King, T. P. (1973) *Arch. Biochem. Biophys. 156,* 509.
King, T. P. (1976) *Adv. Immunol. 23,* 77.
King, T. P., Sobotka, A. K., Kochoumian, L., & Lichtenstein, L. M. (1976) *Arch. Biochem. Biophys. 172,* 661.
Liao, T. H. (1974) *J. Biol. Chem. 249,* 2354.
Liao, T. H., Robinson, G. W., & Salnikow, H. (1973) *Anal. Chem. 45,* 2286.
Lichtenstein, L. M., & Osler, A. G. (1964) *J. Exp. Med. 120,* 507.
Light, W. C., Reisman, R. E., Shimizu, M., & Arbesman, C. E. (1977) *J. Allergy Clin. Immunol. 59,* 247.
Liu, T. Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246,* 2842.
Loveless, M. H., & Fackler, W. R. (1956) *Ann. Allergy 14,* 347.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.
Nair, B. C., Nair, C., Denne, S., Wypych, J., Arbesman, C. E., & Elliott, W. B. (1976) *J. Allergy Clin. Immunol. 58,* 101.
O'Connor, R., & Erickson, R. (1965) *Ann. Allergy 23,* 151.
Ouyang, C. H., & Huang, T. F. (1976) *Biochim. Biophys. Acta 439,* 146.
Parish, H. M. (1965) *Am. J. Med. Sci. 245,* 129.
Reisfeld, R. A., Lewis, U. J., & Williams, D. E. (1962) *Nature (London) 195,* 281.
Reisner, A. H., Nemes, P., & Bucholtz, C. (1975) *Anal. Biochem. 64,* 509.
Rinderknecht, H., Geokas, M. C., Silverman, P., & Haverback, B. J. (1968) *Clin. Chim. Acta 21,* 197.
Settipane, G. A., Newstead, G. J., & Boyd, G. K. (1972) *J. Allergy Clin. Immunol. 50,* 146.
Shiloah, J., Klibansky, C., De Vries, A., & Berger, A. (1973) *J. Lipid. Res. 14,* 267.
Shulman, S. (1968) *Prog. Allergy 12,* 246.
Siriganian, R. P. (1974) *Anal. Biochem. 57,* 383.
Sobotka, A. K., Valentine, M. D., Benton, A. W., & Lichtenstein, L. M. (1974) *J. Allergy Clin. Immunol. 52,* 170.
Sobotka, A. K., Adkinson, N. F., Jr., Valentine, M. D., & Lichtenstein, L. M. (1978) *J. Immunol.* (submitted).
Swank, R. T., & Kundres, K. D. (1971) *Anal. Biochem. 39,* 462.
Tolksdorf, S., McCready, M. H., McCullagh, D. R., & Schwenk, E. (1949) *J. Lab. Clin. Med. 34,* 74.
Wang, D., & Moore, S. (1977) *Biochemistry 16,* 2937.
Weeke, B. (1973) *Scand. J. Immunol. Suppl. 1, 2,* 15.
Yoshida, H., Geller, R. G., & Pisano, J. J. (1976) *Biochemistry 15,* 61.
Zeleznick, L. D., Hunt, K. J., Sobotka, A. K., Valentine, M. D., Tippett, L. O., & Lichtenstein, L. M. (1977) *J. Allergy Clin. Immunol. 59,* 2.

Temporal Relationship of Translation and Glycosylation of Immunoglobulin Heavy and Light Chains†

L. W. Bergman and W. M. Kuehl*

ABSTRACT: The initial glycosylation of MPC 11 γ_{2b} heavy chains occurs quantitatively in vivo when the nascent heavy chains reach a size of approximately 38 000 daltons. Nonglycosylated, completed MPC 11 heavy chains cannot be glycosylated in these cells. Other classes of mouse heavy chains (i.e., μ, α, and γ_1) also appear to be glycosylated as nascent chains; nonglycosylated, completed heavy chains cannot be glycosylated by the cell in any of these cases. In contrast, variant MPC 11 cells synthesizing a heavy chain with a carboxy-terminal deletion appear to glycosylate some heavy chains prior to chain completion and some heavy chains after chain completion and release from the polysomes. Similar to the variant MPC 11 cells, MOPC 46B cells (which synthesize a κ light chain containing an oligosaccharide attached to an asparagine located 28 residues from the amino terminus) glycosylate the majority of light chains prior to chain completion but also some light chains after chain completion and release from the polysomes. In addition, it appears that, although completed MOPC 46B light chains can be glycosylated if they are present in a monomeric form, they cannot be glycosylated if they are present in a covalent dimeric form.

In recent years, a substantial amount of work has been done to characterize the addition of core oligosaccharides to protein. This initial glycosylation event involves the transfer of a large-molecular-weight oligosaccharide containing *N*-acetylglucosamine, mannose, and glucose as a unit from a lipid intermediate to an asparagine acceptor residue on the polypeptide (Waechter et al., 1973; Hsu et al., 1974; Waechter and Lennarz, 1977). Recent evidence has suggested that soon after transfer there is processing of the oligosaccharide (Robbins et al., 1977; Tabas et al., 1978). Previously, we have shown that the transfer of the oligosaccharide can occur while the nascent immunoglobulin heavy chain is still bound to the ribosome, i.e., very soon after the acceptor residue is synthesized and inserted into the lumen of the endoplasmic reticulum (Bergman and Kuehl, 1977). Using various experimental systems and procedures, several laboratories have obtained results which led them to the same conclusion (Kiely et al., 1976; Rothman and Lodish, 1977; Sefton, 1977; Bielinska and Boime, 1978). However, other studies have suggested that glycosylation may occur on completed chains after their release from the ribosome (Shubert, 1970; Buxbaum and Scharff, 1973; Eagon et al.,

† From the Department of Microbiology, Jordan Medical Building, University of Virginia, Charlottesville, Virginia 22908. *Received June 27, 1978.* Research funds were provided through a grant from the National Institutes of Health (AI00293-01) to W.M.K.

0006-2960/78/0417-5174$01.00/0

1975; Tucker and Pestka, 1977).

Results presented in this paper indicate that the cell quantitatively glycosylates the MPC 11 nascent heavy chain soon after the asparagine site becomes available; if glycosylation does not occur while the nascent protein is still bound to the ribosome, the site soon becomes inaccessible for the glycosylation enzymes. We have demonstrated that other classes of mouse myeloma heavy chains also appear to be glycosylated in vivo prior to chain completion. In contrast, it appears that, although most MOPC 46B light chains are glycosylated in vivo as nascent chains, these light chains can be glycosylated in vivo as completed chains.

Materials and Methods

Cells. 45.6, a clone derived from the MPC 11 plasmacytoma cell line (Laskov and Scharff, 1970), synthesizes glycosylated γ_{2b} heavy (H) chains, κ light (L) chains, and a κ light-chain constant-region fragment (F_{CL}). These products represent 12, 8, and 1%, respectively, of proteins synthesized by this clone (Kuehl and Scharff, 1974). Clone M311 was obtained after mutagenesis with Melphalan of the parental clone 45.6 (Birshtein et al., 1974) and was characterized previously (Weitzman and Scharff, 1976; Weitzman et al., 1977). S107 (α, κ), MOPC 21 (γ_1, κ), and MOPC 104E (μ, λ) plasmacytoma cell lines were gifts from Dr. Matthew Scharff, Albert Einstein College of Medicine. The cells were maintained in suspension in Dulbecco's modified Eagle's medium supplemented with 15% heat-inactivated horse serum, 2 mM glutamine, and nonessential amino acids.

The plasmacytoma tumor MOPC 46B was obtained from Litton Bionetics under contract with the National Cancer Institute, National Institutes of Health. The cells were grown in ascitic form by intraperitoneal injection of Balb/c mice which had been primed with Pristane (Potter, 1972).

Cell Labeling, Fractionation, and Nascent Chain Isolation. The methods employed were essentially as described previously (Bergman and Kuehl, 1977). To enrich for "tight" membrane-associated ribosomes, potassium salt buffer was added at final concentrations of 500 mM KCl, 5.5 mM $MgCl_2$, 60 mM Tris[1]-Cl (pH 7.4) to the postnuclear supernatant prior to the isolation of microsomes (Zauderer et al., 1973).

To prepare microsomal immunoglobulin, the microsomal pellet was solubilized with 1% NP40-pH 6.7 buffer [75 mM NaCl-10 mM $MgCl_2$-25 mM phosphate (pH 6.7)] and the ribosomes were removed by centrifugation through 2.0 M sucrose-pH 6.7 buffer at 195 000*g* for 16 h. The supernatant was dialyzed extensively vs. phosphate-buffered saline (138 mM NaCl-3 mM KCl-8 mM $NaH_2PO_4 \cdot 12H_2O$-1 mM K_2HPO_4) and specifically immunoprecipitated.

For short-term pulse-label experiments, $1-3 \times 10^7$ cells were collected by centrifugation at 500*g* for 5 min, washed three times in spinner salts minus methionine, and then labeled in 2.0 mL for the appropriate time period (30 s to 2 min) with [^{35}S]methionine (Amersham/Searle, 800–1200 Ci/mmol) at an isotope concentration of 200–300 μCi/mL. The labeling was terminated by the direct addition of an equal volume of 2% NP40 at 4 °C in a buffer comprised of 150 mM NaCl, 10 mM Tris, and 1.5 mM $MgCl_2$. A postribosomal supernatant was prepared by centrifugation at 160 000*g* for 2 h, and the supernatant was specifically immunoprecipitated.

The isolation of nascent chains was modified in the following manner: The 1.0 M NaCl nascent chain fraction from the QAE-Sephadex column was diluted tenfold with 0.1% Brij 35–6 M urea–0.1 M ammonium formate (pH 4.7) to restore the low NaCl concentration of the QAE starting buffer and then applied to a second QAE-Sephadex column. The column was washed with 4 volumes of QAE starting buffer and eluted by increasing the NaCl concentration from 0.1 to 1.0 M in the pH 4.7 buffer. This eluted fraction from the second column was used as the nascent chain fraction in further studies.

To prepare nonglycosylated immunoglobulin, $1-3 \times 10^7$ cells were preincubated in the presence of 10 mg/mL glucosamine in 25.0 mL of Dulbecco's modified Eagle's medium for 50 min at 37 °C (Bekesi and Winzler, 1969). Then the cells were labeled in 10.0 mL with reconstituted [^{14}C]protein hydrolysate (Amersham/Searle, 54 mCi/matom of carbon) at an isotope concentration of 15–25 μCi/mL in the presence of glucosamine, and the immunoglobulin was isolated by immunoprecipitation of the detergent-lysed postnuclear supernatant.

Antisera. Antibodies directed against γ_{2b} H chain and κ L chain were prepared as described previously (Horwitz and Scharff, 1969). Antibodies directed against: γ_1 H chain, κ L chain (anti-MOPC21); α H chain, κ L chain (anti-S107); and against μ H chain, λ L chain (anti-MOPC 104E) were gifts of Dr. M. Scharff. The immunoprecipitations were performed in antibody excess and incubated overnight at 4 °C. The immunoprecipitates were collected by centrifugation through 1 M sucrose in PBS and washed once with PBS (Rhoads et al., 1972).

Indirect immunoprecipitation utilizing SaCI (*Staphylococcus aureus* Cowans I strain) was carried out according to the procedure of Cullen and Schwartz (1976).

Radioactivity Measurement. Gel-filtration fractions and crushed polyacrylamide gel samples were counted in a Triton X-100/toluene scintillation fluor containing 10% H_2O (v/v) and counted in a Beckman LS 230 scintillation counter.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. The immunoprecipitates were boiled in $NaDodSO_4$ sample buffer and reduced in 0.15 M β-mercaptoethanol at 37 °C. They were then electrophoresed in either 10 or 12.5% polyacrylamide gels using a discontinuous $NaDodSO_4$-Tris-glycine buffer system essentially as described by Laemmli (1970) and Maizel (1971). Cylindrical gels were sliced into 2-mm fractions with a Gilson Aliquogel fractionator and counted as described above. Slab gels were subjected to autoradiographic or photofluorographic procedure (Bonner and Laskey, 1974; Laskey and Mills, 1975) for visualizing the radioactive protein bands.

Densitometry. Autoradiographs and photofluorographs were scanned using a Schoeffel Model SD3000 spectrodensitometer.

Results

Nascent MPC 11 Heavy Chains Are Glycosylated Quantitatively Prior to Chain Completion. We have attempted to determine if MPC 11 heavy chains are glycosylated quantitatively prior to chain completion and release from the ribosome. Figure 1 indicates that after a 30-s or 1-min pulse-labeling period with [^{35}S]methionine all completed heavy-chain polypeptides are glycosylated; no completed heavy-chain polypeptides comigrating with a nonglycosylated heavy-chain marker can be detected. To provide further evidence to support this conclusion and to determine more precisely the temporal relationship between translation and glycosylation, we have compared the ratio of carbohydrate to amino acid in both completed and nascent heavy chains. The completed and nascent heavy chains were isolated from the microsomal fraction of cells labeled for 5 min in the presence of both [^{3}H]glucosamine and [^{35}S]methionine as described under

[1] Abbreviations used: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; QAE, quarternary aminoethyl; PBS, phosphate-buffered saline.

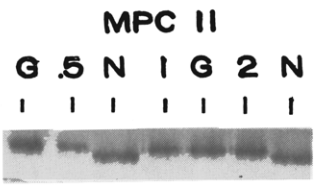

FIGURE 1: Electrophoresis of pulse-labeled MPC 11 completed heavy chains. MPC 11 cells were pulsed with [^{35}S]methionine for 30 s (0.5), 1 min (1), or 2 min (2). Completed heavy chains were immunoprecipitated from the postribosomal supernatant and subjected to NaDodSO$_4$ electrophoresis in 12.5% acrylamide slab gels as described under Materials and Methods. A radioautograph of this gel is shown. Columns G and N are glycosylated and nonglycosylated marker heavy chains, respectively.

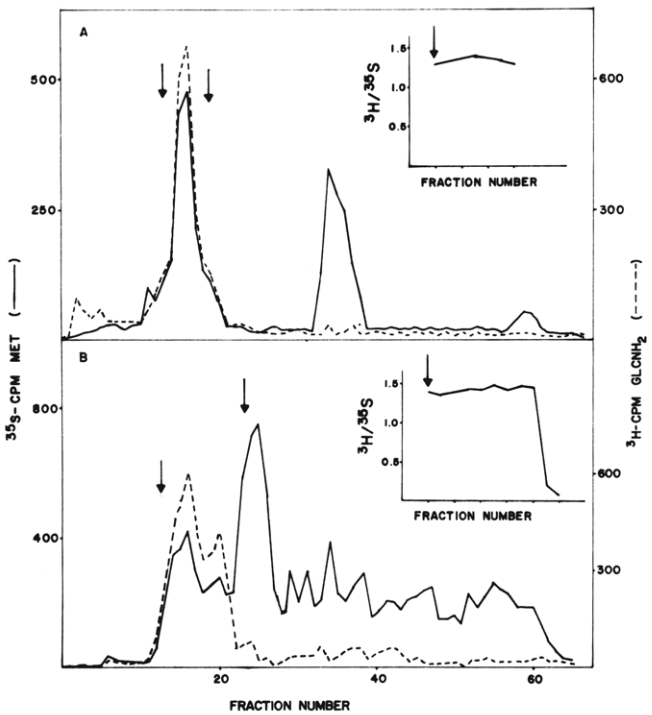

FIGURE 2: Electrophoresis of immunoprecipitated MPC 11 completed and nascent chains. 5–10 × 10^8 MPC 11 cells were collected and labeled for 5 min with [^{3}H]glucosamine (20 Ci/mmol; New England Nuclear) and [^{35}S]methionine at isotope concentrations of 100 and 15 μCi/mL, respectively, in the presence of medium containing all amino acids except methionine and lacking glucose (10 mL total volume). The isolation of completed and nascent chains was as described under Materials and Methods: (A) completed chains; (B) nascent chains; (- - -) [^{3}H]glucosamine; (—) [^{35}S]methionine. The ratio of [^{3}H]glucosamine to [^{35}S]methionine for each fraction (marked by the arrows) was calculated and plotted (insets).

Materials and Methods. As expected, the completed heavy chain (Figure 2A) is homogeneous in size with a nearly constant ratio of ^{3}H-labeled carbohydrate to ^{35}S-labeled amino acid. In contrast, the nascent heavy chains (Figure 2B) are heterogeneous in size, with a range of 38 000–55 000 daltons for the [^{3}H]glucosamine-labeled nascent heavy chains and a range of 10 000–55 000 daltons for the [^{35}S]methionine-labeled nascent heavy and light chains.[2] When compared, the ratios of ^{3}H-labeled carbohydrate to ^{35}S-labeled amino acid

[2] As noted previously (Bergman and Kuehl, 1977), there is a prominent heterogeneous peak of amino acid labeled heavy chain (fraction 25) which migrates slightly faster than the most rapidly migrating glycosylated nascent heavy-chain species. This nonglycosylated nascent polypeptide may accumulate as a result of a translational block, although we have been unable to obtain data which confirms this hypothesis.

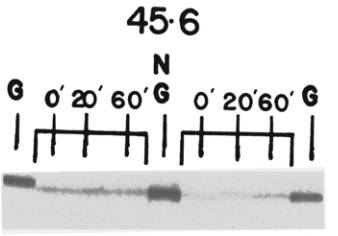

FIGURE 3: Effect of glucosamine inhibition on the glycosylation of MPC-11 heavy chain. One aliquot of MPC-11 cells was incubated for 50 min in the presence of 10 mg/mL glucosamine to inhibit glycosylation. The cells were then labeled for 5 min with ^{14}C-labeled amino acids in the presence of the inhibitor. Samples were removed at various intervals during a chase in medium containing excess glucose and unlabeled amino acids but lacking glucosamine. The samples were immunoprecipitated and analyzed on NaDodSO$_4$-gels as described under Materials and Methods (three time points to left). A second aliquot of cells was incubated for 55 min in the presence of glucosamine and then transferred into medium lacking glucosamine. Samples were removed at various intervals after removal of the glucosamine, pulsed for 5 min with ^{14}C-labeled amino acids, and analyzed as described above (three time points to right). Columns G and NG are glycosylated and nonglycosylated marker heavy chains, respectively.

for the recently completed heavy chains and the glycosylated nascent heavy chains are essentially the same. The slight decrease in the ratio obtained for the nascent glycosylated heavy chain as the species approached full size is probably due to the addition of the final methionine residue onto the growing polypeptide chain (see Discussion). The sharp increase in the ratio obtained for the nascent heavy chains (i.e., a ratio from 0.23 [fraction 21] to 1.49 [fraction 20]) implies that the MPC 11 heavy chains are quantitatively glycosylated as nascent chains soon after the asparaginyl acceptor site becomes accessible and prior to release from the ribosome (see Discussion; Bergman and Kuehl, 1977).

Nonglycosylated Completed MPC 11 Heavy Chains Cannot Be Glycosylated. Although the experiments above demonstrate quantitative glycosylation of nascent MPC 11 heavy chains, we attempted to determine whether it is possible for the cell to glycosylate completed heavy chains after release from the ribosome. MPC 11 cells were incubated in the presence of high levels of glucosamine for 50 min to inhibit glycosylation. The cells were then labeled for 5 min with ^{14}C-labeled amino acids in the presence of glucosamine, and samples were analyzed by immunoprecipitation and NaDodSO$_4$-gels at various times during a chase period in the presence of normal media (lacking both glucosamine and labeled amino acids). The results in Figure 3 indicate that nonglycosylated heavy chains synthesized in the presence of the glucosamine cannot be glycosylated as completed chains during the chase in the absence of glucosamine. As a control, cells were incubated in glucosamine for 55 min and pulsed for 5 min with ^{14}C-labeled amino acids at various times after removal of excess glucosamine to see if the cells had regained the ability to glycosylate newly synthesized heavy chains. The results, also in Figure 3, indicate that the cells regained the ability to synthesize the normal glycosylated heavy chain by 20 min after the removal of glucosamine. As an additional control, we have shown that MPC 11 cells can glycosylate a completed variant heavy chain after excess glucosamine is removed from the medium (see experiments on M311 variant cell line below). These results indicate that wild-type MPC 11 heavy chains cannot be glycosylated as completed chains postrelease from the ribosome.

Other Classes of Mouse Myeloma Heavy Chains Appear to Be Glycosylated Prior to Chain Completion. We examined the glycosylation of other classes of immunoglobulin heavy

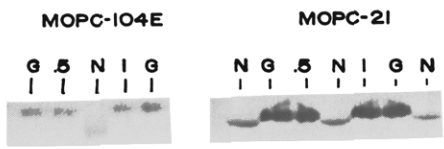

FIGURE 4: Electrophoresis of pulse-labeled MOPC 104E and MOPC 21 completed heavy chains. MOPC 104E and MOPC 21 cells were labeled and analyzed as described in the legend to Figure 1.

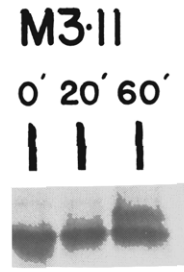

FIGURE 5: Effect of glucosamine inhibition on glycosylation of M311 heavy chain. M311 cells were incubated for 50 min in the presence of 10 mg/mL glucosamine and then labeled for 5 min with ^{14}C-labeled amino acids in the presence of the inhibitor. Samples were removed at various intervals, during a chase in medium containing excess glucose and unlabeled amino acids but lacking glucosamine. The samples were immunoprecipitated and analyzed on NaDodSO$_4$-gels as described under Materials and Methods.

chains synthesized by three mouse plasmacytoma cell lines: MOPC 104E (μ), S107 (α), and MOPC 21 (γ_1). Each cell line was tested for the presence of pulse-labeled nonglycosylated completed heavy chains and for the ability to glycosylate completed heavy chains after inhibition with high levels of glucosamine. With all three cell lines, the 30-s pulse-labeled heavy chain coelectrophoresed with the normal glycosylated marker (i.e., no detectable nonglycosylated heavy chain was present intracellularly). Representative results are shown for MOPC 104E and MOPC 21 in Figure 4. Also, each class of heavy-chain polypeptide remained nonglycosylated after its synthesis in the presence of high levels of glucosamine and subsequent chase in the absence of inhibitor (similar results as those described above for the MPC 11 γ_{2b} heavy chain) (Bergman and Kuehl, unpublished).

A Variant MPC 11 Heavy Chain Which Lacks Carboxy-Terminal Residues Can Be Glycosylated as a Completed Chain. M311 cells synthesize a variant heavy chain which exists in two discrete stable forms, a 38 000-dalton nonglycosylated species and a 42 000-dalton glycosylated species; when glycosylation is blocked in these cells, only the 38 000 dalton species is synthesized (Weitzman and Scharff, 1976; Weitzman et al., 1977). In contrast to wild-type MPC 11 heavy chains, the glycosylation of some M311 heavy chains occurs after completion and release from the ribosome, as demonstrated by the following experiment. M311 cells were pulsed for 1.5 min with [^{35}S]methionine. One aliquot was analyzed immediately and the other aliquot chased for 25 min in the presence of excess unlabeled methionine. After immunoprecipitation and analysis on NaDodSO$_4$-gels, the ratio of nonglycosylated heavy chain to glycosylated heavy chain decreases from 3.5 at the end of the 1.5-min pulse to 1.9 at the end of the 25-min chase. This represents an increase in the glycosylated form of heavy chain from 24% (at end of pulse) to 36% (at end of chase) of the total heavy chain. The amount of radioactivity immunoprecipitated in each aliquot did not differ significantly, thus ruling out degradation of the nonglycosylated heavy chain. These results are consistent with the findings of Weitzman and Scharff (1976). Thus, glycosylation of some M311 heavy chains has occurred in the rough endoplasmic reticulum after release from the ribosome. This result, however, does not rule out that some nascent M311 heavy chains may be glycosylated prior to polypeptide chain completion.

M311 cells were tested for the ability to glycosylate a completed heavy chain synthesized in the presence of high levels of glucosamine. The results, seen in Figure 5, show that nonglycosylated M311 heavy chain can be glycosylated post-release from the ribosome during the chase period in the absence of glucosamine (appearance of the glycosylated form by 60 min into the chase). In the experiment shown, at the end of the chase period 25% of the heavy chains were glycosylated. Since only 30–35% of heavy chains synthesized in the absence of glucosamine are glycosylated (see above), it appears that the glycosylation of completed chains is almost as efficient. Similar to the MPC 11 experiment described previously, the M311 cells had recovered the ability to synthesize a glycosylated heavy chain by 20 min into the chase (Bergman and Kuehl, unpublished). This suggests that the lack of glycosylation of the wild-type MPC 11 heavy chain is a function of the protein itself and is not due to the general metabolic poison effect of the high levels of glucosamine or to spatial separation of the completed polypeptide and the glycosylation enzymes.

MOPC 46B Light Chain Is Glycosylated as a Nascent Chain but Can Be Glycosylated as a Completed Chain. MOPC 46B tumor synthesizes and secretes a 25 000-dalton glycosylated κ light chain which has a single oligosaccharide moiety covalently linked at asparagine-28 in the variable region (Melchers, 1973). MOPC 46B cells were pulse labeled with [^{35}S]methionine, and the completed light chains were immunoprecipitated from the postribosomal supernatant and analyzed on NaDodSO$_4$-gels. The results indicate that approximately 85% of the light chain is glycosylated during this short pulse time of 30 s, while only a glycosylated form of the protein is present in cells pulsed for 2 min and subsequently chased in the presence of excess unlabeled methionine for 20 min (Bergman and Kuehl, unpublished). First, although this result suggests that the cell normally glycosylates some MOPC 46B light chains after completion and release from the ribosome, it is not possible to rule out degradation of the nonglycosylated chains during the chase period. Second, the finding that the majority (85%) of the molecules is glycosylated within 30 s implies that glycosylation of some nascent light chains occurs prior to chain completion and release from the ribosome.

To further examine these questions, MOPC 46B cells were pulsed for 5 min in the presence of both [^{3}H]glucosamine and ^{14}C-labeled amino acids. Subsequently, completed and nascent light chains were isolated from the microsomal fraction and then analyzed on NaDodSO$_4$-gels as described under Materials and Methods. The nascent chain profile, seen in Figure 6B, is heterogeneous in size distribution for both the carbohydrate and amino acid labels, with a size range of approximately 12 000–25 000 daltons for each isotope. The finding that the ratio of ^{3}H-labeled carbohydrate to ^{14}C-labeled amino acid obtained for the nascent chain sample increases from 0.51 (12 000 daltons) to 1.02 (25 000 daltons) as chains approach full size indicates that individual nascent molecules may be glycosylated at various times after the acceptor site is available. This result for MOPC 46B light chain should be contrasted to the result found for MPC 11 heavy chain (Figure 2), as glycosylation appears to occur quantitatively at a precise time in the latter case. The results in Figure 6A show that the completed light chain, as expected, is homogeneous in size and has a constant ratio of ^{3}H-labeled carbohydrate to ^{14}C-labeled amino acid of 1.08. This ratio is slightly greater than that ob-

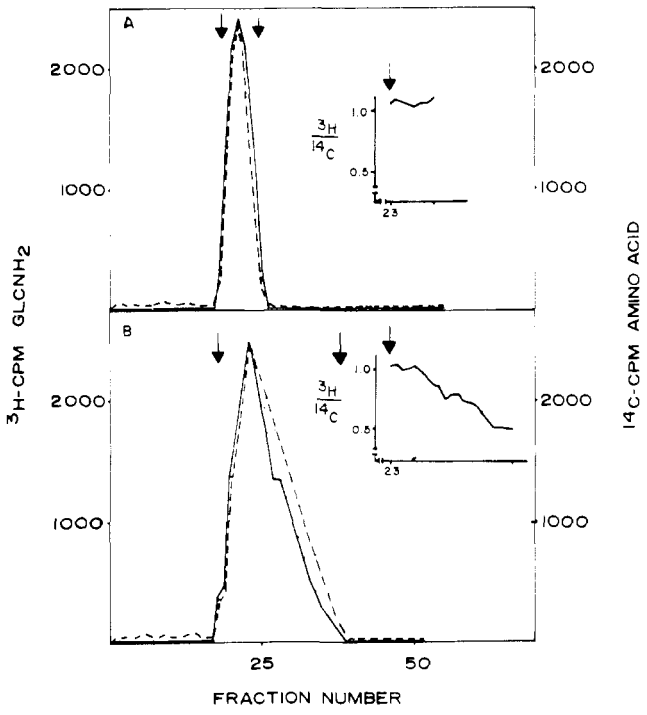

FIGURE 6: Electrophoresis of immunoprecipitated MOPC 46B completed and nascent light chains isolated from the microsomal fraction of cells labeled for 5 min with [^{3}H]glucosamine and ^{14}C-labeled amino acids (isotope concentrations of 125 μCi/mL for [^{3}H] glucosamine and 12.5 μCi/mL for ^{14}C-labeled amino acids). The isolation and analysis are essentially as described in the legend to Figure 2: (A) completed chains; (B) nascent chains; (—) [^{3}H]glucosamine (- - -) ^{14}C-labeled amino acids. The ratio of [^{3}H]glucosamine to ^{14}C-labeled amino acids for each fraction (marked by the arrows) was calculated and plotted (insets).

TABLE I: Effect of Glucosamine Inhibition on the Glycosylation of MOPC 46B Light Chain.[a]

		total light chain (100)	monomer (68)	dimer (32)
T = 0	NG	88	58	30
	G	12	10	2
			monomer (55)	dimer (45)
T = 60	NG	27	0	27
	G	73	55	18

[a] MOPC 46B cells were incubated for 50 min in the presence of high levels of glucosamine. At the level of glucosamine used (10–15 mg/mL in various experiments), we were never able to inhibit glycosylation completely. The cells were then labeled for 5 min with ^{14}C-labeled amino acids in the presence of the inhibitor. Samples were removed at the end of the pulse-labeling period (T = 0) and at the end of a 60-min chase in medium containing excess glucose and unlabeled amino acids but lacking glucosamine (T = 60). Cytoplasmic lysates of the cells were immunoprecipitated and subjected to NaDodSO$_4$-gel electrophoresis in the presence and absence of reducing agent as described under Materials and Methods. The autoradiograph was scanned and the percentage of each form of light chain calculated. In the gel system used, although it was possible to differentiate homodimers consisting of two glycosylated molecules, from homodimers consisting of two nonglycosylated molecules, these forms could not be distinguished from a heterodimer consisting of one glycosylated and one nonglycosylated molecule; therefore the percentage of nonglycosylated and glycosylated light chain in these forms was calculated using the other data in this table.

tained for the nascent chains that are near completion (ratio = 1.02), consistent with the finding that a small amount of glycosylation is taking place on completed and released light chains (see above).

To further investigate this question, we tested the ability of the MOPC 46B light chain to be glycosylated after its synthesis in the presence of high levels of glucosamine. Cells were preincubated in the presence of glucosamine for 50 min and then pulsed for 5 min with ^{14}C-labeled amino acids. Samples were taken at the end of the labeling period (T = 0) and after a 60-min chase in medium containing unlabeled amino acids and excess glucose but lacking glucosamine (T = 60). The samples were immunoprecipitated and subjected to NaDodSO$_4$-gel electrophoresis in the presence and absence of reducing agent to analyze both monomer and dimeric forms of the light chain. The results seen in Table I indicate that completed light chains can be glycosylated postrelease from the ribosome, as seen in the fact that the nonglycosylated form decreased from 88% of the total light chain at T = 0 to 27% at the end of the chase (T = 60). Since the amount of light chain immunoprecipitated at T = 0 and T = 60 was the same, this result cannot be due to selective degradation of the nonglycosylated light chain. At T = 60, the nonglycosylated light chain is present only as a dimer. Moreover, the percentage of light chain present in the nonglycosylated dimeric form did not significantly decrease during the chase (nonglycosylated light chain in the dimer is 30% of the total light chain at T = 0 and 27% of the total at T = 60). This finding suggests that the asparaginyl acceptor site is accessible for glycosylation in the light-chain monomer but is not available for glycosylation if the nonglycosylated molecule has been assembled into a dimer. The increase in the amount of glycosylated L chain dimer at the end of the chase (18%) compared to the beginning of the chase (2%) is due presumably to glycosylation of completed monomeric L chain and subsequent formation of glycosylated L chain dimers.

Discussion

Experiments were performed to determine what percentage of nascent MPC 11 heavy chains are glycosylated prior to release from the ribosome. In short pulse-label experiments, no detectable nonglycosylated heavy chain is present intracellularly. We have obtained the ratios of [^{3}H]glucosamine to [^{35}S]methionine for completed and nascent heavy chains isolated from the microsomal fraction of MPC 11 cells. The decrease seen in the ratio for the nascent chain sample as the chains approach full size is probably due to the addition of the final methionine residue (residue 413; B. Birshtein, personal communication) to the growing nascent molecule. The results in Figure 2 indicate that the carbohydrate to amino acid ratios are very similar for both completed and nascent heavy chains, implying that the glycosylation of the nascent heavy chains is quantitative (i.e., essentially all nascent heavy chains are receiving the oligosaccharide core prior to release from the ribosome). The observation that the ratio obtained for the nascent chain sample increases from 0.23 to 1.49 in a single fraction (approximately 38 000 daltons) suggests that quantitative glycosylation of the nascent chain occurs soon after the asparaginyl acceptor (approximately residue 291)[3] passes through to the lumen of the rough endoplasmic reticulum and becomes available for glycosylation.

Rothman and Lodish (1977), using a synchronized wheat germ cell-free synthesizing system in the presence of exogenous pancreatic rough endoplasmic reticulum membranes, have

[3] Residue 291 is the asparaginyl acceptor site in MOPC 21 heavy chain (Milstein et al., 1975). Since γ_{2b} and γ_1 heavy chains are homologous in sequence, we assume that the acceptor site is at or near residue 291 in MPC 11 heavy chains (Dayhoff, 1974; B. Birshtein, personal communication).

demonstrated a precise sequence of glycosylation of nascent chains of G protein of vesicular stomatitis virus. However, in their studies, the observed translation times ranged from 23 to 43 min, i.e., 20 to 30 times longer than in vivo translation times (Shapiro et al., 1966). Our results provide in vivo evidence that glycosylation occurs quantitatively at a precise time during translation and that this interval may be the only time that transfer of the core oligosaccharide to the MPC 11 heavy chain can occur (see below).

The question of whether glycosylation can occur on completed heavy chains after release from the ribosome was investigated by inhibiting the initial glycosylation event with high concentrations of glucosamine. Glycosylation is inhibited as a result of depletion of uridine nucleotide pools by trapping the cellular uridine nucleotide in the form of UDP-*N*-acetylglucosamine (Bekesi and Winzler, 1969). Figure 3 shows that nonglycosylated, completed heavy chain synthesized in the presence of the inhibitor cannot be glycosylated during a chase period in the absence of the glucosamine, although the cells regain the ability to glycosylate newly synthesized heavy chains by at least 20 min into the chase. Similar experiments using a MPC 11 variant heavy chain provide further evidence that the lack of glycosylation of the wild-type MPC 11 heavy chain is a function of the protein molecule itself and is not due either to the heavy-chain molecules being spatially separated from the glycosylation enzymes or to the general inhibitor effect of the glucosamine. Two possibilities are suggested for the inability of the cell to glycosylate completed MPC 11 heavy chains: (1) intramolecular folding (secondary and tertiary structure) and/or (2) intermolecular assembly (quaternary structure). In support of the first possibility, Pless and Lennarz (1977) reported that the core oligosaccharide can be added in vitro only to denatured ovalbumin and RNase A but not to the native forms of these secretory proteins. Thus, nascent chains may be providing the asparaginyl site with little secondary or tertiary structure, so that glycosylation can occur before the protein folds and can no longer be glycosylated. In support of the second possibility, our experiments with MOPC 46B cells indicate that a completed light chain can be glycosylated if it is in a monomeric form but cannot be glycosylated if it has been assembled into a dimer (see below). It is of interest that crystallographic studies by Davies and co-workers (Silverton et al., 1977) have suggested that the carbohydrate moiety may play a central role as the principal contact between the second constant region domains of the heavy chains in intact immunoglobulin molecules. Therefore, the unavailability of the glycosylation site in complete MPC 11 heavy chains may reflect an intermolecular assembly of the nonglycosylated heavy chain with light chain and/or a second heavy chain. Unfortunately, we cannot distinguish these two possibilities presently because of the very rapid intramolecular folding and intermolecular assembly of newly synthesized heavy chains (Bigelow et al., 1974; Baumal and Scharff, 1973).

Several studies, using pulse–chase amino acid labeling experiments, have demonstrated an apparent increase in the size of intracellular heavy chain during the chase period, suggesting that the addition of core oligosaccharide may occur during a considerable time period following polypeptide chain completion and release from the ribosome (Shubert, 1970; Buxbaum and Scharff, 1973). Using μ-, α-, and γ-producing mouse plasmacytoma cell lines, we have shown that pulse-labeled completed heavy chains coelectrophorese with the respective glycosylated marker heavy chain and that no detectable nonglycosylated heavy chains are present intracellularly; this suggests that glycosylation must occur very early, presumably while the nascent molecule is still bound to the ribosome. It is noteworthy that this is true for μ and α heavy chains as well as γ chains, since the former contain multiple oligosaccharide chains per polypeptide whereas the latter contain only one oligosaccharide per chain. Also, the nonglycosylated heavy chains synthesized in the presence of high levels of glucosamine are unable to be glycosylated during a chase period in the absence of the inhibitor. Therefore, it appears that glycosylation of nascent heavy chains, and other proteins as well, is a general mechanism for transfer of the oligosaccharide core from the lipid intermediate to the protein during glycoprotein biosynthesis. The biological need for the tight coupling of translation and glycosylation could be that the acceptor site(s) for glycosylation may soon become inaccessible for the glycosylation enzymes either by folding of the protein molecule or assembly into a multichain structure.

We have demonstrated that a MPC 11 variant heavy chain (synthesized in the M311 variant cell line) can be glycosylated as a completed chain after release from the ribosome. The data (see Results) indicate that in pulse–chase experiments there is a significant increase in the amount of intracellular glycosylated heavy chain during the chase. Similar results were obtained by Weitzman and Scharff (1976). The glycosylation of the completed M311 heavy chain is also seen in Figure 5, in that there is the appearance of the glycosylated form of the molecule by 60 min after synthesis of the nonglycosylated completed heavy chain in the presence of high levels of glucosamine. It is noteworthy that this variant heavy chain includes only 38 000 daltons of amino acids due to a deletion, starting shortly after the oligosaccharide acceptor site, of the carboxy-terminal region of the molecule (B. Birshtein, personal communication). This carboxy-terminal deletion may account for some of the results seen with this cell line. First, since it is likely that the variant heavy chain is completed at approximately the same time the asparaginyl acceptor site becomes available for glycosylation, it is not surprising that glycosylation is incomplete when the chain is completed and released from the ribosome. Second, this deletion may affect the conformation of the asparaginyl acceptor site, thereby allowing glycosylation to occur—to some extent—after release of the protein from the ribosome. The basis for only one-third of the M311 heavy-chain molecules being glycosylated is not known.

Using an in vitro glycosylation system, Eagon et al. (1975) reported the transfer of the oligosaccharide core from a dolichol lipid intermediate to completed MOPC 46B light chain. Tucker and Pestka (1977) using a wheat germ synthesized MOPC 46B precursor light chain have also shown in vitro glycosylation (2–3% efficient) of the completed precursor light chain. We have investigated the in vivo glycosylation of the light chain to determine the time of addition of the oligosaccharide to the protein. Our results indicate that the majority of the glycosylation is a cotranslational event, occurring while the protein is still bound to the ribosomal complex. Analysis of the pulse-labeled completed light chain reveals that, within 30 s, 85% of the light chain is glycosylated (Bergman and Kuehl, unpublished). Other results (see Figure 6A) demonstrate that glucosamine is incorporated onto nascent chains, as isolated by QAE-Sephadex chromatography, with a heterogeneous size distribution ranging from 12 000 to 25 000 daltons. This indicates that some nascent light chains may be glycosylated soon after the asparaginyl acceptor site (residue 28) reaches the luminal side of the endoplasmic reticulum. The finding that the ratio of [^{3}H]glucosamine to ^{14}C-labeled amino acid increases from 0.51 for 12 000 dalton nascent chains to 1.02 for full size nascent chains (i.e., approximately 23 and 94%, respectively, of 12 000 dalton and 25 000 dalton nascent

chains are glycosylated) indicates that the rate of nascent chain glycosylation is much slower than for the MPC 11 heavy chain. The reason that quantitative glycosylation of the MOPC 46B light chain does not occur at a precise time, as shown above for the MPC 11 heavy chain, may be the presence of the amino-terminal precursor sequence. Several groups have provided evidence that the removal of the signal sequence is also a co-translational event (Blobel and Dobberstein, 1975; Szezesna and Boime, 1976). The binding of the signal sequence to a proteolytic enzyme for processing may interfere with the availability of the acceptor site (residue 28) for glycosylation, thus slowing glycosylation so that individual nascent chains may be glycosylated at various times after the acceptor site is potentially available. It is not known whether or not the precursor sequence is removed before glycosylation can occur.

The findings that 15% of the light chain is nonglycosylated after a 30-s label and the ratio of carbohydrate to amino acid for full size nascent chain is less than that obtained for the microsomal completed chains demonstrate that a small percentage of the glycosylation occurs after release of the polypeptide from the ribosome. We then tested the ability of MOPC 46B light chain to be glycosylated during a chase period after synthesis of the nonglycosylated form in the presence of the inhibitor glucosamine. The data in Table I reveal that the acceptor site is available for glycosylation after release from the ribosome in that there is an increase in the amount of the glycosylated form during the chase period. However, it is apparent that the only nonglycosylated light chain present at the end of the chase period is present in a dimeric form and that the percentage of the light chain present as nonglycosylated dimer did not change during the chase period. This finding suggests that the asparaginyl acceptor site is accessible for glycosylation if the light chain is in the monomeric form but is inaccessbile for glycosylation if the molecule has been assembled into a dimer.

References

Baumal, R., and Scharff, M. D. (1973), *Transplant. Rev. 14,* 163.

Bekesi, J. G., and Winzler, R. J. (1969), *J. Biol. Chem. 244,* 5663.

Bergman, L. W., and Kuehl, W. M. (1977), *Biochemistry 16,* 4490.

Bielinska, M., and Boime, I. (1978), *Proc. Natl. Acad. Sci. U.S.A. 75,* 1768.

Bigelow, C. C., Smith, B. R., and Dorrington, K. J. (1974), *Biochemistry 13,* 4602.

Birshtein, B. K., Preud'homme, J. L., and Scharff, M. D. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3478.

Blobel, G., and Dobberstein, B. (1975), *J. Cell Biol. 67,* 835.

Bonner, W. M., and Laskey, R. A. (1974), *Eur. J. Biochem. 46,* 83.

Buxbaum, J. N., and Scharff, M. D. (1973), *J. Exp. Med. 138,* 278.

Cullen, S. E., and Schwartz, B. D. (1976), *J. Immunol. 117,* 136.

Dayhoff, M. O., Ed. (1974), Atlas of Protein Sequence and Structure, Silver Springs, Md., National Biomedical Research Foundation, p 165.

Eagon, P. K., Hsu, A. F., and Heath, E. C. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 678, abstract.

Horwitz, M., and Scharff, M. D. (1969), in Fundamental Techniques in Virology, Habel, K., and Salzman, N. P., Ed., New York, N.Y., Academic Press, p 253.

Hsu, A., Baynes, J. W., and Heath, E. C. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2391.

Kiely, M. L., McKnight, G. S., and Schimke, R. T. (1976), *J. Biol. Chem. 251,* 5490.

Kuehl, W. M., and Scharff, M. D. (1974), *J. Mol. Biol. 89,* 409.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Laskey, R. A., and Mills, A. D. (1975), *Eur. J. Biochem. 56,* 335.

Laskov, R., and Scharff, M. D. (1970), *J. Exp. Med. 131,* 515.

Maizel, J. V., Jr. (1971), *Methods Virol. 5,* 179.

Melchers, F. (1973), *Biochemistry 12,* 1471.

Milstein, C., Adetugbo, K., Brownlee, G. G., Cowan, N. J., Proud'foot, N. J., Rabbits, T. H., and Seeher, D. S. (1975), *Mol. Approaches Immunol. 9,* 131.

Pless, D. D., and Lennarz, W. J. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 134.

Potter, M. (1972), *Physiol. Rev. 52,* 632.

Rhoads, R. E., McKnight, G. S., and Schimke, R. T. (1972), *J. Biol. Chem. 248,* 2031.

Robbins, P. W., Hubbard, S. C., Turco, S. J., and Wirth, D. F. (1977), *Cell 12,* 893.

Rothman, J. E., and Lodish, H. F. (1977), *Nature (London) 269,* 775.

Sefton, B. M. (1977), *Cell 10,* 659.

Shapiro, A. L., Scharff, M. D., Maizel, J. V., Jr., and Uhr, J. W. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 216.

Shubert, D. J. (1970), *J. Mol. Biol. 51,* 287.

Silverton, E. W., Navia, M. A., and Davies, D. R. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74,* 5140.

Szczesna, E., and Boime, I. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 1179 .

Tabas, I., Schlesinger, S., and Kornfeld, S. (1978), *J. Biol. Chem. 253,* 716.

Tucker, P., and Pestka, S. (1977), *J. Biol. Chem. 252,* 4474.

Waechter, C. J., and Lennarz, W. J. (1977), *Annu. Rev. Biochem. 45,* 95.

Waechter, C. J., Lucas, J. J., and Lennarz, W. J. (1973), *J. Biol. Chem. 248,* 7570.

Weitzman, S., and Scharff, M. D. (1976), *J. Mol. Biol. 102,* 237.

Weitzman, S., Nathanson, S. G., and Scharff, M. D. (1977), *Cell 10,* 679.

Zauderer, M., Liberti, P., and Baglioni, C. (1973), *J. Mol. Biol. 79,* 577.