NH_4Cl treatment was examined by electron microscopy. Plate 1 shows a comparison between control thymocytes and NH_4Cl-treated thymocytes (40min treatment was used to obtain a cell population that is totally permeable to Trypan Blue, as indicated in Fig. 4). It was observed that, although a marked change occurred in the cytoplasm, as shown by the disappearance of ribosomes, the plasma membrane is maintained sufficiently to allow the cell to keep roughly its original shape and to retain the intracellular organelles. More interesting is the fact that cytomembrane structures are still present and clearly identifiable. Thus the NH_4Cl-treated thymocytes appear more as ghosts into which sugar nucleotides can freely enter, substituting the cytosolic pool, and being utilized by the glycosyltransferases of intracellular vesicles.

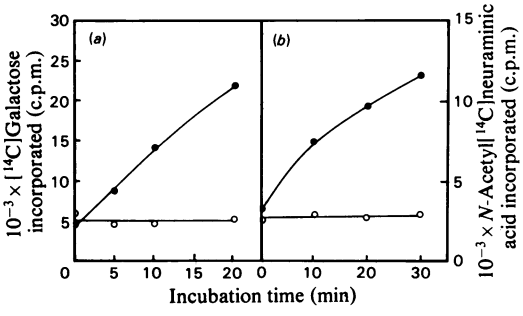


Fig. 5. *Kinetic studies of the incorporation of* [^{14}C]*galactose and N-acetyl*[^{14}C]*neuraminic acid after saturation of cell-surface sites of glycosylation*

Thymocytes were first incubated wtih UDP-[^{14}C]-galactose (*a*) or CMP-*N*-acetyl[^{14}C]neuraminic acid (*b*) as described in the Materials and methods section at 30°C for 40min, in order to saturate cell-surface sites of glycosylation. Cells were then washed in the absence (○) or in the presence (●) of NH_4Cl and then re-incubated with UDP-[^{14}C]-galactose (*a*) or CMP-*N*-acetyl[^{14}C]neuraminic acid (*b*) at 30°C. The incorporation of [^{14}C]galactose and *N*-acetyl[^{14}C]neuraminic acid into endogenous acceptors was measured after acid precipitation.

Conclusions

In the investigation described in the present paper the initial observation was an enhanced utilization of labelled GDP-mannose by NH_4Cl-treated thymocytes for the synthesis of lipid intermediates. This effect was obtained with labelled UDP-galactose and CMP-*N*-acetylneuraminic acid, and further investigations were made with these two sugar nucleotides in which the glycosylation of exogenous macromolecular acceptors could be evaluated. In these studies it was shown that the stimulation observed with NH_4Cl-treated thymocytes did not involve the glycosylation of exogenous glycoproteins. Also, it was proved (1) that NH_4Cl does not stimulate glycosyltransferase activities themselves, (2) that NH_4Cl treatment does not lead to release of glycosyltransferase activities as a result of extensive lysis and (3) that NH_4Cl treatment does not lead to the emergence of additional glycosyltransferases at the cell surface.

Considering that the inclusion of Trypan Blue within the cell reflects the lack of a selective barrier for the entry of exogenous molecules, and showing that the increased incorporation into endogenous acceptors paralleled the number of cells including Trypan Blue, we assumed that the stimulation effect is due to an enhancement of the entry of sugar nucleotides. These results suggest that labelled sugar nucleotides behave as the cellular pool and are utilized for glycosylation by the rough endoplasmic reticulum, leading to a stimulated incorporation into lipid intermediates, or by the smooth-endoplasmic-reticulum membranes, leading to an enhanced incorporation into endogenous acceptors. The fact that no increased incorporation is observed into macromolecular acceptors indicates either that the effect of NH_4Cl is strictly selective for the entry of small-molecular-sized molecules or that macromolecules, even if they penetrate the cell, cannot be glycosylated by the glycosyltransferases, which are known to be located on the luminal side of intracellular vesicles (Fleischer, 1981). More recent studies (R. Cacan, R. Cecchelli, B. Hoflack & A. Verbert, unpublished work) indicate that NH_4Cl has no effect on the penetration of sugar nucleotides within intracellular vesicles. Thus the effect of NH_4Cl seems to be restricted to plasma membrane. The fact that sugar nucleotides entering NH_4Cl-

treated cells can be used for an enhanced endogenous glycosylation indicates they are transported within the lumen of intracellular vesicles.

As observed by electron microscopy, NH_4Cl-treated thymocytes give cellular ghosts that can be utilized as a model for the study, with avoidance of any subcellular fractionation, of how cytoplasmic sugar nucleotides are translocated through intracellular membranes and utilized to glycosylate luminal acceptors.

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