of a gradient for the analysis of single-strand interruptions induced by chemical carcinogens. In combination with the McGrath–Williams technique (1966) this enzyme treatment is a useful analytical tool for exposing single-strand breaks.

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Addition of Glucosamine and Mannose to Nascent Immunoglobulin Heavy Chains†

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

ABSTRACT: We have investigated the process of protein glycosylation in an attempt to answer the question of whether glucosamine and mannose are added to nascent chains prior to chain completion or only to completed chains after release from the ribosome. The MPC 11 mouse plasmacytoma cell line used in these studies synthesizes a glycosylated γ_{2b} heavy chain which accounts for 12% of the total protein synthesis. Nascent chains were separated from completed chains by ion-exchange chromatography of solubilized ribosomes on QAE-Sephadex. Our results indicate that both glucosamine and mannose are incorporated into nascent heavy chains prior to chain completion and release from the ribosome. Gel analysis of specifically immunoprecipitated nascent chains indicates that the carbohydrate moiety can be added to the nascent heavy chains very soon after the presumptive asparaginyl glycosylation site (CH2 domain) is synthesized on the ribosome.

In recent years, a substantial amount of work has been done to determine the structure and biosynthesis of the carbohydrate portion of immunoglobulin (Uhr, 1970; Potter, 1972; Bevan et al., 1972; Melchers and Andersson, 1974; Nisonoff et al., 1975; Kuehl, 1977). One or more carbohydrate groups appear to be attached covalently to the constant region of virtually all heavy chains and to the variable region of several light chains.

Using cell-fractionation methods the subcellular biosynthesis sites and the order of addition of the various carbohydrate residues to the heavy or light chains of different tumors have been determined (Schenkein and Uhr, 1970; Melchers, 1973; Choi et al., 1971). Several groups obtained evidence which prompted them to suggest that the initial glycosylation event occurs while the polypeptide chain is still bound to the

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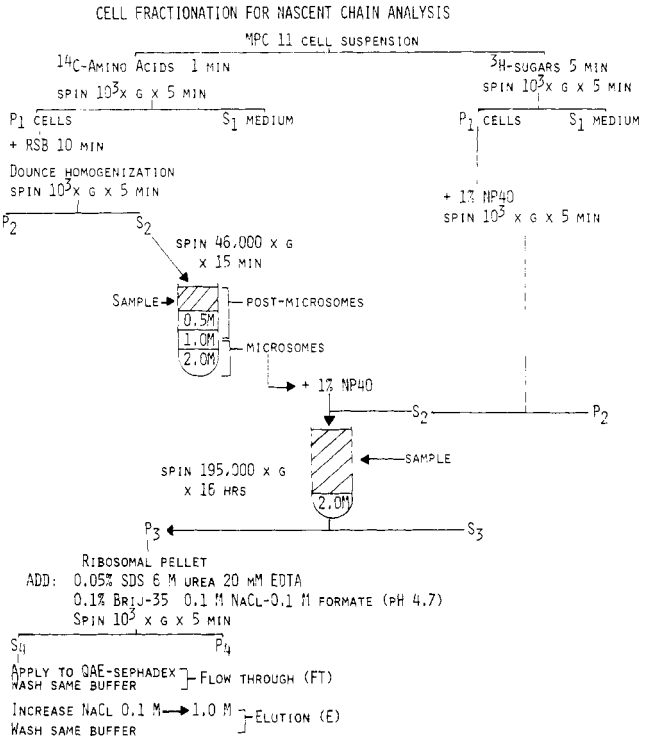

FIGURE 1: Scheme of cell fractionation for the analysis of ribosomes. For details, see Materials and Methods.

ribosomal complex, but the possibility of contamination by completed chains in the preparations cannot be excluded (Melchers and Knopf, 1967, Sherr and Uhr, 1969). Other studies have shown a nonglycosylated completed μ or α heavy chain in pulse-chase labeling experiments, suggesting that in some instances glycosylation occurs at least 2 min after chain completion and release from the ribosome (Schubert, 1970; Buxbaum and Scharff, 1973).

We have examined this question by the use of ion-exchange chromatography to separate completed polypeptides from nascent polypeptides. Immunoprecipitation of the nascent chain fraction has allowed us to demonstrate that glucosamine and mannose can be attached to nascent heavy chains very soon after the site for glycosylation is synthesized, i.e., prior to the completion of translation.

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 three products represent 12, 8, and 1%, respectively, of proteins synthesized by this clone (Kuehl and Scharff, 1974). The cells were maintained in suspension in Dulbecco's modified Eagle's medium supplemented with 16% heat-inactivated horse serum, 2 mM glutamine, and nonessential amino acids.

Cell Labeling and Fractionation. A flow sheet of the cell-fractionation procedures is given in Figure 1. Cells were collected by centrifugation at 500*g* for 5 min and then washed three times with spinner salts (minus glucose for labeling experiments with sugar precursors) prior to labeling with isotopic amino acids or sugar precursors.

For amino acid labeling, the cells were resuspended to a concentration of 2–6 × 10^7 cells per mL in spinner salts and incubated for 1 min at 37 °C with reconstituted [^{14}C]protein hydrolysate (Amersham/Searle, 54 mCi/mAtom of carbon) at an isotope concentration of 30–45 μCi/mL. The incorporation was terminated by chilling in an ice-water bath and subsequent centrifugation at 1000*g* for 5 min. The cells were washed two times with cold spinner salts and then suspended in hypotonic reticulocyte standard buffer (RSB) (0.01 M KCl–0.01 M Tris[1]-HCl, pH 7.2–1.5 mM $MgCl_2$) at 4 °C. After 10 min, the cells were disrupted with ten strokes of a tight Dounce homogenizer and the lysate was spun at 1000*g* for 5 min to prepare a postnuclear supernatant. Microsomes were isolated from the postnuclear supernatant by differential centrifugation through layers of 0.5 and 1.0 M sucrose–pH 6.7 buffer (0.075 M NaCl–0.01 M $MgCl_2$–0.025 M phosphate, pH 6.7) (Becker and Rich, 1966) at 46 000*g* for 15 min onto a 2.0 M sucrose–pH 6.7 buffer cushion (Baglioni et al., 1971). The microsomal pellet was solubilized with 1% NP40–pH 6.7 buffer to disrupt the ribosome–membrane interaction. The ribosomes were then isolated by centrifugation through 2.0 M sucrose–pH 6.7 buffer at 195 000*g* for 16 h.

For sugar labeling, the cells were resuspended to a concentration of 5–10 × 10^7 cells per mL in spinner salts minus glucose plus 10 mM for all amino acids, and incubated for 5 min at 37 °C with [^{3}H]sugars ([^{3}H]glucosamine, New England Nuclear, 20 Ci/mmol; [^{3}H]mannose, New England Nuclear, 13.2 Ci/mmol; [^{3}H]galactose, New England Nuclear, 14.2 Ci/mmol) at an isotope concentration of 50–100 μCi/mL. The incorporation was terminated as above and the cells were lysed in 1% NP-40–pH 6.7 buffer. The postnuclear supernatant was prepared and total ribosomes were isolated by centrifugation through 68% sucrose–pH 6.7 buffer, as described above.

Analysis of the Ribosomal Pellet. Peptidyl-tRNAs were isolated by a modification of a procedure described by Cioli and Lennox (1973). The modifications included: (1) the use of QAE-Sephadex to minimize hydrophobic interactions and (2) the presence of 20 mM EDTA in the resuspension buffer (below) to increase nascent-chain recoveries.

The polyribosomes were resuspended in 0.5% NaDodSO_4–20 mM EDTA–1.0% Brij-35–6 M urea–0.1 M NaCl–0.1 M ammonium formate (pH 4.7). The insoluble material was removed by centrifugation at 1000*g* for 5 min and the supernatant was subsequently diluted tenfold with 0.1% Brij-35–6 M urea–0.1 M NaCl–0.1 M ammonium formate (pH 4.7) (QAE starting buffer). This sample (usually 4–6 mL) was then applied to a 2.0-mL column of QAE-Sephadex (Pharmacia Fine Chemicals) (flow through equals fraction 1). The column was then washed with 4 volumes (fractions 2–5) of QAE starting buffer. The bound material is eluted by increasing the concentration of NaCl from 0.1 to 1.0 M (i.e., QAE elution buffer), with the other ingredients of the QAE buffer remaining unchanged. The column was washed with 5 volumes (fractions 6–10) of the eluting buffer. The radioactivity in each fraction was assayed by precipitation with 20% Cl_3CCOOH after addition of 50 μL of normal rabbit serum to act as carrier. The precipitates were collected on Whatman GF/A filters, washed with 5% Cl_3CCOOH, dried, and subsequently counted in a Beckman scintillation counter after addition of 0.5 mL of NCS (Amersham/Searle) and 7.5 mL of toluene containing 0.4% PPO.

Serological Analysis. Fractions to be serologically analyzed were dialyzed against PBS (138 mM NaCl, 3 mM KCl, 8 mM $NaH_2PO_4 \cdot 12H_2O$, 1 mM K_2HPO_4). During dialysis there was variable loss (ranging from 10 to 40%) of Cl_3CCOOH-precipitable material. The fractions were then immune precipitated directly using rabbit anti-MPC 11 heavy- and light-chain

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethlenedinitrilo)tetraacetic acid; NaDodSO_4, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole.

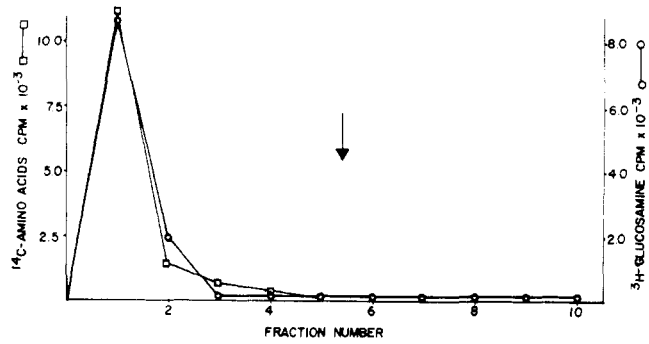

FIGURE 2: Chromatography of amino acid and glucosamine labeled secretion on QAE-Sephadex. Labeled secretion obtained from 24-h incubation of 5×10^6 45.6 cells with isotopic precursor was diazlyed extensively vs. QAE starting buffer (pH 4.7) containing 0.05% $NaDodSO_4$ and 20 mM EDTA and applied to a QAE-Sephadex column. Column fractions were assayed as described under Materials and Methods. (□) [^{14}C]Amino acid label; (○) [^{3}H]glucosamine label.

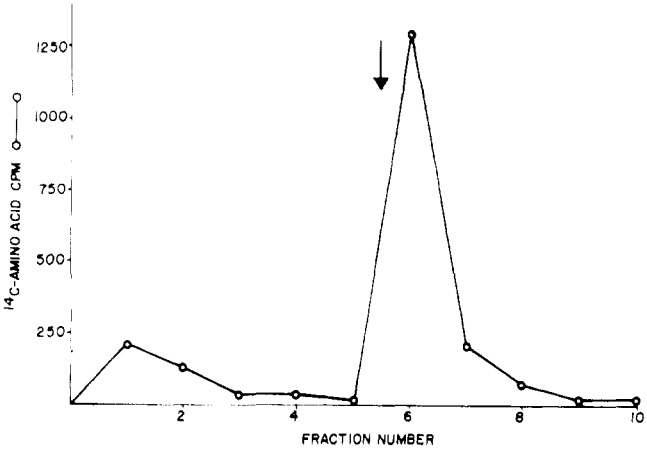

FIGURE 3: Chromatography of membrane-bound ribosomes labeled with [^{14}C]amino acids on QAE-Sephadex. Cells, 3×10^8, were labeled for 1 min with [^{14}C]protein hydrolysate and membrane-bound ribosomes were isolated and QAE-Sephadex chromatography was performed as described under Materials and Methods.

TABLE I: Characterization of Amino Acid Labeled QAE-Sephadex Fractions.[a]

	FT		E	
	cpm	%	cpm	%
Expt 1				
(A) Ribosomal pellet	24 544	17.7	114 125	82.3
(B) FT fraction rerun	16 476	95.9	704	4.1
(C) E fraction rerun	526	11.1	4 196	88.9
(D) E fraction + RNase	4 083	90.9	449	9.1
(E) E fraction + 0.3 M NaOH	4 518	96.1	182	3.9
Expt 2				
(A) Ribosomal pellet	2 596	18.5	11 406	81.5
(B) E fraction rerun	705	24.0	2 227	76.0
(C) E fraction + 1.8 M Tris (pH 8.5)	1 047	81.6	236	18.4

[a] Characterization of QAE-Sephadex fractions. 45.6 cells were labeled for 1 min with [^{14}C]protein hydrolysate and a ribosomal pellet was prepared according to Figure 1. Experiment 1: (A) the ribosomal pellet was fractionated as described under Materials and Methods. (B) The first flow-through fraction from A was reapplied directly to a new QAE-Sephadex column. Three equal aliquots from the first elution fraction were treated in the following manner: (C) The aliquot was kept at 37 °C for 2 h and then diluted with H_2O, 8 M urea, formate buffer, and Brij-35 to lower the NaCl concentration to the original 0.1 M of the QAE-Sephadex starting buffer and leave the other components unchanged, and then reapplied to a new column; (D) treated with 20 μg/mL pancreatic ribonuclease at 37 °C for 2 h before dilution and reloading on a new column; (E) brought to 0.3 M NaOH by addition of 5.0 M NaOH, incubated at 37 °C for 2 h, neutralized by addition of 6 N HCl, diluted and run on a new column. Experiment 2: (A) repeat of Experiment 1A. Two aliquots from the first elution fraction were treated in the following manner: (B) incubated at 37 °C for 2 h, dialyzed for 6 h to establish conditions of the QAE-Sephadex starting buffer and reapplied to new column; (C) brought to 1.8 M Tris (pH 8.5) by addition of 2.4 M Tris (pH 8.5), incubated at 37 °C for 2 h, dialyzed for 6 h as described in Experiment 2B, and reloaded on new column. There was a loss of labeled material during dialysis. All values are total Cl_3CCOOH-precipitable radioactivity. Abbreviations used: FT, flow through; E, elution.

antisera (Horwitz and Scharff, 1969). Controls were done indirectly using normal rabbit serum and a goat antiserum directed against rabbit γ-globulin. All immunoprecipitations were performed in antibody excess at 4 °C. The immunoprecipitates were collected by centrifugation through 1 M sucrose in PBS and washed one time with PBS (Rhoads et al., 1972).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. The immunoprecipitates were boiled in $NaDodSO_4$ sample buffer and then reduced with 0.15 M β-mercaptoethanol at 37 °C. They were then electrophoresed in a 12.5% polyacrylamide 8-cm cylindrical gel using a discontinuous $NaDodSO_4$–Tris–glycine buffer system essentially as described by Laemmli (1970) and Maizel (1971). The gels were sliced into 2-mm fractions with a Gilson Aliquogel fractionator and counted as described above.

Identification of Isotope as Glucosamine or Mannose. Cl_3CCOOH-precipitated [^{3}H]glucosamine-labeled nascent heavy chains were hydrolyzed in 6 N HCl for 18 h at 100 °C, dried, resuspended in 5% acetic acid, dried again, and chromatographed on Whatman No. 1 paper in pyridine–ethyl acetate–H_2O (1:36:1.15, v/v). The chromatography strips were cut into 1.1-cm pieces and counted as described previously. Cl_3CCOOH-precipitated [^{3}H]mannose-labeled nascent heavy chains were treated identically, except hydrolysis was in 2 N HCl for 4 h at 100 °C. Unlabeled glucosamine hydrochloride and mannose were run as standards in the chromatography system.

Chemicals. Pancreatic ribonuclease A was purchased from Worthington Enzymes.

Results

Isolation of Nascent Chains. The separation of completed polypeptides from nascent polypeptides by ion-exchange chromatography is based on the multiple negative charges contributed by the tRNA moiety of the peptidyl-tRNA complex. The specificity of this procedure is seen in Figures 2 and 3 and in Table I. In Figure 2, either amino acid or glucosamine labeled completed polypeptides from 45.6 secretion quantitatively pass through the column in the 0.1 M NaCl–QAE starting buffer. These results indicate that mature immunoglobulin, which accounts for >80% of the label in 45.6 secretion (Laskov and Scharff, 1970), and other completed polypeptides are not bound by the QAE-Sephadex column. Figure 3 shows the chromatographic pattern of a membrane-bound ribosomal fraction isolated from cells labeled for 1 min with [^{14}C]amino acids. These results indicate that approximately 85% of the pulse-labeled material in the ribosomal pellet binds to the column and can be eluted by increasing the concentration of NaCl. (This can also be seen in Table I (expt 1A and expt

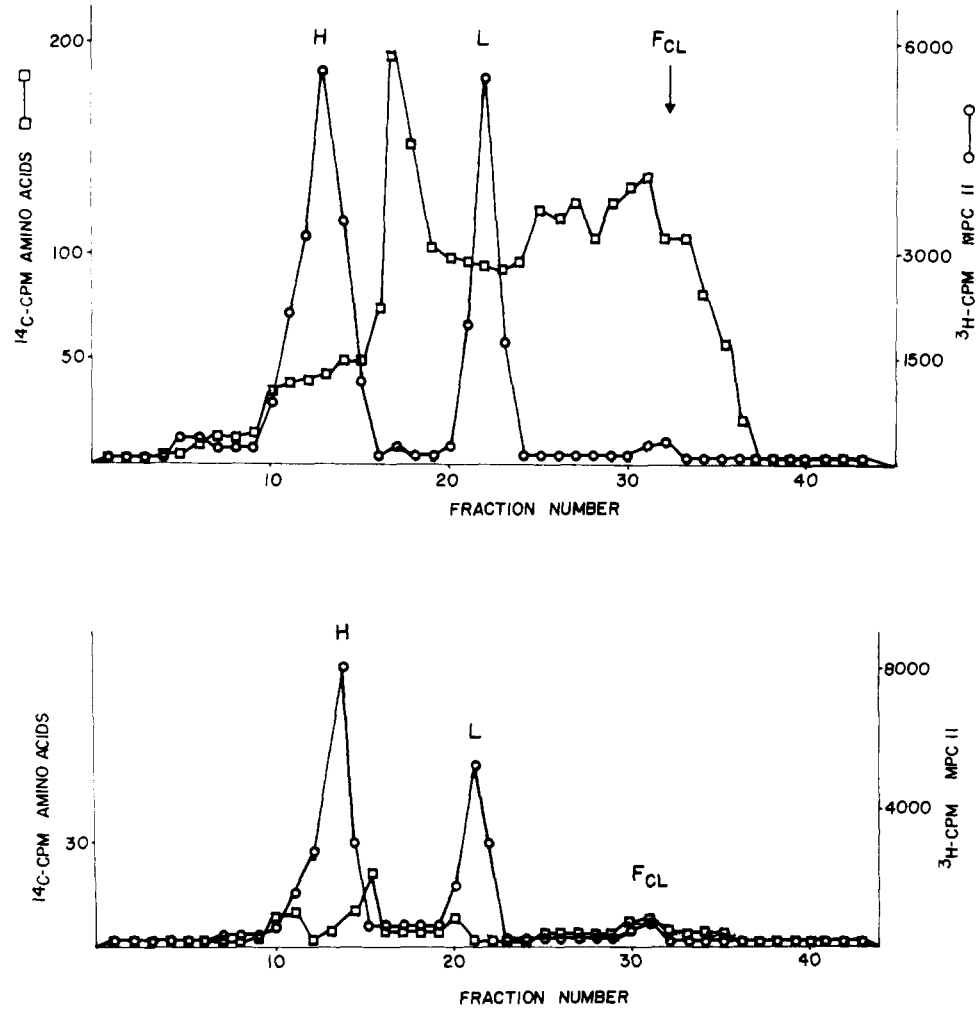

FIGURE 4: Electrophoresis of immunoprecipitated MPC 11 nascent chains labeled with [^{14}C]protein hydrolysate on NaDodSO$_4$-containing acrylamide gels. The labeling and isolation of nascent chains were as described in the legend to Figure 3. The elution fraction was dialyzed against PBS, and immunoprecipitated after the addition of unlabeled MPC 11 heavy and light chain to act as carrier: (A, top) direct immunoprecipitation using anti-heavy and anti-light antiserum; (B, bottom) indirect immunoprecipitation using normal rabbit serum and goat anti-rabbit γ-globulin. (□) [^{14}C]Protein hydrolysate labeled nascent chains; (○) [^{3}H]amino acid labeled MPC 11 intracellular heavy chain, light chain, and F_{CL} marker.

2A).)[2] Table I shows further characterization of the material that elutes from the QAE-Sephadex column. The flow-through fraction was reapplied to QAE-Sephadex and was quantitatively recovered in the flow-through fractions (expt 1B). In contrast, if the eluted material was diluted to restore the low NaCl concentration of the QAE starting buffer and rerun on the QAE-Sephadex column, approximately 90% was bound by the column (expt 1C). However, if the eluted material was digested with pancreatic ribonuclease prior to reapplication, 90% of the material was not retained by the column (expt 1D). Similarly, if the eluted material was treated with 0.3 M NaOH and neutralized prior to reapplication, greater than 95% of the material passed through the column (expt 1E). Finally the material eluted in 1.0 M NaCl was treated with 1.8 M Tris (pH 8.5) to promote deacylation of the peptidyl-tRNA species without degrading the RNA (Sarin and Zamecnik, 1964), and then dialyzed into QAE starting buffer (containing 0.1 M NaCl). Upon reapplication, the material did not bind to the column as compared to the control sample which was incubated in elution buffer, dialyzed to lower the NaCl concentration, and rechromatographed (expt 2B,C). These results indicate that the linkage of the polypeptide to the tRNA molecule is responsible for its chromatographic behavior on QAE-Sephadex; destroying the RNA (RNase or alkali treatment) or the acyl linkage (1.8 M Tris, pH 8.5) drastically affects the retention of the amino acid labeled material by the QAE-Sephadex.

Further characterization of the elution fractions is achieved by specific immunoprecipitation using antiserum directed against both heavy and light chains. Figure 4 shows the gel electrophoretic profiles of the immunoprecipitated 1.0 M NaCl fraction. There is a very heterogeneous size distribution of the specifically immunoprecipitated eluted material (panel A); the gel profile shows an uneven distribution ranging from the size of mature heavy chain to peptides smaller than the constant-region fragment of light chain (F_{CL} 11 500 daltons). The peak (fraction 17) between the marker H and L chains seen in this gel (and in other similar experiments) is not easily explained. It is possible that there is a partial block in translation at this point or it may be possible that there is a selective immunoprecipitation of nascent chains of this size. A control using normal rabbit serum plus goat anti-rabbit γ-globulin is seen in panel B of Figure 4.

From these results, we conclude that peptidyl-tRNA complexes can be selectively isolated from solubilized ribosomes by use of chromatography on QAE-Sephadex.

[2] Overall recoveries of amino acid or sugar labeled material applied to the QAE-Sephadex range between 65 and 80%. Results in the flow-through and elution fractions are expressed as percent of labeled material recovered.

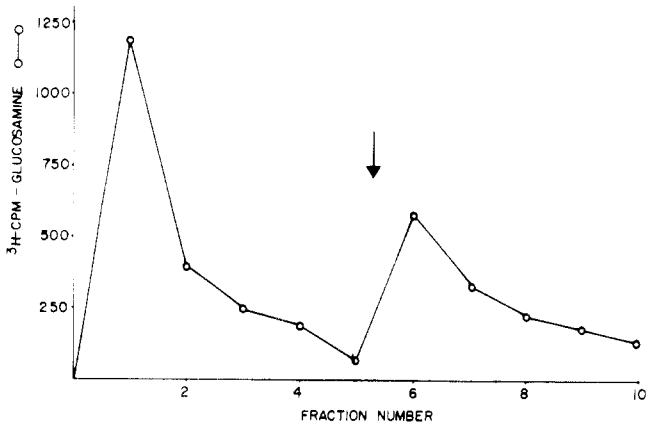

FIGURE 5: Chromatography of total ribosomes labeled with [^{3}H]glucosamine on QAE-Sephadex. Cells, 6.5 × 10^8, were labeled with [^{3}H]-glucosamine for 5 min and total ribosomes were isolated and QAE-Sephadex chromatography was performed as described under Materials and Methods.

Incorporation of Radiolabeled Sugars onto Nascent Chains. To show the addition of glucosamine residues onto nascent polypeptides, 45.6 cells were labeled for 5 min with [^{3}H]glucosamine and total ribosomes (Figure 1) were analyzed by chromatography on QAE-Sephadex. The chromatographic pattern, seen in Figure 5, reveals that approximately 30% of the glucosamine-labeled material that coisolates with ribosomes bound to the column and could be eluted from the column by increasing the NaCl concentration. This eluted fraction of glucosamine-labeled material represents 1.0–1.6% of the total Cl_3CCOOH-precipitable [^{3}H]glucosamine incorporated in 5 min (see Discussion).

Results presented in Table II demonstrate that after pretreatment with RNase (expt 1B), 0.3 M NaOH (expt 1C), or 1.8 M Tris (pH 8.5) (expt 2B) less than 10% of the labeled material was bound to the column and eluted with 1.0 M salt. Although these were the criteria used to define the nascent-chain fraction using amino acid labeled membrane-bound ribosomes, there were several other plausible explanations to account for the presence of the glucosamine-labeled material found in the elution fraction. Pretreatment with 0.15 M β-mercaptoethanol to eliminate any glucosamine-labeled proteins which were disulfide-bonded to unlabeled nascent chains had no effect on the proportion of labeled material in each fraction (expt 3).[3] Finally, treatment of the isolated nascent-chain fraction (fraction E) with 6 M guanidine hydrochloride to promote disaggregation had no effect on the binding of the glucosamine-labeled material upon reapplication to QAE-Sephadex (expt 4).

The glucosamine-labeled nascent-chain fraction was subjected to immunoprecipitation, followed by $NaDodSO_4$ gel coelectrophoresis with ^{14}C-labeled secreted heavy and light chain to serve as internal marker (Figure 6). Panel A is a specific immunoprecipitate (65% of glucosamine-labeled material was precipitated) and panel B is a nonspecific control immunoprecipitate (4% of glucosamine-labeled material was precipitated) of nascent chains. These results indicate that glucosamine residues are incorporated onto the nascent heavy chain; i.e., [^{3}H]glucosamine is present only in the specific immunoprecipitate, and is present in species coelectrophoresing with the marker H chain as well as in species migrating more rapidly than the marker H chain.

[3] Control experiments showed that immunoglobulin could be quantitatively reduced to heavy and light chains under these conditions.

TABLE II: Characterization of Glucosamine-Labeled QAE-Sephadex Fractions.[a]

	FT		E	
	cpm	%	cpm	%
Expt 1				
(A) Ribosomal pellet	2 076	68.6	948	31.4
(B) Ribosomal pellet + RNase	1 250	95.3	62	4.7
(C) Ribosomal pellet + 0.3 M NaOH	1 076	98.6	15	1.4
Expt 2				
(A) Ribosomal pellet	2 580	63.4	1490	36.6
(B) Ribosomal pellet + 1.8 M Tris (pH 8.5)	4 612	92.1	396	7.9
Expt 3				
(A) Ribosomal pellet	2 013	63.9	1 135	36/
(B) Ribosomal pellet + 0.15 M β-ME	3 509	66.2	1 791	33.8
Expt 4				
(A) Ribosomal pellet	10 440	61.4	6 557	39.6
(B) E fraction rerun	629	24.1	1 985	75.9
(C) E fraction + 6.0 M GuCl	328	26.8	894	73.2

[a] Characterization of glucosamine-labeled QAE-Sephadex fractions. 45.6 cells were labeled for 5 min with [^{3}H]glucosamine and a ribosomal pellet was prepared according to Figure 1. Experiment 1: The ribosomal pellet in starting buffer was divided into three aliquots and (A) incubated in starting buffer at 37 °C for 2 h and applied directly to a QAE-Sephadex column; (B) digested with 20 μg/mL pancreatic ribonuclease at 37 °C for 2 h prior to application to a column; (C) treated with 0.3 M NaOH at 37 °C for 2 h, neutralized by addition of 6 N HCl, adjusted to conditions of the QAE-Sephadex buffer, and applied to a column. Experiment 2: The ribosomal pellet was divided into two aliquots and (A) incubated in starting buffer at 37 °C for 2 h, dialyzed for 6 h vs. QAE-buffer (pH 4.7), and applied to a QAE-Sephadex column; (B) brought to 1.8 M Tris, pH 8.5, by addition of Tris-HCl, incubated at 37 °C for 2 h, dialyzed for 6 h vs. QAE starting buffer (pH 4.7), and applied to a QAE-Sephadex column. Experiment 3: The ribosomal pellet was divided into two aliquots and (A) incubated at 37 °C for 2 h and applied to a QAE-Sephadex column and (B) treated with 0.15 M β-mercaptoethanol at 37 °C for 2 h and applied to a column, that was run in the presence of the reducing agent. Experiment 4: (A) The ribosomal pellet was applied directly to a QAE-Sephadex column. The aliquots from the first elution fraction were treated in the following manner: (B) incubated for 4 h at room temperature, dialyzed for 6 h vs. QAE-buffer (pH 4.7), and applied to a QAE-Sephadex column; (C) dialyzed vs. 6 M guanidine hydrochloride for 4 h, dialyzed for 6 h vs. QAE-buffer (pH 4.7), and applied to a column. All values are total Cl_3CCOOH-precipitable radioactivity. Abbreviations used: FT, flow through; E, elution.

Similar experiments were done using total ribosomes isolated from cells labeled for 5 min with [^{3}H]mannose. The chromatographic pattern of the untreated mannose-labeled ribosomes, seen in Figure 7, shows that approximately 50% of the labeled material was bound to the QAE-Sephadex column and was eluted by increasing the NaCl concentration. In this case, the mannose-labeled nascent fraction accounted for 5.2% of the total Cl_3CCOOH-precipitable [^{3}H]mannose incorporated in 5 min (see Discussion). Pretreatment with RNase caused all labeled material to pass through the column (Figure 7). These results indicate that mannose is also incorporated into the nascent-chain fraction which binds to the QAE-Sephadex column. The mannose-labeled nascent-chain fraction was subjected to specific immunoprecipitation (74% of mannose-labeled material was precipitated) followed by gel electrophoresis (Figure 8); a pattern similar to that found with glucosamine-labeled nascent heavy chains is obtained, indicating that mannose is also added to the nascent heavy chain prior to release from the ribosome.

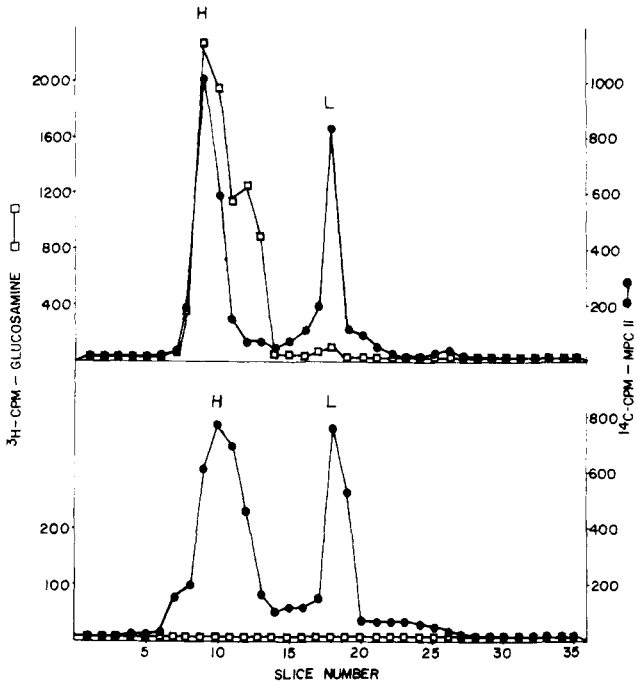

FIGURE 6: Electrophoresis of immunoprecipitated MPC 11 nascent chains labeled with [^{3}H]glucosamine on NaDodSO$_4$-containing acrylamide gels. The labeling and isolation of nascent chains was as described in the legend to Figure 5. The elution fraction was dialyzed against PBS and immunoprecipitated after addition of unlabeled MPC 11 heavy and light chain to act as carrier: (A, top) direct immunoprecipitation using anti-heavy chain and anti-light chain antiserum; (B, bottom) indirect immunoprecipitation using normal rabbit serum plus goat anti-rabbit γ-globulin; (□) [^{3}H]glucosamine-labeled nascent chains; (●) [^{14}C]amino acid labeled MPC 11 H and L chain markers (from secretions).

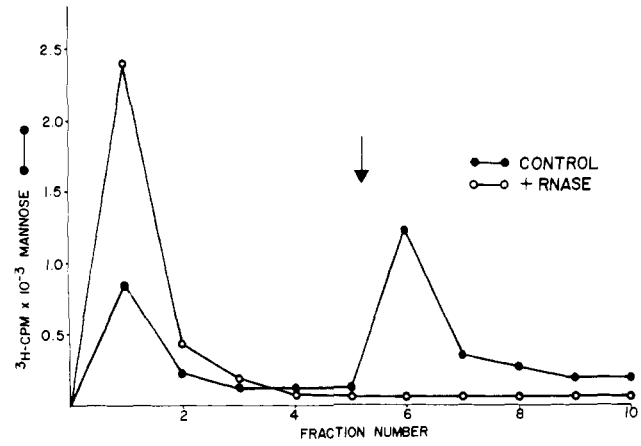

FIGURE 7: Chromatography of total ribosomes labeled with [^{3}H]mannose on QAE-Sephadex. Cells, 8.0×10^8, were labeled with [^{3}H]mannose for 5 min and total ribosomes were isolated and QAE-Sephadex chromatography was performed as described under Materials and Methods: (●) incubated for 2 h and applied directly to QAE-Sephadex; (○) treated with 20 μg/mL pancreatic ribonuclease at 37 °C for 2 h and applied directly to column.

To show that the incorporated [^{3}H]glucosamine and [^{3}H]mannose recovered in nascent heavy chains was present as glucosamine and mannose and not as a metabolic product, samples of specifically immunoprecipitated [^{3}H]glucosamine- and [^{3}H]mannose-labeled nascent chains were acid hydrolyzed and subjected to paper chromatography, as described under Materials and Methods. For the [^{3}H]glucosamine-labeled sample, >98% of the isotope cochromatographed with a glucosamine standard; in the case of the [^{3}H]mannose-labeled sample, 85% of the isotope cochromatographed with the mannose standard. (It is likely that some of the mannose may be degraded during the acid hydrolysis (Bergman, Volk, and Kuehl, unpublished).) These results provide definitive evidence that glucosamine and mannose are incorporated into nascent heavy chains.

Finally, as a control to show the absence of nonspecific binding (of sugar-labeled material) to QAE-Sephadex, total ribosomes isolated from cells labeled for 5 min with [^{3}H]galactose were subjected to chromatography on QAE-Sephadex. The results (Bergman and Kuehl, unpublished) indicate that galactose-labeled material is present only in the flow-through fraction of the column. The material coisolating with the ribosomes accounts for 1.4% of the total Cl_3CCOOH-precipitable [^{3}H]galactose incorporated in 5 min.

Discussion

From the results presented here, we conclude that some glucosamine and mannose residues are attached to nascent MPC 11 heavy chains prior to polypeptide chain completion and release from the ribosomes. The isolation of the nascent chains is achieved by ion-exchange chromatography as described previously by Cioli and Lennox (1973). The specificity of the isolation procedure is seen in the following results: (1) greater than 95% of the amino acid or glucosamine labeled secretion (Figure 2) or galactose-labeled ribosomal fraction (Bergman and Kuehl, unpublished) passed through the QAE-Sephadex column, while (2) greater than 80% of the labeled material from membrane-bound ribosomes isolated from cells labeled with amino acids for 1 min was retained by the column and was eluted by increasing the NaCl concentration (Figure 3). The binding of the amino acid labeled material (or of glucosamine- or mannose-labeled material) to the column was sensitive to agents that destroyed either the tRNA moiety or the acyl bond between the tRNA molecule and the peptide chain (Tables I and II). The pattern of specifically immunoprecipitated amino acid labeled nascent chains that results from NaDodSO$_4$ gel electrophoresis (Figure 4) shows the heterogeneous size distribution expected from nascent polypeptides.

Melchers and Knopf (1967) and Uhr (1970) using IgG-secreting plasmacytomas, Robinson (1969) using liver cells, and O'Brien (1977) using bovine retina have suggested that glucosamine and possibly mannose are added to nascent polypeptides prior to chain completion. However, the possibility of contamination of membrane-bound ribosomes by completed polypeptide chains cannot be excluded from their results. Using pulse–chase amino acid and/or carbohydrate labeling experiments with IgM- and IgA-secreting mouse plasmacytomas, several groups (Schubert, 1970; Buxbaum and Scharff, 1973) have demonstrated an apparent increase in the size of completed intracellular heavy chain during the chase period, suggesting that addition of the core sugars may occur a considerable time after release from the ribosome. However, this increase in size of heavy chain following completion has not been observed in other studies with IgM- or IgA-secreting plasmacytomas (Bevan, 1971; Parkhouse and Melchers, 1971; Bargellesi et al., 1972) or with IgG-secreting plasmacytomas (Kuehl, 1977).

Our results are consistent with the initial glycosylation event occurring while the polypeptide is bound to the ribosome. Immunoprecipitation of the nascent-chain fraction using antiserum directed against heavy and light chains reveals that heavy chain, which accounts for more than 70% of the total glycoprotein synthesis in 45.6 (Bergman and Kuehl, unpublished), is glycosylated with glucosamine and mannose residues

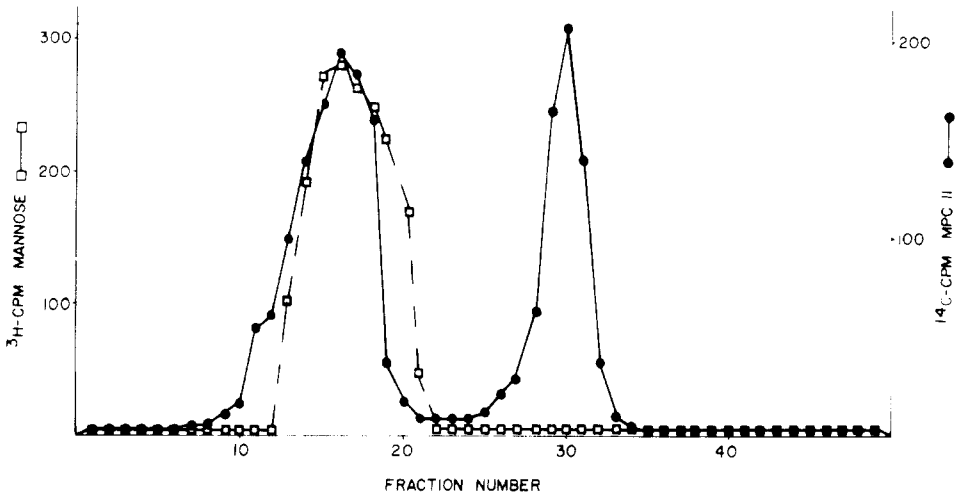

FIGURE 8: Electrophoresis of immunoprecipitated MPC 11 nascent chains labeled with [^{3}H]mannose on $NaDodSO_4$-containing acrylamide gels. The labeling and isolation of nascent chains were as described in the legend to Figure 7. The elution fraction was dialyzed against PBS and directly immunoprecipitated with anti-heavy chain and anti-light chain antiserum after addition of carrier: (□) [^{3}H]mannose-labeled nascent chains; (●) [^{14}C]amino acid labeled MPC 11 heavy and light chain marker (from secretions).

prior to completion of heavy-chain translation. In addition, since immunoprecipitation did not precipitate all the glucosamine- or mannose-labeled nascent chains, it is possible that other glycoproteins, as well as heavy chain, are being glycosylated as nascent chains. By comparison with the known amino acid sequence of MOPC 21 (IgG1) heavy chain (Milstein et al., 1975), it should be an asparagine at position 291 (CH2 domain) that is glycosylated. Therefore, approximately two-thirds or 32 000 daltons of heavy chain must be synthesized before the glycosylation site is potentially available for attachment of the sugar residues. Analysis of the $NaDodSO_4$ gel electrophoresis patterns of immunoprecipitated nascent chains labeled with [^{3}H]glucosamine or [^{3}H]mannose (Figures 6 and 8, respectively), reveals an apparent size range of 38 000 to 55 000 (full size) daltons, suggesting that the glucosamine and mannose residues can be attached to the nascent heavy chains very soon after the attachment site is synthesized on the ribosomal complex. These results, though, do not reveal what fraction of the heavy-chain population is being glycosylated prior to completion of translation and release from the ribosome. Preliminary evidence from this laboratory (Bergman and Kuehl, unpublished) analyzing completed MPC 11 (IgG_{2b}) heavy chains in cells pulse labeled for 30 s or 1 min reveals that no detectable nonglycosylated heavy chain is present.

The translation time for heavy chain has been shown to be approximately 60 s (Shapiro et al., 1966). Thus, assuming a constant ratio of heavy chain elongation, glycosylation of the nascent heavy chains can occur only during the last 10–15 s of translation (i.e., after the presumptive asparaginyl glycosylation site in the CH2 domain is synthesized). Consequently, if incorporation of labeled sugar precursor into heavy chain is linear in time, one expects approximately 3–5% of the total Cl_3CCOOH-precipitable carbohydrate-labeled heavy chain synthesized during a 5-min pulse to be in the nascent-chain fraction. We obtained a 1.0–1.5% overall yield of glucosamine-labeled nascent chains after a 5-min labeling time. On the basis of the theoretical calculations above, these low yields of labeled nascent chains are reasonable, since the relative yield of glucosamine-labeled nascent chains may be lowered due to the incorporation of additional glucosamine (but not mannose) to completed chains in the smooth-membrane fraction (Melchers, 1973).

Recently, Schimke and coworkers (Kiely et al., 1976), using similar methods, reported that glucosamine and mannose residues are added to ovalbumin nascent chains.

Experiments from the laboratories of Lennarz (Waechter et al., 1973), using hen oviduct, and Heath (Hsu et al., 1974), using a MOPC 46B plasmacytoma, suggested that the addition of "core" sugar to glycoproteins occurs via a carbohydrate–phosphoryl polyisoprenol intermediate. Recent experiments by Pless and Lennarz (1977) reported that the "core" carbohydrate (i.e., glucosamine and mannose) is added as a unit to denatured but not native secretory proteins, as opposed to a stepwise addition of peripheral sugar residues catalyzed by specific glycosyl transferases (Schachter, 1974). We are presently investigating the size homogeneity of the carbohydrate unit attached to the nascent heavy chains by column chromatography of exhaustively pronase-digested mannose-labeled nascent chains (Knopf et al., 1975). If the nascent glycopeptide is homogeneous in size distribution and cochromatographs with intracellular heavy-chain glycopeptides that contain only glucosamine and mannose (Knopf et al., 1975), it would tend to support the addition of the core residues as a unit.

The attachment of the oligosaccharide chain to secreted glycoprotein appears to be a process that involves several steps: (1) addition of glucosamine and mannose residues to nascent polypeptides or polypeptides soon after chain completion and release from the ribosome; (2) sequential addition of additional glucosamine and initial galactose residues via specific transferases in the smooth-membrane fraction; and (3) the addition of the terminal sugars at the time of secretion.

Acknowledgments

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Synthesis in Vitro of Ribosomal Protein S20 and Its Precursor†

George A. Mackie

ABSTRACT: I have purified and characterized two products synthesized in vitro in a system for coupled transcription and translation programmed by DNA from a transducing bacteriophage carrying the gene for ribosomal protein S20. One of these polypeptides appears to be identical with authentic S20 by several criteria, including its electrophoretic and chromatographic mobilities, and its ability to bind to 16S RNA. The second polypeptide is less basic than S20, but exhibits all the structural and functional properties of a precursor to S20, including the presence of an additional methionine residue, apparently as *N*-formylmethionine. Moreover, it is converted, albeit slowly, to S20 in cell-free extracts. The persistence of the precursor form of S20 may be functionally significant as well.

The mechanism of regulation of ribosomal biogenesis remains one of the outstanding unanswered questions of the molecular biology of *Escherichia coli.* I am approaching this problem by exploring the possibility that covalent modification of ribosomal proteins during their assembly into functional ribosomes plays a significant role in governing the rate and/or specificity of this process. I have previously shown that several ribosomal proteins, among them, S20 (Mackie, 1976, 1977), exhibit kinetics of labeling in vivo compatible with the processing of precursors into mature forms of the protein. To confirm this prediction, I have characterized the products synthesized in vitro from the DNA of bacteriophage λddapB2 (Friesen et al., 1976) which carries the gene for ribosomal protein S20 in a system allowing coupled transcription and translation (Zubay, 1973). Among the products are both S20 and, in larger quantities, a protein with structural and functional properties of S20 which has an N-blocked methionine residue, apparently as *N*-formylmethionine. This latter polypeptide is converted to S20 in the crude extracts used for protein synthesis, confirming its likely role as the precursor to S20. L. Reis, L. Lindahl, and M. Nomura (personal communication) have also isolated such a molecule and come to similar conclusions. These findings correct my earlier suggestion that the precursor to S20 might be considerably larger than mature S20 (see the Discussion).

Materials and Methods

Bacterial and Bacteriophage Strains. The following strains were obtained from the named investigators: C600 (P. Leder),

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