Synthesis of the Glycoprotein Hormone α Subunit and Placental Alkaline Phosphatase by HeLa Cells: Effect of Tunicamycin, 2-Deoxyglucose, and Sodium Butyrate†

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ABSTRACT: The glycoprotein hormone α subunit is secreted by human cervical carcinoma cells (HeLa) as an ectopic gene product. Studies were undertaken to determine whether the tumor protein is glycosylated, as is the normal placental protein, and whether protein glycosylation is a prerequisite for secretion. About 80% of the HeLa protein was adsorbed to concanavalin A–Sepharose (Con A–Sepharose) and could be eluted with 0.2 M methyl α-glucoside, demonstrating its glycoprotein character. Tunicamycin (TM) at concentrations between 0.25 and 2 μg/mL and 2-deoxy-D-glucose (dGlc) at concentrations between 1 and 10 mM caused only a marginal (20–40%) reduction in extracellular α. Neither TM nor dGlc caused a concomitant rise in intracellular α as the level of extracellular hormone decreased, suggesting that the inhibitors were not specifically reducing protein secretion. The possibility that α secreted by HeLa cells in the presence of TM was glycosylated prior to the drug's addition and subsequently released seems unlikely in view of the kinetics of α secretion following TM addition. The carbohydrate content of α synthesized in the presence of TM was examined by immunoprecipitation of culture media from cells grown in the presence of [^{3}H]glucosamine and ^{14}C-labeled amino acids and by chromatography of media on Con A–Sepharose. In the presence of TM at 2 μg/mL, α polypeptide synthesis was decreased by about 40% while α glycosylation was inhibited by 92%. These results suggest that glycosylation of α is not required for secretion since almost total inhibition of glucosamine incorporation caused a reduction in α levels no greater than could be accounted for by the moderate inhibition of general protein synthesis caused by this agent. The proportion of α not bound to Con A–Sepharose increased when TM was present, but a fraction of the α secreted in the presence of TM contained at least some carbohydrate since it was still retained by the lectin. Thus, the possible contribution of a minor oligosaccharide component, resistant to TM, in the facilitation of α release cannot be totally excluded. Addition of sodium butyrate to cultures resulted in a 10-fold increase in α production and a 5-fold increase in the synthesis of placental alkaline phosphatase (PAP), a membrane-bound glycoprotein. Deoxyglucose caused a drastic reduction in the butyrate-induced synthesis of both α and PAP down to uninduced levels. That the glycosylation inhibitors reduced the butyrate-stimulated synthesis of α and alkaline phosphatase to a greater extent than their basal levels of synthesis suggests the possibility that butyrate may enhance the accumulation of these proteins by a mechanism that is particularly sensitive to these compounds.

Ectopic proteins are those gene products elaborated by tumors which are not characteristic of the cell type from which the tumor was derived [for a review, see Odell & Wolfsen (1975)]. Synthesis of ectopic proteins has been documented for tumors growing in vivo and for continuous tumor cell lines growing in vitro (Rees & Ratcliffe, 1974; Braunstein et al., 1973; Rabson et al., 1973; Orth, 1973). The tumor cell product has usually been found to be similar to the product of the normal cell of origin (Kaganowicz et al., 1979), although in some cases immunologic or physicochemical differences have been noted (Weintraub et al., 1975).

HeLa, a continuous cervical carcinoma cell line, has long been known to elaborate an alkaline phosphatase similar in properties to the placental isoform of this enzyme (Fishman et al., 1968; Elson & Cox, 1969). More recently, it has been observed that certain strains of HeLa also synthesize and secrete human chorionic gonadotropin (hCG)[1] (Ghosh & Cox, 1976; Lieblich et al., 1976), a placental polypeptide hormone containing two nonidentical subunits, α and β. The alkaline phosphatases from placenta and HeLa are membrane-bound glycoproteins, and both subunits of placental hCG are glycosylated, containing about 30% carbohydrate (Endo et al., 1979; Bahl, 1968; Kennedy & Chaplin, 1976).

Sodium butyrate has pleiotropic effects on a wide variety of cell types as reviewed by Prasad & Sinha (1976). Ghosh et al. (1977) have shown that gonadotropin β production can be increased in HeLa$_{65}$ cells by sodium butyrate, and the results of Chou et al. (1977) and Griffin et al. (1974) show that the gonadotropin α subunit and alkaline phosphatase are also enhanced by millimolar concentrations of this fatty acid. Though the mechanism of induction is unknown, these authors suggest that chorionic gonadotropin synthesis may be related to the inhibition of DNA synthesis caused by this compound.

Tunicamycin inhibits the formation of *N*-acetylglucosamine-containing lipid intermediates involved in the assembly of the core regions of oligosaccharide chains linked N-glycosidically to protein (Struck & Lennarz, 1975). Inhibition by the antibiotic seems to involve its binding to the enzyme that transfers *N*-acetylglucosamine 1-phosphate from UDP-*N*-acetylglucosamine to dolichyl phosphate, forming *N*-

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[1] Abbreviations used: hCG, human chorionic gonadotropin; PAP, placental alkaline phosphatase; Btr, butyrate; dGlc, 2-deoxy-D-glucose; TM, tunicamycin; α-MG, methyl α-D-glucopyranoside (methyl α-glucoside); Con A–Sepharose, concanavalin A covalently attached to agarose; MEM, minimum essential medium; NaDodSO$_4$, sodium dodecyl sulfate.

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acetylglucosaminylpyrophosphoryldolichol (Heifetz et al., 1979). The exact mechanism of 2-deoxyglucose inhibition of glycosylation has not been established, though it is likely that disruption of glycolysis by inhibition of phosphohexose isomerase (Kuo & Lampen, 1972) in concert with the trapping of uridine and guanosine nucleotides as their 2-deoxyglucose derivatives (Biely & Bauer, 1968) may account for the suppression of the capacity to synthesize the normal complement of sugar nucleotides required for the glycosylation of proteins.

The studies described below were undertaken to determine whether the tumor α protein is glycosylated, as is the normal placental hormone, and whether protein glycosylation is a prerequisite for secretion. The effects of inhibiting protein glycosylation with TM and dGlc on the synthesis of α and PAP have been investigated in HeLa cells in the absence and presence of sodium butyrate.

Materials and Methods

Materials. Crude human chorionic gonadotropin was purchased from Organon, Inc. (West Orange, NJ), and purified as described previously (Morgan & Canfield, 1971). Concanavalin A covalently attached to agarose (Con A–Sepharose) was a product of Pharmacia. Sodium butyrate was purchased from Matheson Coleman and Bell, and Sigma Chemical Co. supplied 2-deoxy-D-glucose (grade III) and methyl α-D-glucopyranoside. New England Nuclear was the source of all radioisotopes, including a ^{14}C-labeled L-amino acid mixture (113–536 mCi/mmol), D-[6-^{3}H]glucosamine hydrochloride (18.8 Ci/mmol), and [1-^{14}C]butyrate (15 mCi/mmol). Culture media and serum were obtained from Grand Island Biological Co., and tunicamycin was obtained through the courtesy of Eli Lilly. Goat antirabbit immunoglobulin serum was purchased from Cappel Laboratories.

Cell Culture. HeLa S3 cells were maintained in spinner culture in Eagle's minimal essential medium (MEM) with spinner salts and supplemented with 6 mM L-glutamine, 6% fetal calf serum, 80 units/mL penicillin, and 80 μg/mL streptomycin sulfate. Cells were plated in MEM (containing 5.5 mM glucose) into 25- or 75-cm^2 flasks at a density of about 10^5 cells/mL, and growth was continued at 37 °C under 5% CO_2 in a humidified incubator until cells were nearly confluent, generally 2 or 3 days. At that time, media were replaced, and where indicated, glycosylation inhibitors, butyrate, and/or radioactive precursors were added. After incubation, the media and cells were assayed for α and PAP as described below.

Preparation of Cell Extracts. Media were aspirated from the culture flasks and cleared of detached cells by centrifugation at 1500*g* for 10 min. The cell sheet was removed with trypsin–EDTA and combined with cells recovered from the media. Cells were washed several times in 50 mM Tris-HCl (pH 7.4) buffer containing 0.9% (w/v) NaCl, resuspended in a small volume of the same buffer, and frozen at −20 °C. Cells, after rapid thawing, were disrupted on ice by sonication with a Branson sonifier (microtip, setting 6, 1–2 min, 50% duty cycle). The resulting sonicates were centrifuged at 15000*g* for 15 min, and the supernatant fluid was assayed for α and PAP as described below. Protein was determined by the method of Lowry et al. (1957) using bovine serum albumin as standard.

Incorporation of Radioisotopes. Cells were grown to a high density, after which time the medium was replaced with MEM containing 0.1 the concentration of essential amino acids, 6% dialyzed fetal calf serum, 2.5 μCi/mL [^{3}H]glucosamine, 0.5 μCi/mL ^{14}C-labeled amino acid mixture, and the amounts of butyrate, TM, and dGlc as indicated in the table and figure legends. At the desired time, media were removed and cleared of floating cells by centrifugation. The cell sheet was removed by trypsinization, and extracts were prepared as described above. Proteins were precipitated from 0.5 mL of media or 0.1 mL of cell sonicate by the addition of 1 mL of 10% trichloroacetic acid. The precipitates were collected on nitrocellulose filters (Millipore, 0.45 μm), washed with 20 mL of 5% trichloroacetic acid, dried, and counted in toluene scintillation fluid. Counting efficiencies were 77% for ^{14}C and 29% for ^{3}H, with 4% spill of ^{14}C into the ^{3}H channel. The scintillation fluid contained 5 g of 2,5-diphenyloxazole (PPO) and 0.25 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) per L of toluene.

α Radioimmunoassay. Immunization of rabbits with hCG-α and complete Freunds adjuvant was performed according to standard immunologic techniques. The antigen had been purified to homogeneity (as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis) from commerical preparations of hCG (Organon, Inc.). The resulting antisera showed no cross-reactivity with an unrelated hormone, placental lactogen, no cross-reactivity with hCG-β, and 2.7% cross-reactivity with hCG.

Purified hCG-α was iodinated by the lactoperoxidase method as described by Roth (1975). Monomeric hormone was obtained by chromatography of iodination reaction mixtures on Sephadex G-100. The labeled protein used had an average specific activity of 40–95 μCi/μg. Incubation mixtures for radioimmunoassay contained 0.1–0.5 mL of culture media or 0.025–0.10 mL of cell extract, rabbit anti-α serum (1:10000 final dilution), 1 μL of normal rabbit serum, ^{125}I-labeled α (approximately 50000 cpm), and 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% (w/v) bovine serum albumin and 0.02% (w/v) NaN_3 to a final volume of 1 mL. Mixtures were incubated at room temperature for 16–20 h at which time 30 μL of goat antirabbit IgG serum was added and incubation continued at 37 °C. The immune precipitates were collected by centrifugation and washed once with 1 mL of cold 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% (w/v) Brij 58 detergent. The wash solution was combined with the original supernatant and counted along with the pellets in a well-type manual solid scintillation counter.

Assay for Alkaline Phosphatase. Alkaline phosphatase in cell extracts was assayed by the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at pH 10.7 and 37 °C as described by Edlow et al. (1975). Conditions of protein concentration and time of incubation were chosen such that the increase in absorbance at 400 nm was proportional to the amount of activity present. One unit is that activity catalyzing the hydrolysis of 1 μmol of *p*-nitrophenyl phosphate per min at 37 °C.

Con A Chromatography. Columns of Con A–Sepharose (5-mL bed volume) were washed extensively first with 0.1 M sodium acetate buffer (pH 6) containing 1 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 0.02% (w/v) NaN_3 and then with 0.05 M potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl and 0.02% (w/v) NaN_3. After the medium was dialyzed against phosphate–saline buffer to remove glucose, it was applied to the column at room temperature. The flow was stopped for 30–60 min, and then the lectin was washed with 6–8 column volumes of phosphate–saline buffer. Adsorbed material was eluted with the phosphate–saline buffer containing 0.2 M α-MG. α was determined in 0.1–0.5 mL of selected fractions by radioimmunoassay (RIA) as described above. Bound and unbound materials were quantitated by determining the area under the peaks (paper weighing). Control experiments showed that

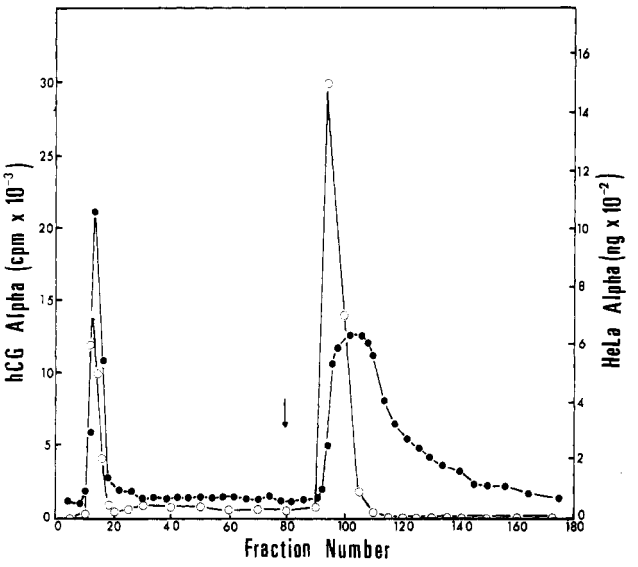

FIGURE 1: Chromatography of HeLa α on Con A–Sepharose. α was partially purified from HeLa media by organic solvent fractionation (G. S. Cox, unpublished experiments) and cochromatographed on a column of Con A–Sepharose (10-mL bed volume) with ^{125}I-labeled hCG-α (4 × 10^5 cpm) as described under Materials and Methods. The sample volume was 2 mL; fractions of 1 mL were collected and counted directly for ^{125}I and then assayed for α by RIA. The arrow denotes elution with phosphate–saline buffer containing 0.2 M α-MG. (●) ^{125}I-labeled hCG-α; (○) HeLa α.

^{125}I-labeled human placental lactogen, a nonglycosylated peptide hormone, did not bind to the Con A columns.

Results

Lectin Chromatography of HeLa α. For determination of whether the α protein secreted by HeLa cells in culture was glycosylated, conditioned media were dialyzed extensively against 0.05 M potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl to remove glucose and applied to a column containing concanavalin A covalently attached to agarose. The results (Figure 1) show that about 20% of the immunoreactive α eluted in the application buffer and that about 80% of the material was retained by the lectin and could be eluted with 0.2 M α-MG. These results indicate that the ectopic hormone is glycosylated and that the oligosaccharide side chain on HeLa α contains at least mannose or glucose residues. Shown for comparison is the profile obtained with ^{125}I-labeled hCG-α; little if any difference can be noted.

Effect of Tunicamycin on the Accumulation of HeLa α. Preliminary experiments examined the incorporation of [^{3}H]glucosamine and ^{14}C-labeled amino acids into trichloroacetic acid insoluble material in the presence of TM. Figure 2A shows that TM at concentrations of 1–2 μg/mL inhibited [^{3}H]glucosamine incorporation into HeLa-secreted proteins by 90–95%. However, it also reduced ^{14}C-labeled amino acid incorporation into secreted proteins by about 30–40% (panel B). For both glucosamine and amino acid incorporation, the inhibition of labeling in the presence of TM was somewhat greater for extracellular (secreted) proteins than for intracellular proteins. Moreover, the ratio of glucosamine to amino acid incorporated was decreased by a factor of 26 for extracellular proteins and by a factor of 8 for intracellular proteins (panel C). Similar effects of TM on the incorporation by rat hepatocytes of carbohydrate and amino acid precursors into secreted proteins have been reported by Struck et al. (1978).

The effects of TM on α synthesis and secretion are presented in Table I. Drug concentrations between 0.5 and 2 μg per mL of culture media decreased the intracellular and extracellular levels of α by about 25% and 40%, respectively. These reductions in α accumulation are comparable to the antibiotic's inhibition of general protein synthesis as discussed above (Figure 2B) and are considerably less than those expected for a direct correlation with reductions in protein glycosylation (Figure 2A).

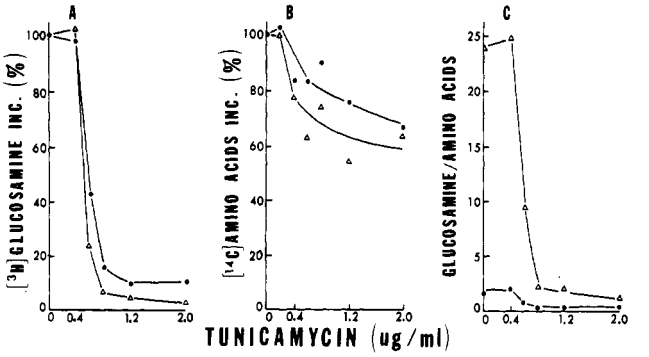

FIGURE 2: Effect of tunicamycin on the incorporation of [^{3}H]-glucosamine and ^{14}C-labeled amino acids into HeLa proteins. Cells (5×10^5) were plated in 25-cm^2 flasks containing 5 mL of MEM and grown for 2 days, after which time the medium was replaced. Following a 24-h preincubation with TM at the concentrations indicated, cells were further incubated for 6 h with [^{3}H]glucosamine (5 μCi/mL) and a mixture of ^{14}C-labeled amino acids (0.5 μCi/mL). Radioactivity was determined in media and cell extracts as described under Materials and Methods except that precipitates were collected on glass fiber filters (Whatman GF/C) and sonicates were heated at 90 °C for 10 min after the addition of trichloroacetic acid to hydrolyze aminoacyl-tRNA. Radioactivity was normalized to the amount of cell protein (Lowry et al., 1951) in each flask. (A) [^{3}H]Glucosamine incorporation; (B) ^{14}C-labeled amino acid incorporation; (C) ^{3}H/^{14}C ratio. The incorporation (cpm/mg of cell protein) of [^{3}H]glucosamine was 114 000 (intracellular) and 48 400 (extracellular), and the incorporation (cpm/mg of cell protein) of ^{14}C-labeled amino acids was 70 800 (intracellular) and 2000 (extracellular) in the absence of TM. (●) Acid-insoluble radioactivity in cell sonicates; (△) acid-insoluble radioactivity in culture media.

Table I: Effect of Tunicamycin on the Accumulation of α in the Absence and Presence of Sodium Butyrate[a]

TM (μg/mL)	sodium butyrate (3 mM)	α (ng/mg of cell protein)[b] intracellular	α (ng/mg of cell protein)[b] extracellular
0	–	39.3 (100)	392 (100)
0.25	–	27.4 (70)	326 (83)
0.50	–	27.8 (71)	268 (69)
1	–	27.7 (71)	263 (67)
2	–	30.5 (78)	164 (42)
0	+	168 (100)	3790 (100)
0.25	+	198 (118)	3890 (102)
0.50	+	184 (100)	3380 (89)
1	+	118 (70)	2150 (57)
2	+	106 (63)	1320 (35)

[a] Cells were grown to a high density in 75-cm^2 flasks. The medium was replaced, and TM was added to the final concentrations as indicated in the table. One set of flasks received no further additions (–), while a duplicate set received 3 mM sodium butyrate (+). Growth was continued for 2 days after which time α content was determined in both cell extracts (intracellular) and the media (extracellular) as described under Materials and Methods. [b] Values in parentheses are percentages.

Sodium butyrate has been shown to cause a variety of changes in cultured mammalian cells (Prasad & Sinha, 1976). In addition to the stimulation of PAP and α synthesis as described above, butyrate has also been shown to induce a glycolipid sialyltransferase (Fishman et al., 1974), indicating an involvement in cellular glycosylations. Thus, it was of interest to examine the effect of TM on butyrate-induced α. Table I shows that 3 mM sodium butyrate caused about a

Table II: Incorporation of [^{3}H]Glucosamine and ^{14}C-Labeled Amino Acids in the Presence of 2-Deoxyglucose[a]

dGlc (mM)	intracellular protein (cpm/mg of cell protein)[b]		extracellular protein (cpm/mg of cell protein)[b]	
	[^{3}H]glucosamine	^{14}C-labeled amino acids	[^{3}H]glucosamine	^{14}C-labeled amino acids
0	141 700 (100)	123 700 (100)	282 700 (100)	12 200 (100)
5	31 300 (22)	69 400 (56)	29 900 (11)	7 100 (58)
20	24 500 (17)	49 600 (40)	19 300 (7)	7 300 (60)

[a] Cells were plated in 25-cm^2 flasks. When the cells approached confluency, 5 mL of fresh media supplemented with dGlc at the concentrations indicated was added. After 2 days, 1.5 μCi of a ^{14}C-labeled amino acid mixture and 15 μCi of [^{3}H]glucosamine were added, and incubation was continued for 22 h. At that time, acid-precipitable radioactivity was determined in both cell extracts (intracellular) and media (extracellular) as described under Materials and Methods. [b] Values in parentheses are percentages.

Table III: Effect of 2-Deoxyglucose on the Accumulation of α in the Absence and Presence of Sodium Butyrate[a]

dGlc (mM)	sodium butyrate (3 mM)	α (ng/mg of cell protein)[b]	
		intracellular	extracellular
0	–	18 (100)	235 (100)
1	–	11.3 (63)	180 (77)
2	–	19.8 (110)	160 (68)
4	–	18.5 (103)	139 (59)
10	–	19.6 (109)	117 (50)
0	+	263 (100)	2944 (100)
1	+	116 (44)	1107 (38)
2	+	96 (37)	721 (25)
4	+	87 (33)	330 (11)
10	+	68 (25)	238 (8)

[a] Cells were grown in 75-cm^2 flasks containing MEM (5.5 mM glucose). Three days after being plated, cultures received fresh media containing the concentrations of dGlc indicated in the table. One set of duplicate flasks also received 3 mM sodium butyrate (+) while the other set had no further additions (–). After 72 h, media and cells were collected and assayed for α as described under Materials and Methods. [b] Values in parentheses are percentages.

10-fold increase in α synthesis which was diminished in the presence of TM. The effect of the inhibitor on both intracellular and extracellular protein was similar, except perhaps at the higher drug concentrations where a somewhat greater inhibition was observed in the extracellular fraction. It should be noted that there was not a concomitant rise in intracellular α as the level of extracellular α decreased, which might be expected if the drug were acting to inhibit protein secretion.

Effect of Deoxyglucose on the Accumulation of HeLa α. As in the case of TM, dGlc (20 mM) inhibited the incorporation of [^{3}H]glucosamine into intracellular and extracellular proteins by 83% and 93%, respectively, while it reduced the incorporation of ^{14}C-labeled amino acids into these same fractions by only 40–60% (Table II). These results are quite similar to those reported previously by Eagon & Heath (1977). Thus, although TM and dGlc have some effect on total protein synthesis in HeLa cells, their major effect is to block protein glycosylation (glucosamine incorporation into acid-precipitable material).

The effect of dGlc on α synthesis is presented in Table III. As seen, the reduction in both intracellular and extracellular α concentrations by dGlc was only marginal (50% or less) in the range of 1–10 mM. This inhibition of α synthesis in the presence of the inhibitor can probably be accounted for by the overall decrease in protein synthesis (Table II).

Table IV: Expression of Alkaline Phosphatase in the Presence of Tunicamycin or 2-Deoxyglucose[a]

additions	alkaline phosphatase (milliunits/mg of cell protein)[b]	
	no butyrate	3 mM butyrate
TM (μg/mL)		
0	2.5 (100)	8.4 (100)
0.25	2.8 (111)	7.4 (88)
0.50	1.7 (68)	7.3 (87)
1	1.7 (68)	6.3 (75)
2	1.4 (56)	4.4 (52)
dGlc (mM)		
0	5.4 (100)	44.5 (100)
1	4.8 (89)	22.3 (50)
2	5.2 (96)	18.3 (41)
4	5.7 (105)	9.5 (21)
10	4.6 (85)	6.4 (14)

[a] Cells were plated in 75-cm^2 flasks and maintained for 3 days. At that time, fresh medium was added and supplemented with TM or dGlc at the concentrations indicated. One set of duplicate cultures also received 3 mM sodium butyrate. Cells were harvested by trypsinization after 72 h and assayed for alkaline phosphatase as described under Materials and Methods. [b] Values in parentheses are percentages.

The accumulation of α in response to 3 mM butyrate was also inhibited by dGlc (Table III). The inhibition here, however, was markedly greater than that observed with TM, as both intracellular and extracellular α were reduced 75–92% by 10 mM dGlc. It is of interest that the concentration of butyrate-induced hormone never declined below the uninduced level. These results would suggest that synthesis of the protein in response to butyrate is more sensitive to inhibition by dGlc than synthesis of the hormone in the absence of inducer.

Levels of Alkaline Phosphatase in the Presence of TM and dGlc. The glycosylation inhibitors were also examined for their effect on the activity in HeLa cells of PAP, a membrane glycoprotein. Tunicamycin caused a reduction in both uninduced and butyrate-induced levels of PAP (Table IV) to a value that was comparable to the inhibition of general protein synthesis caused by the antibiotic (Figure 2B). In contrast, there was little if any effect of dGlc on PAP synthesis in control cells, but there was over an 80% reduction in activity of the enzyme in butyrate-stimulated cultures at sugar concentrations of 4–10 mM. Control experiments have shown that 5 mM dGlc does not inhibit phosphatase activity when added to enzyme assay mixtures.

Carbohydrate Content and Con A Chromatography of α from TM-Treated Cultures. The carbohydrate content of α synthesized in the presence of TM was examined by immunoprecipitation of culture media from cells grown in the presence of [^{3}H]glucosamine and ^{14}C-labeled amino acids and by Con A chromatography of media from cultures containing TM. It can be concluded from the data presented in Table V that in the presence of TM at 2 μg/mL, α polypeptide synthesis (immunoprecipitable ^{14}C) was decreased by about 40% while α glycosylation (immunoprecipitable ^{3}H) was inhibited by over 90%. The amount of α in the media as determined by RIA was also reduced, the levels being similar to those determined by the immunoprecipitation of ^{14}C-labeled amino acid labeled material and considerably greater than that estimated by the immunoprecipitation of [^{3}H]glucosamine-labeled material. These results suggest that the reduction of α in the media of TM-treated cultures can be accounted for by the reduction in α polypeptide synthesis. These data also

Table V: Effect of Tunicamycin on the Incorporation of [^{3}H]Glucosamine and ^{14}C-Labeled Amino Acids into HeLa α[a]

TM (μg/mL)	total secreted protein (cpm/mg of protein)[b] [^{3}H]glucosamine	total secreted protein (cpm/mg of protein)[b] ^{14}C-labeled amino acids	^{3}H/^{14}C	immunoprecipitable protein (cpm/mg of protein)[b] [^{3}H]glucosamine	immunoprecipitable protein (cpm/mg of protein)[b] ^{14}C-labeled amino acids	^{3}H/^{14}C	α (RIA) (ng/mL)[b]
0	185 000 (100)	38 200 (100)	4.84	2050 (100)	350 (100)	5.86	30 (100)
1	77 700 (42)	29 700 (78)	2.62	480 (19)	235 (67)	2.04	14.8 (49)
2	36 600 (20)	27 300 (72)	1.34	175 (8)	145 (58)	1.21	11.4 (38)

[a] Cells were grown and labeled for 18 h as described under Materials and Methods. Total secreted protein denotes acid-insoluble material in the culture media and was determined as described under Materials and Methods. Immunoprecipitable protein denotes that radioactivity which could be specifically precipitated by anti-α serum and was determined as follows: Triton X-100 and NaCl were added to 2 mL of media at final concentrations of 1% (v/v) and 0.5 M, respectively. Anti-α serum or preimmune serum (10 μL) was added to duplicate samples and incubated overnight at room temperature. Goat antirabbit IgG (200 μL) was then added and the incubation continued at room temperature overnight. The immune precipitates were collected by centrifugation, washed 3 times with 50 mM phosphate buffer (pH 7.4) containing 0.1% Brij 35, precipitated with 10% trichloroacetic acid, and counted on Millipore filters as described under Materials and Methods. Counts were corrected for ^{14}C spill into the ^{3}H channel, and preimmune values were subtracted from anti-α values to correct for nonspecific absorption. The RIA values for α were determined on separate aliquots of the labeled media. [b] Values in parentheses are percentages.

Table VI: Distribution of α following Chromatography of Media on Con A–Sepharose[a]

additions to culture	Con A unbound (%)	Con A bound (%)
none	23	77
TM	68	32
Btr	22	78
Btr + TM	42	58

[a] Cells were grown to a high density in four 75-cm^2 flasks in 20 mL of MEM. The media were changed and supplemented with either tunicamycin (2 μg/mL), butyrate (3 mM), tunicamycin (2 μg/mL) plus butyrate (3 mM), or no further additions, and cultivation was continued for 72 h. The media were removed by aspiration, cleared of cells, and chromatographed on Con A–Sepharose as described under Materials and Methods. Con A unbound refers to that material which eluted in the application buffer, while Con A bound denotes the fraction eluted from the lectin by 0.2 M α-MG. The data presented represent the average of two separate experiments.

show that the incorporation of radiolabeled precursors into α (^{3}H/^{14}C ratio) was similar to that for total secreted protein, indicating that this ectopic product is processed like other cellular proteins.

As presented in Table VI, it is evident that the proportion of α eluting in the application buffer during Con A chromatography increased for both control and butyrate-treated cultures when TM was present, but a fraction of the α secreted in the presence of TM contained at least some carbohydrate since it was still retarded by the lectin. Whether the material not retained by the Con A columns is devoid of carbohydrate is unknown, though the bound and unbound peaks maintain their elution characteristics during rechromatography of the isolated fractions (data not shown).

Kinetics of α Release in the Presence of TM. It was possible that the α secreted by HeLa cells in the presence of TM was glycosylated prior to the drug's addition and subsequently released. The kinetics of α secretion following TM addition, however, indicate that this was probably not the case since the level of hormone increased gradually over a 3-day period, indistinguishable from untreated cells (Figure 3). Release of presynthesized material would more likely have exhibited an early rise in α concentration to a level comparable to that observed on day 3 in control cultures. These data also show a greater inhibition by TM on the expression of α in butyrate-treated cells than in control cells.

Effect of TM and dGlc on [1-^{14}C]Butyrate Uptake. The fact that the levels of α and PAP in cultures containing butyrate were never reduced to basal levels (over the concentration ranges examined) suggested the possibility that the inhibitors might alter the cell's permeability to butyrate, thereby preventing induction. This explanation was also suggested by the report of Olden et al. (1979) which describes the inhibition by tunicamycin of glucose, uridine, and amino acid uptake in chick embryo fibroblasts, indicating a role of glycoprotein carbohydrates in membrane transport. The accumulation of [1-^{14}C]butyrate by HeLa cells in the presence of TM and dGlc was examined to test this possibility. It was found, however, that under the conditions employed [^{14}C]-butyrate uptake by HeLa S3 was reduced only slightly (25%) by dGlc at 20 mM and not at all by the sugar at concentrations less than 10 mM nor by TM up to 2 μg/mL (Figure 4). Since butyrate-stimulated increases in α and PAP were reduced 70–90% by the sugar at a concentration of 5 mM, it seems unlikely that these agents are causing the dramatic reduction of ectopic synthesis in butyrate-treated cultures by simply

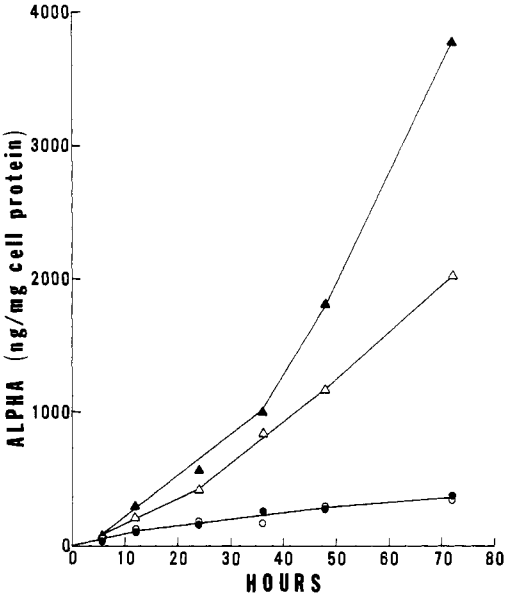

FIGURE 3: Kinetics of α secretion in HeLa cultures. Cells (2 × 10^6) were plated from spinner culture into four 75-cm^2 flasks containing 20 mL of MEM. After 3 days, the medium was aspirated, and the cell sheet was washed twice with sterile 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl. Fresh medium (20 mL) was added containing no additions (●), 1 μg/mL TM (○), 3 mM Btr (▲), or 3 mM Btr plus 1 μg/mL TM (△). Media (1 mL) were removed at various times and frozen until assayed for α. At the 72-h point, the cells were trypsinized, and the amount of cell protein per flask was determined: control, 6.4 mg; TM, 6.6 mg; Btr, 5.2 mg; Btr plus TM, 5.0 mg.

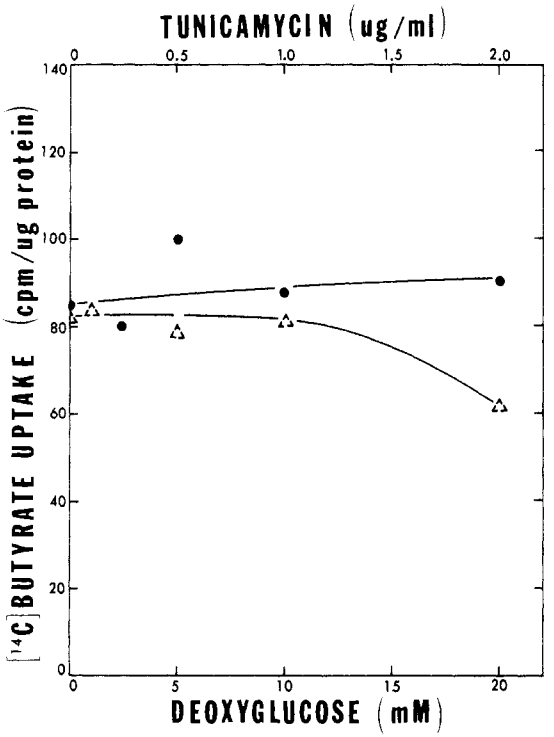

FIGURE 4: Effect of tunicamycin and 2-deoxyglucose on [1-^{14}C]-butyrate uptake in HeLa cells. Cells were plated in 25-cm^2 flasks and grown for 3 days. Fresh medium (5 mL), containing the indicated concentrations of tunicamycin (●) or deoxyglucose (△), was then added. Twenty-four hours later, [1-^{14}C]butyrate (0.5 μCi/mL) was added to each flask and incubation continued for 4 h. Cells were removed by trypsinization, washed 3 times with 3 mL of cold 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl, and resuspended in 3 mL of the same buffer. Cells from 1 mL of this suspension were collected on Amicon microporous filters, washed with 15 mL of cold phosphate–saline buffer, digested with NCS tissue solubilizer (Amersham/Searle), and counted in toluene scintillation fluid.

restricting uptake of butyrate into the cells.

Discussion

It has been suggested that most proteins secreted by eukaryotic cells are glycoproteins (Eylar, 1965). Although the biological function of oligosaccharide chains of secretory glycoproteins is not known, it has been proposed that the attachment of carbohydrate to these proteins is essential for their secretion (Melchers, 1973). However, it is clear that not all secretory proteins are glycosylated. The major secretory product of liver, serum albumin, contains no bound carbohydrate (Peters et al., 1971). Furthermore, studies by Kraekenbuhl et al. (1977) indicate that in bovine pancreatic exocrine cells all secretory proteins travel through the Golgi cisternae, are intermixed and packaged in zymogen granules, and are then cosecreted, suggesting an identical route for the intracellular transport and secretion of both glycosylated and unglycosylated proteins.

In a number of instances, inhibitors of glycosylation block the secretion of glycoproteins. Deoxyglucose inhibits the secretion of yeast invertase (Kuo & Lampen, 1972), though this block has been suggested to result from a general depression in the synthesis of extracellular proteins rather than a direct effect on the secretory process (Kratky et al., 1975). In the case of certain complete immunoglobulins, there is evidence implicating the carbohydrate chains in their secretion. It has been found that the secretion of IgG, IgA, and IgE by myeloma tumor cells is prevented if the addition of the oligosaccharide chain to the immunoglobulin heavy chain is blocked by dGlc (Melchers, 1973) or TM (Hickman et al., 1977).

In contrast, Struck et al. (1978) have shown that tunicamycin did not affect the secretion of transferrin or very low density lipoprotein from chick hepatocytes even though the drug inhibited glucosamine incorporation by 75–90% into the same proteins. Similarly, Eagon & Heath (1977) demonstrated that dGlc had little effect on the secretion of the normally glycosylated K-46 immunoglobulin light chain by myeloma tumor cells. Moreover, the proteins bovine pancreatic ribonuclease and bovine α-lactalbumin are normally secreted as both glycosylated and unglycosylated forms (Plummer & Hirs, 1964; Hill & Brew, 1975), indicating that secretion is not necessarily dependent on glycosylation even for a particular polypeptide. Thus, it seems that no generalization can be made about the role of carbohydrate chains in secretion. In certain glycoproteins, the carbohydrate may indeed by involved in the secretory process, whereas in others, it may serve an entirely different function.

Weintraub et al. (1980) have investigated the relationship of glycosylation to the synthesis of thyroid-stimulating hormone (TSH) in mouse pituitary tumor cells and found that a nonglycosylated α subunit was secreted in the presence of tunicamycin which was incompetent for combination with TSH-β. They concluded that specific glycosylation may be required for α–β-subunit combination but not for secretion. Likewise, Ruddon et al. (1979) have reported that doses of tunicamycin up to 5 μg/mL for 16 h did not appear to inhibit the synthesis or secretion of hCG subunits by choriocarcinoma cells (JAR). The results obtained in the present study suggest that the HeLa α protein, shown to be glycosylated by its adsorption to Con A–Sepharose, is not dependent for secretion on complete oligosaccharide side chains since the glucosamine content of the subunit could be reduced by 92% with a reduction in secreted hormone levels no greater than would be expected from the decrease in general protein synthesis caused by the glycosylation inhibitor (Table V).

Both tunicamycin and deoxyglucose caused a decrease in the levels of total (extracellular plus intracellular) HeLa α, the greatest decrease occurring in butyrate-treated cells in the presence of dGlc. These inhibitors reduced the levels of secreted α somewhat more than they did the intracellular levels of the protein. That is, a slight decrease in the ratio of media to lysate α was observed at the highest concentrations of inhibitor (Tables I and III). Nevertheless, in all cases, the extracellular subunit represented 78–92% of the total α in a flask, under conditions where glycosylation was inhibited by over 90%. Thus, the quantities of extracellular hormone were considerably greater than would be anticipated for a direct correlation between subunit glycosylation and secretion. The fact that dGlc had a similar effect on the accumulation of α subunit (a secreted protein) and PAP (a membrane protein) also suggests that the sugar was not acting primarily on protein secretion. Thus, taken together, these results suggest that glycosylation may facilitate subunit release but seems not to be an absolute requirement.

The possibility that TM and dGlc treatment resulted in retention and rapid intracellular degradation of certain nonglycosylated forms has not yet been examined. Moreover, the data do not rule out the possible contribution to α secretion of a minor oligosaccharide component that is resistant to TM and dGlc since a fraction of the extracellular subunit could still be adsorbed by Con A (Table VI). It will be of interest to determine whether or not such Con A bound material contains normal carbohydrate side chains.

As stated above, the mechanism by which sodium butyrate enhances α and PAP levels in HeLa cells is not known. The present results demonstrate that the inhibition of α and PAP synthesis by dGlc and TM (though to a lesser extent) was generally greater in butyrate-induced cultures, indicating that the cellular response to butyrate which leads to an increased accumulation of these proteins is quite sensitive to the glycosylation inhibitors. Moreover, reduction of the butyrate-induced proteins by dGlc was similar to the inhibition of glycosylation caused by this sugar and was greater than can be accounted for by its effect on polypeptide synthesis (Tables II and III).

Although the inhibition of protein glycosylation in chick embryo fibroblasts by TM or other inhibitors of glycosylation resulted in defective transport of several metabolites (Olden et al., 1979), the results reported here indicate that TM up to 2 μg/mL had no inhibitory effect on sodium butyrate uptake and that dGlc reduced its uptake only slightly (25%) at 20 mM but had no effect at those concentrations which markedly reduced α and PAP induction (i.e., 1–5 mM). This would suggest that the reduced induction cannot be explained as the simple inhibition of butyrate uptake by the cells.

Sodium butyrate has been shown to increase the activity of a sialyltransferase in HeLa cells (Fishman et al., 1974), and the secretion of thryoglobulin has been shown to be enhanced by sialylation (Monaco & Robbins, 1973), thus suggesting a possible involvement of the fatty acid in these reactions. As noted above, however, butyrate increased not only the extracellular levels but also the intracellular levels of HeLa α (Tables I and III), suggesting that overall synthesis and not just secretion was affected. Moreover, a concomitant rise in intracellular α was not observed when the levels of extracellular α decreased following TM or dGlc addition as might be expected if secretion were the rate-limiting step. This last interpretation could be complicated by the possibility that an intracellular pool of apoprotein may not accumulate if the nonglycosylated protein is rapidly degraded. In this regard, Braatz & Heath (1974) have demonstrated that while *Micrococcus sodonensis* secretes extracellular enzymes such as alkaline phosphatase under conditions of glucosamine inhibition, the nonglycosylated proteins were rapidly degraded. Similarly, Olden et al. (1978) observed that a major effect of tunicamycin treatment on the predominant cell-surface glycoprotein of chick embryo fibroblasts was to accelerate its rate of degradation 2–3-fold.

It should be noted that the experiments described above demonstrate an accumulation of α subunit and alkaline phosphatase activity in the presence of sodium butyrate and a decrease in the induced levels as a result of dGlc addition. They do not, however, differentiate between an effect of these compounds on increased synthesis or decreased degradation of the ectopic proteins. Experiments are currently under way to examine these alternatives and to determine whether effects of butyrate other than protein induction can be prevented or reversed by dGlc.

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Reconstitution of Binding Protein Dependent Active Transport of Glutamine in Spheroplasts of *Escherichia coli*†

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ABSTRACT: In order to directly prove that the periplasmic glutamine binding protein is an essential component of the osmotic shock sensitive active transport system for glutamine in *Escherichia coli*, we demonstrated the reconstitution of binding protein dependent glutamine transport in spheroplasts of that organism. It was shown by arsenate inhibition that the reconstituted transport system was energy dependent, and the use of azaserine, an inhibitor of glutamine-utilizing enzymes, indicated that the restoration of transport by binding protein did not require the metabolizing of the transport substrate. Furthermore, the binding protein dependent transport of glutamine was shown to require at least one other macromolecular component, presumably membrane bound, which was absent in a strain containing a deletion of the genes coding for the glutamine transport system but was present in a strain carrying a mutation only in the structural gene for the glutamine binding protein.

Despite a wealth of genetic evidence that the periplasmic binding proteins of *Escherichia coli* and *Salmonella typhimurium* are associated with particular active transport systems [for examples, see Ames & Lever (1970), Boos (1972), Rahmanian et al. (1973), and Weiner & Heppel (1971)], direct demonstrations of this fact have been scarce. Shortly after the discovery of many of the binding proteins, a number of studies appeared reporting the restoration of various transport systems in osmotically shocked cells by the addition of concentrated shock fluid or purified binding proteins (Wilson & Holden, 1969; Anraku, 1968; Medveczky & Rosenberg, 1970; Nishimune & Hayashi, 1971). However, the extent of the binding protein stimulated transport in shocked cells was, in most cases, not substantial, and proper control experiments were not demonstrated. In addition, some of these results could not be reproduced by other investigators (Rosen, 1973) or even by the same laboratory (Gerdes et al., 1977). In order to avoid these ambiguities, we examined the possibility of restoring shock-sensitive glutamine transport in spheroplasts of *E. coli*. In this paper we discuss the reconstitution of binding protein dependent active transport of glutamine in spheroplasts and show that the reconstituted transport system is energy dependent and does not require the metabolizing of the transport substrate. Evidence is also presented that glutamine transport requires at least one other macromolecular component in addition to the glutamine binding protein.

Materials and Methods

Materials. Radioactive glutamine was from New England Nuclear; radioactive proline was from Schwarz/Mann. Cellulose acetate filters, used in transport assays, were obtained from Millipore; nitrocellulose filters, used in glutamine binding protein assays, were from Schleicher & Schuell. Sucrose was Schwarz/Mann special enzyme grade. Azaserine (*O*-diazoacetyl-L-serine) was obtained from Calbiochem.

Bacterial Strains and Growth Media. The three *E. coli* K-12 strains used in this study were PSM2 (F⁻ *thi metC glnPo*), PSM223 (F⁻ *thi metC ΔnadA glnP*), and PSM116 (F⁻ *thi metC glnPo glnP*), which will be described in detail elsewhere (unpublished results). PSM2 contains a regulatory mutation, *glnPo*, that causes overproduction of the glutamine transport system. PSM223, derived from PSM2, contains a deletion extending entirely through the genes coding for the glutamine transport system. PSM116, also derived from PSM2, has a point mutation in the structural gene for the glutamine binding protein.

Minimal salts medium E (Vogel & Bonner, 1956) containing 0.5% succinate (sodium salt) was used for growth of cells. Methionine was added to 0.4 mM, thiamine to 40 μM, nicotinamide to 30 μM, and biotin to 20 nM.

Purification of the Glutamine Binding Protein. Glutamine binding protein was purified as described previously (Willis & Seegmiller, 1976), except that the final column (SP-Sephadex) was eluted with 4 column volumes of a linear gradient of 0.05-0.20 M NaCl in 10 mM Tris[1]-acetate, pH

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