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Incorporation of Glycosidically Linked Sialic Acid from Radiolabeled Free Sialic Acid and Cytidine Monophosphate–Sialic Acid by Intact Hamster Fibroblasts: A Reexamination†

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ABSTRACT: When incubated with cytidine monophosphate (CMP)–[^{14}C]sialic acid or [^{14}C]sialic acid, intact hamster fibroblasts incorporate labeled sialic acid into glycosidic linkage to cellular acceptors. A comparative study under strictly identical conditions shows that the two substrates behave differently in the following ways: (a) incorporation of sialic acid into glycosidic linkage from CMP–sialic acid is immediate and shows no lag, whereas a distinct lag is seen with the free sugar; (b) intact cells pretreated with neuraminidase incorporate 4–8 times more sialic acid from CMP–sialic acid (as compared to untreated control), but no difference in incorporation is seen by using free sialic acid with or without neuraminidase pretreatment of the cells; (c) when cells are prelabeled with CMP–sialic acid, greater than 50% of acceptor-bound sialic acid is released by neuraminidase; however, only a negligible fraction is released from labeled cells preincubated with free sialic acid; (d) CMP, a competitive inhibitor of sialyltransferase activity and which does not penetrate intact cells, inhibits sialic acid incorporation from CMP–sialic acid but not from the free sugar. Additionally, by employment of a gentle extraction procedure, a significant amount of sialyltransferase which exhibits some unique properties is extracted from intact cells. We conclude from these data that, at least in hamster fibroblasts, acceptors on the cell surface are readily glycosylated by direct transfer of the sialic acid moiety from CMP–sialic acid and that the loosely attached cell surface sialyltransferase may play a role in catalyzing the transfer reaction.

A variety of mammalian glycoproteins and glycolipids contain sialic acids at the terminal nonreducing end of the oligosaccharide chains (Kornfeld & Kornfeld, 1976; Fishman & Brady, 1976). The enzyme sialyltransferase (EC 2.4.99.1) catalyzes the incorporation of sialic acid onto these macromolecules by transferring the sialic acid moiety from the nucleotide sugar CMP–sialic acid[1] (Roseman, 1970). Most sialyltransferases are membrane-bound enzymes, the major cellular site being the Golgi apparatus (Roseman, 1970; Fleischer, 1977); other cellular organelles such as mitochondria (Bosmann, 1971), synaptosomes (Den et al., 1975), and rough endoplasmic reticulum (Bernacki, 1975; Shier & Trotter, 1976; Jarnefelt, 1976) also appear to contain significant amounts of enzyme activity. Several recent studies have shown that incubation of intact cells with radiolabeled CMP–sialic acid results in the incorporation of radioactivity into acid-precipitable macromolecular components. The following lines of evidence indicate that a major fraction of the cell-associated radioactivity can be accounted for by the labeling of the external acceptors on the cell surface: treatment of intact labeled cells with exogenously added neuraminidase released a large fraction of the acceptor-bound sialic acid (Datta, 1974; Bernacki, 1974; Patt & Grimes, 1974; Porter & Bernacki, 1975; Painter & White, 1976; Cervén, 1977); intact cells pretreated with neuraminidase incorporated three- to eightfold more sialic acid as compared to untreated cells (Datta, 1974; Bernacki, 1974; Porter & Bernacki, 1975; Cervén, 1977); exposure of intact cells to galactose oxidase to modify terminal galactose residues before incubation with CMP–sialic acid diminished sialic acid incorporation (Cervén, 1977); autoradiography of labeled cells revealed that greater than 80% of the radioactivity was associated with the plasma membrane (Porter & Bernacki, 1975; Cervén, 1977). Additional experimental data that are consistent with the above notion show the following: (a) addition of a 100–1000-fold excess of nonradioactive free sialic acid in the incubation fluid containing labeled CMP–[^{14}C]sialic acid did not reduce the incorporation of labeled sialic acid into acid-precipitable material (Datta, 1974; Porter & Bernacki, 1975; Painter & White, 1976; Cervén, 1977), although very high concentrations (e.g., 20 mM) of unlabeled sialic acid abolished incorporation of radioactivity from CMP–sialic acid (Hirschberg et al., 1976; Deppert & Walter, 1978) presumably because of contaminating inhibitor or due to the toxic effect of sialic acid itself [see Deppert & Walter (1978)]; (b) the initial rate of uptake of radioactivity from CMP–sialic acid into the *acid-soluble* fraction was ~25% of that found incorporated into the *acid-precipitable* fraction (Datta, 1974; Painter & White, 1976); (c) inhibitors that block transport of sugars, amino acids, and nucleosides did not influence the

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[1] Abbreviations used: CMP–sialic acid, cytidine monophosphate–sialic acid; Con A, concanavalin A; CMP, cytidine monophosphate; UDP, uridine diphosphate; α-methyl mannoside, methyl α-D-mannopyranoside.

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rate of incorporation from the nucleotide sugar (Datta, 1974). Taken together, these results led to the general conclusion that direct transfer of the sialic acid moiety from CMP–sialic acid to cell surface acceptors in glycosidic linkage may be operative in intact cells and that a part of the cellular sialyltransferase activity must reside on the cell surface for such labeling to occur.

Hirschberg et al. (1976), on the other hand, recently concluded that incubation of intact hamster and mouse fibroblasts with CMP–sialic acid results in hydrolysis of the nucleotide sugar, uptake of free sialic acid inside of the cells, and subsequent incorporation of the sugar moiety to internal acceptors. Thus, very little, if any, direct incorporation of sialic acid occurred on the cell surface acceptors, indicating lack of sialyltransferase activity on the plasma membrane. The evidence in support of their conclusion came from the measurement of uptake and incorporation of labeled sialic acid and inhibition of these processes by sodium azide and cytidine, a comparison of the isotope ratio in the mixture of [^{3}H]CMP–sialic acid and CMP–[^{14}C]sialic acid between the outside medium and the cells, and an estimation of the distribution of radioactivity within the cells after incubation with labeled [^{3}H]CMP–sialic acid and free [^{14}C]sialic acid (Hirschberg et al., 1976; Hirschberg & Yeh, 1977). Based on an enzyme assay with exogenously added high molecular weight acceptors, Deppert & Walter (1978) concluded that no detectable cell surface associated sialyltransferase activity was present in hamster cells.

It is difficult to provide a rational explanation for the apparent discrepancies between the results of Hirschberg et al. (1976), Deppert & Walter (1978), and those obtained by others; the use of different cell lines, a variety of incubation conditions and experimental protocols to distinguish between soluble and glycosidically linked sialic acid, and the lack of strict comparative kinetic studies using sialic acid and CMP–sialic acid as the substrates, especially in terms of characterization of sialoacceptors, may have been the contributing factors. These considerations prompted us to reinvestigate the questions of whether glycosidically linked sialic acids are formed by direct glycosylation of cell surface acceptors from the nucleotide sugar, CMP–sialic acid, and whether there is sufficient evidence for the existence of cell surface sialyltransferase. We now provide data to show that, at least in hamster fibroblasts, incorporation of sialic acid from free sialic acid and CMP–sialic acid in glycosidic linkage proceeds with different kinetics, and, depending on the time of incubation and other conditions, different classes of cellular acceptors are glycosylated by these sugar substrates; further, by actual enzyme extraction we show that a significant fraction of cellular sialyltransferase activity resides on the cell surface.

Materials and Methods

Materials. BHK-21 clone 13, an established line of hamster fibroblasts, was obtained from the American Type Culture Collection. Eagle's minimal essential medium and normal and heat-inactivated calf sera were supplied by GIBCO. CMP–[^{14}C]sialic acid (4,5,6,7,8,9-^{14}C, specific activity 200 mCi/mmol, and 4-^{14}C, specific activity 0.9 mCi/mmol) and [^{14}C]sialic acid (4,5,6,7,8,9-^{14}C, specific activity 214 mCi/mmol) were purchased from New England Nuclear. Unlabeled sialic acid was obtained from Pfanstiehl. Highly purified *Clostridium perfringens* neuraminidase completely free of protease, β-galactosidase, and *N*-acetylneuraminic acid aldolase (Cassidy et al., 1965) was a gift from Dr. G. W. Jourdian of this department. Concanavalin A (Con A) was purified from Jack bean meal according to the method of Agrawal & Goldstein (1967). Triton X-45 was a product of Rohm and Haas. Cytidine 5′-monophosphate and methyl α-D-mannopyranoside (α-methyl mannoside) were obtained from Sigma and Pfanstiehl, respectively. Desialyzed fetuin was prepared by the method of Spiro (Kim et al., 1971). All other chemicals were of analytical grade.

Cell Culture. BHK-21 cells were routinely grown in Eagle's modified minimal essential medium (Eagle, 1965) supplemented with 10% calf serum, 1% tryptose phosphate broth, 200 units of penicillin, 4 mg of streptomycin sulfate, and 1000 units of mycostatin per 100 mL of medium. The culture dishes (Lux) were incubated at 37 °C in 10% CO_2 in a humidified incubator. Cells were detached in divalent cation-free phosphate-buffered saline containing 0.02% ethylenediaminetetraacetic acid and 0.025% trypsin, and the cell number was determined by a Coulter counter. Cultures were routinely monitored for mycoplasma by radioautography and were free from contamination. Cell viability was examined by an increase in cell number and by trypan blue exclusion (Phillips, 1973).

Incorporation of Glycosidically Linked Sialic Acid by Intact Cells. Cells grown in monolayer to the desired confluency in 60-mm dishes were washed with warm phosphate-buffered saline and incubated at 37 °C in 1.5 mL of phosphate-buffered saline containing 10 nmol/mL CMP–[^{14}C]sialic acid or [^{14}C]sialic acid. The reaction was terminated by adding 2 mL of a solution of a 1:1 mixture of 2% phosphotungstic acid in 1 N HCl and 50% trichloroacetic acid, and the radioactivity in the acid-precipitable material was determined as described earlier (Datta, 1974). Pretreatment of cells with neuraminidase or exposure of cells to the enzyme after labeling with radioactive precursors and identification of product were carried out according to the published procedures (Datta, 1974).

Assay of Sialyltransferase Activity. Enzyme activity was routinely assayed in a final volume of 0.1 mL containing 50 μmol of cacodylate buffer, pH 6.0, 0.4 μmol of $MnCl_2$, 10 nmol of CMP–[^{14}C]sialic acid, 1 mg of desialyzed fetuin, and 1 to 2 mg of protein (Fan, 1978). After incubation at 30 °C for 30 min, aliquots of the mixture were adsorbed onto GF/C filter papers and precipitated with 2% phosphotungstic acid in 1 N HCl. The filters were washed with trichloroacetic acid, dried, and counted as described (Datta, 1974). The assay was linear with respect to time and enzyme concentration. For determination of the K_m values, the concentration of CMP–[^{14}C]sialic acid (specific activity 0.9 mCi/mmol) was varied from 0.0025 to 3 mM; the concentration of desialyzed fetuin required for saturation at two different levels of CMP-sialic acid (13 and 340 nmol/mL reaction mixtures) was approximatley 10 mg/mL.

Sialic Acid Determination. Two separate procedures were employed to determine the total sialic acid content of BHK cells: the thiobarbituric acid methods of Aminoff (1961) and the modified resorcinol method of Jourdian et al. (1971). The amount of glycosidically linked sialic acid was also determined by treating a washed cell suspension with periodic acid at 37 °C for 100 min and heating the mixture with the resorcinol reagent for 15 min in a boiling water bath followed by extraction of the resorcinol-reactive chromogen with *tert*-butyl alcohol (Jourdian et al., 1971). Less than 2% of the total sialic acid in BHK cells was found to be present as free sugar. For examination of the amount of sialic acid in intact cells that is susceptible to neuraminidase, cells at different stages of confluency were treated in situ with purified enzyme (0.2 unit/mL in phosphate-buffered saline) for 10 min at 37 °C,

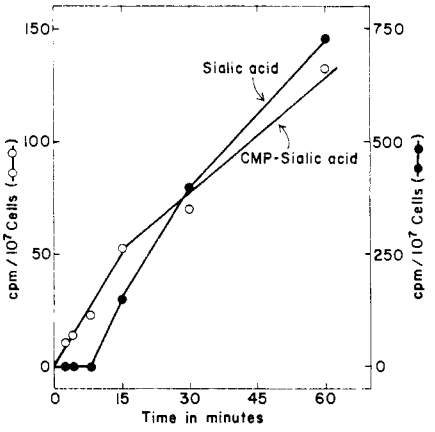

FIGURE 1: Kinetics of sialic acid incorporation into acid-precipitable material from CMP–sialic acid and sialic acid. Cells grown to confluency in monolayer were washed and incubated separately at 37 °C with CMP–[^{14}C]sialic acid (8 nmol/mL; 1.6 μCi) and [^{14}C]sialic acid (8 nmol/mL; 1.7 μCi) in phosphate-buffered saline. At times specified duplicate plates were withdrawn and the radioactivity in acid-precipitable material was determined as described under Materials and Methods. Cell number was determined in duplicate parallel cultures at each time point.

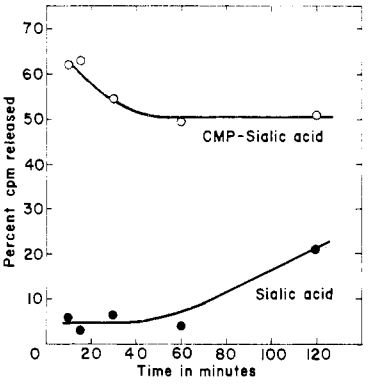

FIGURE 2: Neuraminidase-susceptible radioactivity in cells incubated with CMP–[^{14}C]sialic acid (○) and [^{14}C]sialic acid (●) as a function of time. Cells grown to confluency in monolayer were incubated at 37 °C with the radiolabeled precursors. At times specified, incubation fluid was removed from duplicate plates and the cells were washed with warm phosphate-buffered saline and treated with 0.2 unit/mL purified neuraminidase in phosphate-buffered saline for 10 min; duplicate control plates were treated in an identical manner except that no neuraminidase was added. The cells were then precipitated, and radioactivity in acid-precipitable material was determined as described under Materials and Methods. The data are expressed as the percent of count released by neuraminidase as compared to untreated control.

at which time the release of sialic acid was essentially complete. Following neuraminidase treatment, the cells were washed and the residual sialic acid was analyzed. Authentic sialic acid was used to construct the standard curves.

Preparation of Con A–Sepharose. Purified Con A was coupled onto Sepharose 4B activated by cyanogen bromide according to the method of Lloyd (1970) and poured into a column (8 × 1 cm). Con A–Sepharose was saturated with a 10% solution of heat-inactivated calf serum in 0.05 M cacodylate buffer, pH 6.0, and washed with 0.1 M α-methyl mannoside in the cacodylate buffer at 4 °C to decrease nonspecific binding of proteins (C. V. Natraj and P. Datta, unpublished experiments). The column was extensively washed with cacodylate buffer to remove α-methyl mannoside prior to loading the sample. Following sample addition, the column was washed with 4 bed volumes of buffer to elute materials not bound to the lectin. About 10 bed volumes of buffer containing 0.1 M α-methyl mannoside was generally adequate to elute materials that were bound to the lectin.

Results and Discussion

Kinetics of Sialic Acid Incorporation into the Glycosidic Linkage. Incubation in situ of hamster fibroblasts with CMP–[^{14}C]sialic acid and [^{14}C]sialic acid (specific activities of 200 and 214 mCi/mmol, respectively) under identical conditions resulted in a time-dependent incorporation of radioactivity into acid-precipitable fractions. By two independent techniques [see Datta (1974)], incorporated radioactivity was chemically identified as sialic acid. A comparison of the kinetics of incorporation (Figure 1) clearly shows that with CMP–[^{14}C]sialic acid as the substrate the incorporation was immediate, showed no lag, and tended to slow down after ~15 min (open circles), whereas a distinct lag of ~8 min followed by a high rate of incorporation was seen with [^{14}C]sialic acid (closed circles). In several experiments, the lag period with free sialic acid varied between 4 and 8 min and the initial rate of incorporation was approximately three- to fivefold higher than that observed with CMP–[^{14}C]sialic acid. These results are consistent with the observation that free sialic acid is not a substrate recognized by sialyltransferase (Roseman, 1970); thus, for incorporation into glycosidic linkage to occur, [^{14}C]sialic acid must be transported inside the cell and converted to CMP–[^{14}C]sialic acid. If, as proposed by Hirschberg et al. (1976), incorporation of radioactivity from CMP–[^{14}C]sialic acid were *preceded* by the hydrolysis of the nucleotide sugar to free sugar and transport of free sugar inside the cell, we would have found a similar lag in kinetics as seen with free [^{14}C]sialic acid.

Nature of the Labeled Acceptors. Hirschberg et al. (1976) have reported that after incubation for 90 min of hamster and mouse fibroblasts with [^{3}H]sialic acid, between 16 and 25% of the total radioactivity in the cell was found in acid-precipitable material; similar experiments with CMP–[^{14}C]sialic acid showed that between 28 and 40% of the total label in the cell was acid precipitable. A closer examination of their data especially with BHK and 3T3 cells revealed a twofold increase in the percentage of total radioactivity incorporated into the acid-precipitable pellets from CMP–[^{14}C]sialic acid as compared to that found with [^{3}H]sialic acid as the substrate. Unfortunately, no data were presented to establish what fraction of the acid-insoluble radioactivity from these two precursors was localized on the cell surface.

The experiments depicted in Figure 2 (closed circles) show that at early times of incubation with [^{14}C]sialic acid, a large fraction of the labeled acceptors appears to be *intracellular* because a negligible amount (~5%) of acceptor-bound radioactivity was released from labeled intact cells after treatment with purified neuraminidase; only after 2 h of incubation with the free sugar did a significant fraction (~20%) of acid-precipitable radioactivity become susceptible to neuraminidase. In contrast, with CMP–[^{14}C]sialic acid as the substrate, the fraction of neuraminidase-susceptible acceptor-bound radioactivity was initially higher and remained constant at ~50% during the entire incubation period (Figure 2, open circles).

A separate set of experiments revealed that incubation in situ for 60 min with [^{14}C]sialic acid of intact cells *pretreated* with neuraminidase led to incorporation into the acid-precipitable fraction of 648 ± 50 cpm/10^7 cells as compared to 693 ± 30 cpm/10^7 cells for an untreated control. Under identical conditions with the same batch of cells, the incorporation of radioactivity from CMP–[^{14}C]sialic acid into acid-precipitable material was 2325 ± 105 and 273 ± 22

cpm/10^7 cells, respectively, for neuraminidase-pretreated and untreated cultures. Thus, an increase in the available sialoacceptors by neuraminidase treatment resulted in enhanced incorporation of radioactivity from CMP–sialic acid and not from free sialic acid.

We can make one general conclusion from the above results: during *short-term incubation*, incorporation of sialic acid from the free sugar into glycosidic linkage is predominantly (~95%) intracellular, whereas with CMP–sialic acid at least 50% of the radioactivity was incorporated into glycosidic linkage to acceptors on the cell surface. Prolonged incubation of intact cells in situ with free [^{14}C]sialic acid, however, led to the appearance of some labeled material on the cell surface (see Figure 2, closed circles) presumably because of the time needed for the export of labeled glycoproteins and their insertion into plasma membrane. A similar observation has been made (Yurchenco & Atkinson, 1977) for the incorporation of L-fucose into intact HeLa cells.

The results described above [also those of Hirschberg et al. (1976)] do not provide an answer as to why in almost all experiments the *total amount* of radioactivity (and not the percent of total counts) found in the acid-precipitable fraction was significantly lower (20–50%) when CMP–sialic acid was the substrate as compared to free sialic acid, especially when equimolar concentrations of these precursors with similar specific radioactivities were used; a low level of hydrolysis of CMP–sialic acid seen in various buffers (in the presence or absence of cells) for up to an incubation period of 2 h (Bernacki, 1974; Patt & Grimes, 1974; Painter & White, 1976; Hirschberg et al., 1976) cannot account for this reduction in labeling. One plausible explanation may be that more sialoacceptors are available for glycosylation *inside* the cell than on the cell surface and a rapid uptake of free sialic acid may lead to a higher extent of labeling of intracellular acceptors. It is difficult to analyze quantitatively the amount of asialoacceptors because of the dynamic situation in terms of synthesis and turnover of both intracellular and plasma membrane components. Nevertheless, direct chemical analysis of glycosidically linked sialic acids to cellular acceptors can be carried out to estimate approximately their distribution between the cell surface and inside of the cells. By two different methods (Aminoff, 1961; Jourdian et al., 1971) using cells at 50 and at 100% confluency, the amount of total bound sialic acid was on the average 10.0 ± 1.0 nmol/mg of cell protein (four determinations); a different aliquot of the same batch of cells pretreated in situ with neuraminidase had 7.7 ± 0.7 nmol of bound sialic acid per mg of cell protein (four determinations), indicating that at least 25% of the bound sialic acid on hamster fibroblasts is susceptible to enzymic cleavage. Because stimulation of sialic acid incorporation from CMP–sialic acid by intact cells after neuraminidase treatment has been variously reported to be between three- and eightfold (Datta, 1974; Bernacki, 1974; Cervěn, 1977), from the ratio of endogenous acceptors with and without terminal sialic acid residues we can estimate that between 0.31 and 1.2 nmol of sialic acid (per mg of cell protein) may be potentially incorporated into glycosidic linkage on the cell surface. If we were to assume a similar range of ratios of asialoacceptors over sialoacceptors inside the cell, the amount of sialic acid that may be incorporated onto internal acceptors is between 1.3 and 5 nmol/mg of cell protein.[2] Thus, in quantitative terms, more endogenous acceptors appear to be free for potential sialylation inside the cell than on the cell surface.

[2] This is probably an underestimate because the glycoconjugates on the cell surface are presumably "mature" with a higher degree of sialylation as compared to the intracellular nascent molecules, some of which may not be fully glycosylated.

Table I: Inhibition of Sialic Acid Incorporation in Situ by CMP[a]

CMP concn (nM)	% sialic acid incorpn from	
	CMP-sialic acid	sialic acid
0	100	100
0.5	96	92
1.0	61	95
2.0	37	94

[a] Intact cells in monolayer were incubated for 60 min at 37 °C with CMP-[^{14}C] sialic acid or [^{14}C] sialic acid in phosphate-buffered saline containing CMP as indicated. Following incubation, cells were precipitated and acid-insoluble radioactivity was counted as described under Materials and Methods.

Modulation of Sialic Acid Incorporation in Intact Cells. Two independent studies (Bernacki, 1975; Maca & Hakes, 1977) have shown that CMP is a potent competitive inhibitor of sialyltransferase activity; with rat liver microsomal enzyme an apparent K_i of 0.62 mM has been calculated (Bernacki, 1975). Experiments on the regeneration of surface sialic acid on cultured lymphoma cells in the presence or absence of CMP (Maca & Hakes, 1977) indicated that synthesis of intracellular sialoproteins and their subsequent export to plasma membrane were not influenced by CMP. For a direct measurement of the effect of CMP on the incorporation of radioactivity into the acid-precipitable fraction from CMP-[^{14}C]sialic acid and free [^{14}C]sialic acid, hamster cells were incubated in situ with these substrates with various concentrations of CMP. As shown in Table I, there was a progressive inhibition in the sialic acid incorporation from CMP–sialic acid with increasing levels of CMP in the incubation fluid; no inhibition was detected with [^{14}C]sialic acid as the substrate. These results are predicted if CMP–sialic acid were the glycosyl donor for the transfer of sialic acid onto external acceptors catalyzed by cell surface associated sialyltransferase.

It has been reported that upon incubation for 2 h of NIL hamster fibroblasts with [^{3}H]CMP–sialic acid in buffer ~12% of radioactivity was recovered as [^{3}H]cytidine, indicating cleavage of the sugar nucleotide to CMP followed by its conversion to cytidine, presumably by cell surface phosphatases (Hirschberg et al., 1976). Thus, it is possible to argue that whereas CMP itself cannot be transported across the plasma membrane, the reduction of sialic acid incorporation by CMP may actually be due to cytidine, a breakdown product of the nucleotide. However, this seems unlikely because 1 mM cytidine by itself had no inhibitory effect on the activities of membrane-bound enzymes or the sialyltransferases solubilized by Triton X-45 (data not shown).

Log phase growing cells (50–60% confluent) and confluent cells were washed with warm phosphate-buffered saline and incubated in situ for 60 min at 37 °C in the same medium containing various concentrations of the lectin Con A to examine whether incorporation of sialic acid into acceptors in intact cells from CMP–sialic acid can be influenced by agents that bind to the cell surface. Following incubation, the cells were again washed with warm phosphate-buffered saline to remove free Con A and incorporation of radioactivity in situ from CMP-[^{14}C]sialic acid was measured. The results displayed in Table II show that Con A stimulated sialic acid incorporation into the acid-precipitable fraction. Control experiments (see Table II) revealed that (a) the stimulatory effect of the lectin was lost when α-methyl mannoside was present during incubation with Con A and (b) α-methyl mannoside by itself did not influence sialic acid incorporation.

Table II: Stimulation of Sialic Acid Incorporation by Concanavalin A[a]

	% sialic acid incorpn by	
	growing cells	confluent cells
0 μg/mL Con A	100	100
1 μg/mL Con A	99	88
50 μg/mL Con A	144	148
500 μg/mL Con A	147	164
50 μg/mL Con A + 0.1 M α-methyl mannoside	100	
0.1 M α-methyl mannoside		105

[a] Intact cells in monolayer were incubated for 60 min at 37 °C with various concentrations of Con A with or without α-methyl mannoside. Following incubation, cells were washed with warm phosphate-buffered saline and incorporation of radioactivity into acid-precipitable material from CMP-[^{14}C]sialic acid was measured as described under Materials and Methods. The data are averages from four separate experiments, with a maximum variation of ±7%.

Similar results have been reported for mouse thymocytes (Painter & White, 1976). Because Con A is known to cause alterations in the membrane structure by binding to cell surface carbohydrates (Bittiger & Schnebli, 1976), one plausible interpretation of the lectin-mediated stimulation of sialic acid incorporation is that Con A may somehow alter the enzyme–acceptor interactions on the cell surface. It is noteworthy that Con A can bind to sialyltransferase; however, the lectin does not inhibit the activity of detergent-solubilized enzyme (see below).

Evidence for Cell Surface Associated Sialyltransferase. The cumulative results discussed thus far clearly show that intact BHK cells incubated with CMP–sialic acid incorporated sialic acid into glycosidic linkage onto acceptors external to the cell surface. These results are in agreement with the previously published reports using a variety of animal cells in culture and argue against the interpretation provided by Hirschberg et al. (1976) from their experiments on sialic acid uptake from the free sugar and the nucleotide sugar. Although direct transfer of sialic acid from CMP–sialic acid is a priori evidence for the existence of cell surface associated sialyltransferase, nevertheless, it seems desirable to obtain experimental evidence that will strengthen this conclusion. Lack of transfer of sialic acid from CMP–sialic acid into glycosidic linkage to exogenously added high molecular weight acceptor by intact cells [see Deppert & Walter (1978)] is not compelling evidence to rule out the existence of cell surface enzyme.

In a series of experiments with crude "membrane fraction" (sedimented at 480*g* for 10 min from cell homogenate) as well as Triton X-45 solubilized preparations (not sedimented at 40000*g* for 60 min), Fan (1978) detected more than one form of sialyltransferase in hamster fibroblasts based on the following criteria: (a) heat inactivation kinetics exhibited multiphasic components; (b) three distinct pH optima were around pH 6.2–6.4, 6.9, and 7.6; (c) polyacrylamide gel electrophoresis followed by an activity assay of sliced gels revealed three distinct peaks of enzyme activity; (d) the substrate saturation curve for CMP–sialic acid with a saturating concentration of asialofetuin as the acceptor showed more than one component with apparent K_m values of 6, 40, and 230 μM. As shown in Table III, experiments 1 and 2, passage of detergent-solubilized enzyme through a Con A–Sepharose column indicated the presence of at least two classes of enzyme, one not bound to Con A and comprising 70–80% of the enzyme recovered and a second one (20–30%) bound to Con A which was specifically eluted with α-methyl mannoside. An aliquot

Table III: Elution of Sialyltransferase through Concanavalin A–Sepharose Columns[a]

	% enzyme act. eluted with	
source of enzyme	buffer	buffer + α-methyl mannoside
(1) detergent-solubilized 40000*g* supernatant	49 (80)	12 (20)
(2) detergent-solubilized 150000*g* supernatant	37 (71)	15 (29)
(3) urea-extracted enzyme	0 (0)	80 (100)
(4) released enzyme	7 (14)	43 (86)

[a] Samples of various enzyme preparations were layered on columns and eluted with cacodylate buffer followed by a solution of 0.1 M α-methyl mannoside in buffer as described under Materials and Methods. Detergent-treated samples were obtained as follows [see Fan (1978)]: cells growing in monolayer were washed, detached with a rubber policeman, disrupted by argon cavitation at 4 °C in 0.05 M cacodylate, pH 6.0, and centrifuged at 800*g* for 10 min. The pellet obtained was solubilized at 4 °C for 60 min in the cacodylate buffer containing 0.04% Triton X-45. The suspension was subjected to sonic oscillations (2 × 3 s) and centrifuged at either 40000*g* or 150000*g* for 60 min, and the supernatant was used as the source of enzyme. Urea-extracted enzyme was prepared by incubating intact cells in monolayer for 2 h at 37 °C with serum-free medium containing 0.2 M freshly prepared urea and centrifuging the incubation fluid at 25000*g* for 60 min at 4 °C followed by dialysis of the supernatant against 0.05 M cacodylate buffer (pH 6.0)–4 mM $MnCl_2$. For spontaneously released enzyme, cells grown to 80% confluency in heat-inactivated serum were washed and incubated for 15 h at 37 °C in serum-free medium–1% crystalline bovine serum albumin. The incubation medium was centrifuged at 23000*g* for 10 min, and the total enzyme activity was precipitated by 80% saturation of $(NH_4)_2SO_4$ and dialyzed against cacodylate buffer. The numbers in parentheses indicate the percent of total activity recovered from the column.

of enzyme which was eluted with buffer only was passed through a freshly prepared Con A–Sepharose column to test for overloading of the column; greater than 90% of the added activity again eluted with buffer only.

BHK cells grown in complete medium in the presence of normal or heat-inactivated calf serum were washed with phosphate-buffered saline and incubated in situ for 2 h at 37 °C in serum-free medium containing 0.2 M freshly prepared urea [see Yamada & Weston (1974) and Natraj & Datta (1978a,b)] to examine which one, if any, of these two classes of the enzyme is localized on the cell surface. The extract was centrifuged at 25000*g* for 60 min at 4 °C, and the supernatant was dialyzed against 0.05 M cacodylate buffer, pH 6.0, containing 4 mM $MnCl_2$. The results displayed in Table IV show that on the average ~20% (range 15–28%) of total cellular sialyltransferase activity was extracted under these mild conditions. Because normal calf serum (from GIBCO) has a variable amount of sialyltransferase activity depending on the lot number (Fan, 1978), several such urea extracts were prepared from cells passaged in heat-inactivated serum (which contained no enzyme activity); the amounts of extractable enzyme in these preparations were similar to those found in cells grown in untreated serum (Table IV). When subjected to high-speed centrifugation at 130000*g* for 3 h, all of the enzyme activity remained in the supernatant fluid, indicating that the enzyme was not associated with cell debris or membrane fragments precipitable under these conditions.

It was important to determine whether incubation of cells with serum-free medium plus 0.2 M urea caused damage to cells and/or cell death and lysis, thus releasing enzyme activity in the incubation medium. Three separate lines of evidence indicated that the small percent of damaged or dead cells

Table IV: Extraction of Sialyltransferase Activity from Intact Hamster Fibroblasts Grown in Monolayer[a]

source of enzyme	% total act.
Expt I: Cells Grown in Normal Serum	
cell homogenate before urea treatment	100
urea extract	23
cell homogenate after urea treatment	63
Expt II: Cells Grown in Heat-Inactivated Serum	
urea extract 1	15
urea extract 2	28
urea extract 3	16
serum-free medium extract	5
phosphate-buffered saline extract	3

[a] Cells were grown to the desired density in either normal or heat-inactivated calf serum. To extract enzyme activity, washed cells were incubated in phosphate-buffered saline, serum-free medium, or serum-free medium plus 0.2 M freshly prepared urea as described in Table III. In experiment II, urea extract 1 and urea extract 2 were obtained, respectively, from cells passaged through one or two transfers in heat-inactivated serum. Urea extract 3 was from cells at 80% confluency. The data are normalized with respect to total enzyme activity found in cells as measured after cells were homogenized from a parallel culture in 0.05 M cacodylate buffer (pH 6.0)–4 mM $MnCl_2$.

observed in some instances cannot account for the release of 20–25% of total enzyme activity: (a) growth of cells in complete medium with 0.2 M urea had no effect on the increase in cell number, and under the phase microscope, no abnormal morphology was observed; (b) cells pretreated with the urea solution showed greater than 90% viability when allowed to grow in complete medium with 10% serum; (c) trypan blue exclusion studies showed that only 5% of the cells were stained after extraction in situ with the urea solution, whereas in control cultures treated with phosphate-buffered saline ~3% of the cells took up the dye. With NIL hamster fibroblasts and a sensitive chromium-51 release assay, it has been estimated (Hirschberg et al., 1976) that between 4 and 8% of cells may show leakage of chromium after incubation for 2 h in growth medium or various buffers. Some biochemical evidence provided below on the nature of the enzyme extracted from intact cells by serum-free medium containing 0.2 M urea also argues against significant leakage of enzyme by cell damage or lysis.

It has been mentioned already that between 20 and 30% of enzyme activity in the detergent-solubilized fraction of the cell homogenate was retained by Con A–Sepharose and was specifically eluted with α-methyl mannoside (Table III). The data of experiment 3, Table III, clearly show that enzyme extracted in situ with the urea solution from intact cells (also comprising ~20–25% of total cellular enzyme, see Table IV) remained tightly bound to Con A, and all of the activity recovered from the column was eluted with α-methyl mannoside. Further, the linear Lineweaver–Burk plot (Figure 3A) of the urea-extracted enzyme shows one apparent K_m value of 50 μM for CMP–sialic acid; the pH activity profile (not shown) also reveals one peak in the pH range 6.0–6.2 and a shoulder at pH 6.8. These results appear to suggest that only one species of the multiple isoenzymic forms of the enzyme was being extracted from intact cells. If cell death and lysis were the results of leakage of enzyme in the incubation medium, we would have found a mixture of enzymes and not what appears to be of one class.

Spontaneous Release of Sialyltransferase into the Culture Medium. Because of the facile extraction of enzyme from intact cells under mild conditions, it seemed plausible that some fraction of the loosely bound cell surface associated enzyme

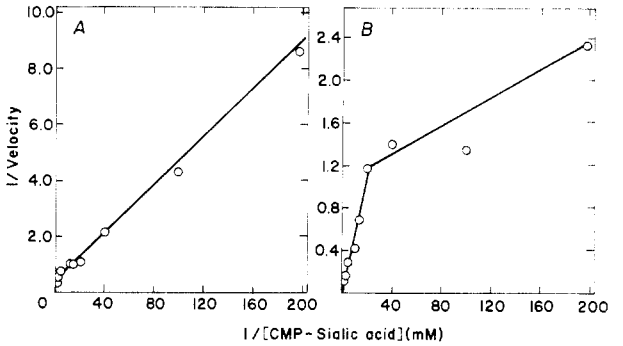

FIGURE 3: CMP–sialic acid saturation curves of urea-extracted and spontaneously released sialyltransferases. The initial velocities of sialic acid incorporation (cpm/min) with increasing concentrations of CMP–[^{14}C]sialic acid were measured at a saturating concentration (10 mg/mL) of desialyzed fetuin. The data are expressed as reciprocal initial velocity vs. reciprocal CMP–sialic acid concentration. (A) Enzyme extracted with urea; (B) enzyme released into the culture medium.

Table V: Release of Sialyltransferase Activity into the Culture Medium as a Function of the Time of Growth[a]

time of growth (h)	cells/plate ($\times 10^{-6}$)	cpm/plate ($\times 10^{-3}$)	cpm/10^6 cells ($\times 10^{-3}$)
0	0.51	0	
18	0.55	85	154
42	1.44	140	97
66	3.19	340	106
89	5.74	517	90

[a] Cells were plated out in 10% heat-inactivated serum and allowed to grown at 37 °C in 10% CO_2. At times specified, two plates were removed, the culture medium was decanted and centrifuged at 25000g for 10 min, and an aliquot was assayed for enzyme activity in duplicate. The results are expressed as total radioactivity incorporated in 30 min (average of two plates). The number of cells per plate was also determined by a Coulter counter at each time point.

may be released into the medium spontaneously during cell growth. Cells were grown in heat-inactivated calf serum for various lengths of time and the enzyme activity was monitored after centrifugation of the culture medium at 23000*g* for 10 min to test this experimentally. The results displayed in Table V indicate that a progressively larger amount of enzyme activity was released as a function of the age of the culture; when normalized to a constant number of cells, the amount of enzyme released was initially higher but remained approximately constant at a lower value up to 89 h of incubation. In several independent experiments, the total enzyme activity released into the medium was approximately equal to that found in the total cell population remaining on the dishes (as measured after homogenization of cells in the cacodylate buffer); the presence of an activator in the medium or of an inhibitor in the cell homogenate was ruled out by mixing a known volume of each with a known enzyme sample and assaying for enzyme activity. One interpretation of these data is that, during growth, the loosely attached enzyme continuously sloughs off the cell surface as the plasma membrane is continually replenished with new enzyme molecules by de novo synthesis. It has been reported recently (Bernacki & Kim, 1977) that metastasizing mammary tumor cells show an increased release of sialyltransferase activity in the serum, presumably during shedding of the cell surface components.

Preliminary characterization of the enzyme released spontaneously into the medium suggested that more than one species may be involved. For example, (a) the precipitate obtained after centrifugation of the culture medium at 130000*g*

for 3 h (previously clarified at 23000*g* for 10 min to remove debris) contained ~40% of the total activity, (b) the substrate saturation kinetics revealed a biphasic plot with two apparent K_m components (Figure 3B), and (c) upon passage through a Con A–Sepharose column a small fraction of the enzyme activity did not bind to the lectin; greater than 85% of the activity was eluted by α-methyl mannoside (Table III, experiment 4). It may be recalled that enzyme extracted by serum-free medium containing a low concentration of urea was not precipitable by centrifugation at 130000*g* for 3 h, showed one K_m component, and was completely retained by Con A. These results taken together appear to indicate that the major enzyme activity released spontaneously into the culture medium is similar to that localized on the cell surface; the minor species may have originated from a few lysed dead cells.

Conclusions

Incubation of hamster and mouse fibroblasts in monolayer cultures with labeled CMP–sialic acid results in the incorporation of radioactivity into the acid-precipitable fraction. The central question here is whether the nucleotide sugar is involved in the direct transfer of the sialic acid moiety into glycosidic linkage to acceptors that are external to the cell surface. On the other hand, if little or no cell surface acceptors are found to contain labeled sialic acid in glycosidic linkage, should we conclude that, during incubation with cells, CMP–sialic acid was hydrolyzed and the free sugar transported inside the cell where it is "activated" to CMP–sialic acid prior to the incorporation of sialic acid onto intracellular acceptors. Research claiming support for each of these two possibilities has been reported in the literature. Unfortunately, no single study was carried out that included experiments to (a) analyze the kinetics of incorporation of sialic acid into acid-precipitable material from the free sugar and CMP–sialic acid, (b) localize the distribution of labeled acceptors inside the cell or on the plasma membrane, and (c) demonstrate directly the presence or absence of sialyltransferase on the cell surface. By combining all of these approaches, we have now shown that, at least in hamster fibroblasts, acceptors on the cell surface are rapidly glycosylated by direct transfer of the sialic acid moiety from CMP–sialic acid. The direct demonstration of cell surface associated sialyltransferase also strengthens this conclusion.

It should be emphasized, however, that upon incubation of intact cells with CMP–sialic acid some *intracellular* acceptors are also labeled with sialic acid in glycosidic linkage because of hydrolysis of CMP–sialic acid followed by uptake of free sialic acid inside of cells. Thus, the ratio of glycosidically linked sialic acid on intracellular acceptors over that found on cell surface acceptors should vary significantly depending on the time of incubation, the rate of hydrolysis of CMP–sialic acid, and the number of total asialoacceptors present inside the cell and on the cell surface.

Incubation of intact cells with free sialic acid, on the other hand, leads predominantly to glycosylation of intracellular acceptors; the reaction proceeds with a lag because of transport of the free sugar inside of the cells and its subsequent "activation" to the nucleotide sugar, the true substrate for sialyltransferase. Nevertheless, it is also expected that upon long-term incubation with [^{14}C]sialic acid a variable fraction of the *cell surface acceptors* will have labeled sialic acid in glycosidic linkage. This is best explained by the currently accepted model of biosynthesis of membrane components (Wickner, 1979): de novo synthesis of "high-mannose" glycoproteins occurs in the rough endoplasmic reticulum with the terminal sugars most likely being added on in the Golgi apparatus; eventually, the completed glycoproteins are inserted in the plasma membrane or excreted into the medium. A detailed quantitative analysis of the temporal sequence of the conversion of free fucose to fucosylglycoproteins in HeLa cells (Yurchenko & Atkinson, 1977) shows a 10–15-min lag between incorporation of fucose into total cell protein and plasma membrane presumably due to the time needed for the transit of intracellular glycoproteins to the cell surface.

The results described here with CMP–sialic acid and those with UDP-*N*-acetylglucosamine (Natraj & Datta, 1978a,b) clearly suggest that the acceptors on the cell surface may be directly glycosylated in intact cells from the nucleotide sugar substrates under extremely mild conditions, and it would be feasible to investigate the effect of membrane glycosylation on cellular metabolism and growth behavior.

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Synthesis of Glycosaminoglycans by Cultured Rabbit Smooth Muscle Cells†

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ABSTRACT: Rabbit aortic smooth muscle cells were evaluated for their ability to synthesize and accumulate glycosaminoglycans (GAGs). Because of the sensitivity of the microtechniques utilized, it is possible to determine the specific radioactivity of the GAGs obtained after radioactive incorporation of [^{35}S]SO_4^{2-} and [^{14}C]glucosamine. Data obtained at various incubation times indicate that the distribution of the GAGs secreted by the cells into the medium is different from that retained by the cell layer. Hyaluronic acid was shown to be the most abundantly produced GAG, and much of this GAG does not appear to be incorporated into the extracellular matrix. Also, a high percentage of the total chondroitin sulfate B synthesized was secreted into the medium. On the other hand, most of the heparan sulfate and chondroitin sulfate C/A synthesized seems to be associated with the cell layer. These results are consistent with those found in whole rabbit aorta.

Evidence for the biosynthesis of glycosaminoglycans (GAGs)[1] by vascular smooth muscle cells has recently been established (Wight & Ross, 1975). In these studies the GAGs were measured only in terms of radioactivity present in the culture medium and at a single incubation time. Cultured fibroblasts and arterial endothelial cells (Buonassisi, 1973) have been examined as well. More recently, kinetic data of total GAG synthesis have been evaluated (Gamse et al., 1978).

In the present report we describe the synthesis of GAGs by cultured rabbit aortic smooth muscle cells. The types of GAGs formed and their rates of synthesis and secretion were examined. The micromethod employed in these studies permits simultaneous determination of two essential aspects of GAG biosynthesis. It is possible to measure the total amount and distribution of the GAGs synthesized as well as to quantify the radioactivity of the [^{35}S]SO_4^{2-} or [^{14}C]glucosamine incorporated into each of these mucopolysaccharides during a prescribed pulse period. Such a procedure allows for long-term investigations as well as short-term pulse–chase experiments.

Although many studies on collagen and elastin biosynthesis have been carried out on vascular smooth muscle cells in culture (Ross, 1971; Faris et al., 1976; Burke et al., 1977), little is known of the role the GAGs play in the synthesis and secretion of these proteins. By developing methods to determine GAG distribution and synthesis in cell cultures, one should now be able to evaluate the influence of these mucopolysaccharides on connective tissue protein synthesis. The present communication suggests that such experiments are feasible and will be the subject of further investigations.

Materials and Methods

Preparation of Smooth Cell Cultures. Smooth muscle cells were isolated and grown from the medial layer of the aortic arch of weanling rabbits as described previously (Faris et al., 1976). Cells were seeded into second passage at a density of 1.5×10^6 cells/flask (75 cm^2). These cells were maintained in 20 mL of Dulbecco's Modified Eagle's Medium supplemented with fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL). Flasks were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed 2 times/week. Previous electron microscopic studies revealed that cells prepared in this manner have the ultrastructure characteristics of smooth muscle cells (Mazurkowitz et al., 1980).

Pulse-Label Experiments. Cells used for pulse experiments were grown for 19 days in the second passage [approximately (4–5) $\times 10^6$ cells/flask]. Before the cells were pulsed, the spent medium was aspirated off and the cell layers were washed 2 times with Ca^{2+}- and Mg^{2+}-free Puck's saline G followed by one wash with serum-free Dulbecco medium. The cells to be pulsed with [^{35}S]SO_4^{2-} were then preincubated for 1 h in the same medium. The cells to be pulsed with [^{14}C]glucosamine were preincubated for 1 h with serum-free medium containing 10 mM pyruvate in place of glucose. In each case this medium was replaced with 10 mL of serum-free medium per flask that contained 1 μCi/mL of either [^{35}S]SO_4^{2-} or [^{14}C]glucosamine (New England Nuclear, Boston, MA). Control flasks did not contain any radioactive labeled precursors.

At specified incubation times the spent radioactive medium from pairs of flasks containing the same isotope was pooled and the cells were washed 3 times with 2.0 mL of Puck's saline G. These wash solutions were added to the corresponding pooled medium, and the resulting solutions were dialyzed vs. cold 0.01 M Na_2SO_4 and then against water. In the case of [^{14}C]glucosamine, dialysis was carried out vs. water. The cell layers of each pair of flasks were harvested in 1.5 mL of Puck's saline G with the aid of a rubber policeman and pooled.

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[1] Abbreviations used: GAG, glycosaminoglycan; CSB, chondroitin sulfate B; CSC, chondroitin sulfate C; HA, hyaluronic acid; HS, heparan sulfate. CSC may also contain a minute amount of CSA.

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