Localization of the Carbohydrate within the Variable Region of Light and Heavy Chains of Human γG Myeloma Proteins*

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ABSTRACT: Six human γG_1 myeloma proteins having a carbohydrate moiety on the Fab fragment were analyzed. The carbohydrate was located on the light chain in four of the proteins (three of λ and one of κ type), on the Fd fragment in one, and one protein had carbohydrate on both the Fd fragment and light chain (κ type). The amino acid composition and sequence analysis of the glycopeptides indicated that the point of attachment of the carbohydrate varied from protein to protein. However, in all six proteins studied, the localization of the carbohydrate appeared to be restricted to two regions within the variable portion of the light chains and Fd fragments. One was near the N terminus between positions 22 and 32 and the other near the C-terminal end of the variable portion between positions 63 and 93. Of the six patients studied, three formed both γG myeloma protein and Bence Jones protein. The carbohydrate composition of the Bence Jones proteins was similar to that of the light chains of the corresponding myeloma proteins, except for the sialic acid content. Whereas the light chains contained 1 mole of sialic acid per mole of protein, in each preparation of Bence Jones protein, the sialic acid content was higher and varied in individual molecules between 1 and 6 moles per mole of protein. Reconstitution experiments revealed no apparent relationship between sialic acid content and the capacity of the light chains to combine with heavy chains to form a 7S γG protein.

It is well established that human γG-immunoglobulins contain a carbohydrate moiety on the Fc fragment (Franklin, 1960; Grey and Abel, 1970). Whereas it was previously believed that the carbohydrate was restricted to only the Fc fragment, in a recent study of 76 human γG myeloma proteins, it was demonstrated that approximately 25% contained a carbohydrate moiety on the Fab fragment in addition to the one on the Fc fragment (Abel *et al.*, 1968). The carbohydrate was linked to either the light chains or the Fd fragments, and in a few of the proteins, to both. There was no correlation between the presence of carbohydrate on the Fab fragment with the light-chain type nor with the heavy-chain subclass of the myeloma proteins. Two human λ- and two κ-type Bence Jones proteins having a carbohydrate moiety have also recently been reported by Edmunson *et al.* (1968) and by Hood *et al.* (1969), respectively. The purpose of the present investigation was to localize the point of attachment of the carbohydrate on either the light chains or Fd fragment and to determine whether a single point of attachment was involved as is the case with the Fc fragment, or if the carbohydrate was attached at multiple sites. In addition, the carbohydrate of three λ Bence Jones proteins was studied and compared to the carbohydrate of the light chains isolated from the corresponding myeloma proteins.

Materials and Methods

Protein Isolation. Human γG myeloma proteins were isolated from the serum and Bence Jones proteins from the urine of patients with multiple myeloma by either DEAE-cellulose chromatography or by Pevikon block electrophoresis as previously described (Abel *et al.*, 1968). The Bence Jones proteins were further purified by Sephadex G-100 gel filtration in 1 N acetic acid. Two major peaks of protein were eluted from the G-100 column. The first peak contained dimer and the second monomer Bence Jones protein as shown by urea acid starch gel electrophoresis (Poulik, 1960). The isolated Bence Jones proteins were analyzed by immunoelectrophoresis using antisera specific for κ and λ light chains (Fahey and McLaughlin, 1963), and an antihuman serum obtained from Hyland Laboratories, Los Angeles, Calif. By this method, a small amount of a contaminating protein of β-electrophoretic mobility was demonstrated in the preparations of dimer Bence Jones protein, and no contaminating proteins were demonstrable in the monomer Bence Jones protein. The heavy-chain subclass of the myeloma proteins was determined by Ouchterlony analysis, using rabbit antihuman subclass antisera as previously described (Spiegelberg and Weigle, 1968).

Isolation of Heavy and Light Polypeptide Chains. The γG myeloma proteins were dissolved in 0.5 M Tris-HCl buffer (pH 8.2) and were partially reduced by treatment with dithiothreitol at a concentration of 0.02 M for 1 hr at room temperature and alkylated with 0.05 M iodoacetamide. After standing for 1 hr at 4° the heavy chains and light chains were dialyzed

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against 1 N acetic acid and separated in the cold by gel filtration on columns of Sephadex G-100 equilibrated with 1 N acetic acid.

Recombination of Heavy Chains with Light Chains. Recombination experiments of heavy chains with light chains and Bence Jones protein from one of the patients (Pet) were performed as previously described (Grey and Mannik, 1965). Partially reduced and alkylated Bence Jones protein Pet was fractionated by Pevikon block electrophoresis into an electrophoretically fast and slow moving fraction. The different Bence Jones protein preparations were labeled with ^{131}I and the light chains with ^{125}I according to a modified chloramine-T method (McConahey and Dixon, 1966), using 20 μg of chloramine-T/mg of protein. The heavy chains, light chains, and Bence Jones protein at a protein concentration between 0.5 and 1 mg per ml were dialyzed against 0.01 M propionic acid and appropriate mixtures were prepared. Following dialysis first against distilled water and then against 0.02 M Tris-HCl buffer (pH 8.0), the mixtures were applied to a Sephadex G-200 column equilibrated with Tris-HCl buffer (pH 8.0). The quantitative distribution of the radioactive light chains in the reconstituted γG and the free light chains was determined by γ scintillation counting.

Starch Gel Electrophoresis. Vertical starch gel electrophoresis at pH 8.8 was performed using a discontinuous buffer system described by Fahey (1963) and allowed to proceed at 450 V for 4–5 hr at 4°. Starch gel electrophoresis in 8 M urea was performed using the urea–formate buffer (pH 3.5) described by Poulik (1960).

Peptide Mapping and Isolation of Peptides. Analytical and preparative peptide mapping and elution of the peptides from the paper were carried out as previously described (Grey and Kunkel, 1967). The proteins were reduced and either carboxymethylated or aminoethylated and digested with trypsin or trypsin followed by chymotrypsin at a w/w ratio of 1:100. Trypsin treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone and chymotrypsin were obtained from Worthington Biochemicals. The Bence Jones proteins were treated with neuraminidase (*Vibrio cholerae*, Behringwerke) before tryptic or chymotryptic digestion in order to avoid the formation of multiple peptide spots or lines on the paper resulting from the heterogeneity in the sialic acid content of individual molecules. Digestion with neuraminidase was carried out by incubation at 37° for 24 hr with 20 units of neuraminidase/mg of protein dissolved in 0.05 M acetate buffer (pH 5.5), containing 0.01 M calcium chloride. Some of the isolated glycopeptides dissolved in 0.1 M NH_4HCO_3 were digested with either subtilisin or pronase for 4 hr at room temperature using a w/w enzyme to substrate ratio of 1:50.

Carbohydrate Analysis. All carbohydrate analyses of both the Bence Jones proteins and light chains isolated from the myeloma proteins were performed on the peaks of protein eluted from the columns of Sephadex G-100 equilibrated with 1 N acetic acid. The acetic acid effluent from the column prior to the elution of the protein was used as a blank. The methods used to determine the various sugars have been described previously (Abel *et al.*, 1968). Hexoses were determined by the orcinol reaction (Svennerholm, 1956), fucose by the cysteine reaction (Dische and Schettles, 1948), glucosamine using a 12-cm column of "Aminex A-5" on the short column of a Beckman amino acid analyzer (Abel *et al.*, 1968), and sialic acid by the thiobarbituric acid assay (Warren, 1959). The standards used consisted of 0.058–0.233 μmole/ml of an equimolar mixture of galactose and mannose, 0.05 and 0.1 μmole per ml of D-glucosamine, 0.012–0.048 μmole/ml of L-fucose, and 0.0485 and 0.194 μmole per ml of crystalline *N*-acetylneuraminic acid. All values given in the tables represent average values of duplicate determinations of two different protein preparations.

Determination of Amino Acid Composition and Sequence of Peptides. The amino acid composition of the glycopeptides was determined using a Beckman Model 120C amino acid analyzer. The peptides were hydrolyzed in 1 ml of doubly distilled 6 N HCl for 24 hr at 110° and dried in a desiccator over sodium hydroxide and concentrated sulfuric acid. The presence or absence of tryptophan was determined by examining the unstained peptide maps under ultraviolet light. The amino acid sequence analyses were carried out either by the dansyl-Edman procedure (Gray, 1967) utilizing polyamide thin-layer chromatographic sheets to identify the dansylamino acids (Woods and Wang, 1967) or by the subtractive Edman procedure (Konigsberg, 1967) and by carboxypeptidase digestion as previously described (Abel and Grey, 1967).

Results

Sugar Contents of γG Myeloma Proteins and Bence Jones Proteins. Six γG_1 myeloma proteins having carbohydrate covalently linked to the Fab fragment were studied. The carbohydrate was attached in four proteins to the light chains, three of which were of λ type (Bla, Hug, and Pet) and one of κ type (Ste); in one protein (Wil) to the Fd fragment; and in one protein (Bou), carbohydrate was found on both the Fd fragment and κ light chain. The presence of a glycopeptide on the Fd fragment was demonstrated by isolation of the glycopeptide from the Fab fragment and by the failure to demonstrate the glycopeptide on the light chain which had been isolated from the intact myeloma protein. The sugar composition of the carbohydrate moiety of all six proteins was similar and consisted of 5–6 moles of hexoses, 4–5 moles of glucosamine, 1 mole of fucose, and 1 mole of sialic acid per mole of either light chain or Fd fragment. A carbohydrate moiety of similar composition was present on the Fc fragment in all proteins studied. The sum of the carbohydrate recovered on the Fab and Fc fragments did not differ significantly from the carbohydrate determined for the intact myeloma proteins Bou, Pet, Stev, and Wil of which sufficient material was available to accurately determine the individual sugars. The three patients having λ, γG_1 myeloma proteins in the serum (Bla, Hug, and Pet), in addition had a λ Bence Jones protein in the urine. The sugar content of the Bence Jones proteins and of the light chains isolated from the corresponding myeloma proteins is shown in Table I. There was no significant difference in the hexose, glucosamine, and fucose content between the Bence Jones proteins and the corresponding light chains. In contrast, the Bence Jones proteins contained about three times more sialic acid than the light chains. When examined by starch gel electrophoresis the Bence Jones proteins of all three patients showed multiple bands. An example of this obtained with the protein Pet is shown in Figure 1. Following the removal of the sialic acid with neuraminidase, all of the bands were reduced to one major band, indicating that the multiple banding was the result of heterogeneity in the sialic acid content. In contrast to the Bence Jones proteins, the untreated light

TABLE I: Carbohydrate Content of λ Bence Jones Proteins and Corresponding Light Chains.

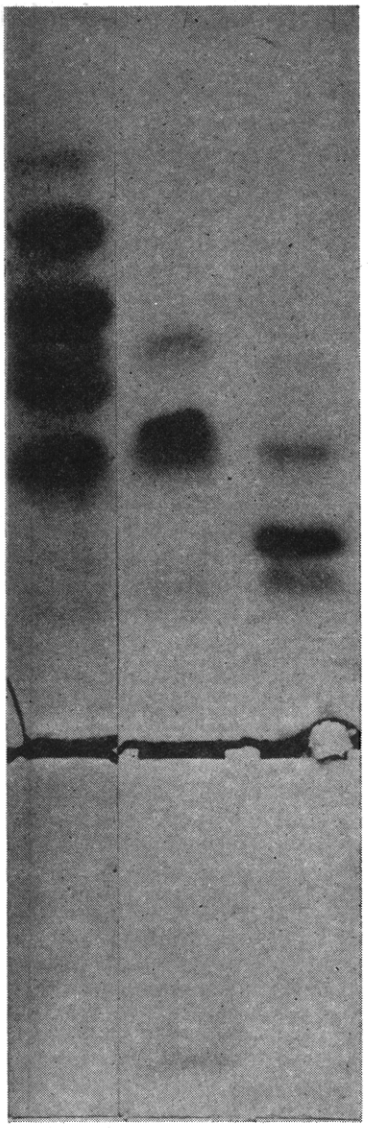
Protein	Moles/22,500 g			
	Hexoses	Glucosamine	Fucose	Sialic acid
BJP mono[a] (Pet)	6.3	6.2	1.3	3.0
BJP dim (Pet)	6.1	6.0	1.2	3.5
Light chain (Pet)	6.2	6.5	1.4	1.0
BJP mono (Hug)	4.8	4.6	n.t.	3.7
Light chain (Hug)	4.6	4.3	n.t.	1.1
BJP mono (Bla)	5.5	n.t.	n.t.	3.0
Light chain (Bla)	5.1	n.t.	n.t.	1.2

[a] BJP = Bence Jones protein, mono = monomer, and dim = dimer.

chains showed one major band indicating a more homogeneous distribution of the sialic acid. The appearance of the Bence Jones protein in starch gel before and after neuraminidase treatment together with the finding of an average of approximately 3 moles of sialic acid/mole of protein is consistent with the assumption that the Bence Jones protein consisted of molecules having from 1 to 6 moles of sialic acid per mole of protein. In contrast, the light chains which contained 1 mole of sialic acid/mole of protein showed a single major band on starch gel electrophoresis, a finding which indicated an essentially homogenous distribution of the sialic acid among this population of molecules.

Recombination of Heavy Chains with Light Chains of Low and High Sialic Acid Content. In order to determine whether the low sialic acid content of the light chains as compared to that of the Bence Jones proteins could have been the result of selective combination of the heavy chains with light chains of low sialic acid content, a recombination experiment was performed. Heavy chains isolated from the γG protein Pet were allowed to combine under competitive conditions with ^{125}I-labeled light chains and different preparations of ^{131}I-labeled Bence Jones proteins having an average of 3.8, 2.5, or 0.2 moles of sialic acid per mole of protein. When the recombined γG was isolated, the ratio of ^{131}I to ^{125}I was found to be the same irrespective of the sialic acid content of the Bence Jones protein. In several different experiments, the ratio of light chains to Bence Jones protein in the combined γG varied from 0.9 to 1.2, indicating that the Bence Jones protein, even of the highest sialic acid content was capable of recombining with the heavy chains equally as well as the homologous light-chain preparations.

Amino Acid Composition and Sequence Analyses of the Glycopeptides. In order to localize the point of attachment of the carbohydrate on either the light chains or Fd fragments, the amino acid composition, and amino acid sequence of the glycopeptides was determined. These data were then compared to the published sequence data available on Bence Jones proteins and Fd fragments in order to determine the regions of maximum homology, although the positioning of the glycopeptides by compositional similarity to portions of the sequence of other proteins does not allow positioning the peptides with absolute certainty. Only a limited quantity of the proteins was available for study and therefore, only incomplete amino acid sequence analysis was obtained on most glycopeptides. The nature of the linkage of the carbohydrate was not investigated in this study. All glycopeptides contained glucosamine as the sole amino sugar and it was assumed that the sugar was linked to the peptide through a *N*-glycosidic linkage to an Asx residue as is the case in all immunoglobulins thus far studied (Rosevear and Smith, 1961; Dawson and Clamp, 1968). The glycopeptides contained 4–5 moles of glucosamine/mole of peptide, a figure similar to that found for the light chains and Fab fragments from which they were isolated. As shown in Table II, the tryptic glycopeptides obtained from each of the reduced and alkylated Bence Jones



(1) (2) (3)

FIGURE 1: Starch gel electrophoresis employing a glycine buffer (pH 8.8), anode at top. (1) Bence Jones protein Pet; (2) light chains isolated from myeloma protein Pet; (3) Bence Jones protein Pet treated with neuraminidase. The point of application appears as a solid bar.

TABLE II: Partial Amino Acid Sequence of the Glycopeptides of Three λ Bence Jones Proteins (Bla, Pet, Hug).[a]

	18	22	25	30	33
New λ_1	Val-	Thr-Ile-Ser-	Cys-Ser-	Gly-Gly-Ser- Thr-Asn-Ile-	Gly-Asn- Asn-Tyr
Kern λ_4	Ala-	Val-Ile-Thr-	Cys-Ser-	Gly-Asp-Asp-Leu-Glu-Lys-Phe-Thr-	Val-Ser
Bal λ	(Val,Thr,Ile,Ser)	*Cys*	(Ser, Gly,Asx, Asx, Thr, Glx, Ile,	Gly,Ala,	Gly)Lys
Pet λ	*Val-*	*Thr-Ile-Ser-Cys-*	Thr-*Gly-Asx-Asx-* Ser-	*Asx-Ile-* Ala-Gly-	Gly-Arg
			(Thr,Gly,Asx,Asx, Ser)(Asx-Ile-	Ala-Gly)(Gly-Arg)	
			← P-G →	← S-2 →	← S-1 →

	83	87	90	94
New λ_1	Ala-	Asp-Tyr-Tyr-Cys-	Ala-Thr-Trp-Asp-Ser-	Ser-Leu
Kern λ_4	Ala-	Asp-Tyr-Phe-Cys-	Glu-Thr-Trp-Asp-Thr-Ile-	Thr
Hug λ	(Glu,Ile,	Tyr,Tyr)*Cys*	(Ala, Thr,Trp,Asx, Ser, Ser)*Leu*	

[a] The amino acid sequence of the two λ Bence Jones proteins of known sequence are given for comparative reasons: New (Langer *et al.*, 1968) and Kern (Prostigl *et al.*, 1968). Numbering of the residues proceeds from the N terminus. Vertical arrows indicate tryptic split following reduction and aminoethylation of the proteins. Arrows pointing to the right indicate identification of the residues by Edman degradation and dansylation and arrows pointing to the left by carboxypeptidase digestion. P-G: glycopeptide obtained following digestion of protein Pet with pronase; S-1 and S-2: peptides isolated from the tryptic glycopeptide Pet following digestion with subtilisin. The carbohydrate was probably attached to an Asx residue in either position 25 or 26 in proteins Bla and Pet and to Asx residue 91 in protein Hug.

proteins contained 1 mole/mole of carboxymethylcysteine. Since there are only five cysteine residues per light chain, the position of which within the chains are constant, the presence of this amino acid in the glycopeptides made the positioning of the peptides extremely easy. The tryptic glycopeptides of the reduced and aminoethylated proteins contained no aminoethylcysteine indicating that the carbohydrate was attached on the C-terminal side of the half-cystine residue. The amino acid composition of the glycopeptides excluded the possiblity that they were derived from the constant region of the λ chains. In contrast, the amino acid composition and sequence of the glycopeptides showed strong homology to the amino acid sequence surrounding the variable region cysteines. The glycopeptides of the proteins Bla and Pet showed maximum homology between positions 18 and 33 (Table II). The carbohydrate was probably attached to either the Asx residue 25 or 26 in protein Pet since digestion of the tryptic glycopeptide with pronase yielded a glycopeptide having the composition (Thr,Gly,Asx,Asx,Ser) and digestion with subtilisin yielded a peptide having the sequence Asx-Ile-Ala-Gly, which did not contain carbohydrate. The tryptic glycopeptide obtained from the carboxymethylated protein Hug contained 36 amino acids and that of the aminoethylated protein 31 amino acids. The composition of the glycopeptides was similar to that of λ chains between residues 83 and 112 which includes the second cysteine of the variable region in position 87. The chymotryptic glycopeptide obtained from the aminoethylated protein Hug contained 7 amino acids which were identical to those of protein New between positions 88 and 94 (Table II), suggesting that the carbohydrate was probably attached to the Asx residue in position 91.

TABLE III: Partial Amino Acid Sequence of the Glycopeptides of Two κ Light Chains (Bou and Ste).[a]

		62	65	70
Roy	κ_1	Phe-Ser-Gly-Thr-Gly-Ser-Gly-Thr-Asp-Phe		
Fr 4	κ_3	Phe-Ser-Gly-Ser-Gly-Ser-Gly-Thr-Asp-Phe		
Bou	κ_3	*Phe*(Ser, Gly, Ser, Gly, Ser, Gly, Thr, Asx)*Phe*		
Ste	κ	*Phe*(Ser, Gly, Ser, Gly, Ser, Gly, Thr, Asx)*Phe*		

[a] The amino acid sequence of the κ_1 protein Roy (Hilschman, 1967) and the κ_3 protein Fr 4 (Milstein, 1967) are given for comparative reasons. Numbering of the residues proceeds from the N terminus. Arrows pointing to the right indicate identification of the residues by Edman degradation and dansylation and arrows pointing to the left by carboxypeptidase digestion. The carbohydrate was most likely attached to the Asx-70 in proteins Bou and Ste.

The partial amino acid sequence of the chymotryptic glycopeptides of the κ-light chains Bou and Ste are shown in Table III. The amino acid composition of both glycopeptides was identical and when compared to known κ-chain sequences were found to have a striking resemblence to the reported sequence between positions 62 and 71. The κ-subtype 3 protein F4 is given in Table III for comparative reasons since the κ light-chain Bou belonged to this subgroup (kindly determined by Dr. Leroy Hood). By homology, the carbohydrate was probably attached to the Asx residue 70 in both proteins.

The amino acid sequence of the glycopeptides obtained from the Fd fragment Bou and Wil are shown in Table IV. The amino acid sequence of the glycopeptide obtained from the protein Bou following digestion with chymotrypsin showed maximum homology between positions 28 and 34 to two Fd fragments (He and Ou) belonging to the Fd subtype II (Cun-

TABLE IV: Amino Acid Sequence of the Glycopeptides of Two Fd Fragments (Bou and Wil).[a]

		26	28	34
He	Fd-II		Ser-Leu-Thr-Thr-Asp-Gly-Met	
Ou	Fd-II		Ser-Leu-Ser-Thr-Ser-Arg-Met	
Bou	Fd		*Ser-Leu*-Asx-*Thr-Ser-Gly-Met*	
Vin	Fd(γG_4)	Gly-Phe —	Thr-Val-Ser-Thr-Asn-Trp-Met	
Wil	Fd	*Gly-Phe*(Asx,Gly,Tyr)	Phe-*Thr-Asn-Tyr*-	

[a] The amino acid sequence of two Fd fragments of subtype II (He: Cunningham *et al.*, 1969, and Ou: Wikler *et al.*, 1969) and of a Fd fragment obtained from a γG_4 myeloma protein (Vin: Pink and Milstein, 1969) are given for comparative reasons. Numbering proceeds from the N terminus. Arrows pointing to the right indicate identification of the residues by Edman degradation and dansylation and arrows pointing to the left by carboxypeptidase digestion. Vertical arrow indicates chymotryptic split of the tryptic glycopeptide Wil. The carbohydrate was most likely attached to the Asx residue in position 30 in protein Bou and to the Asx residue localized between positions 27 and 30 in protein Wil.

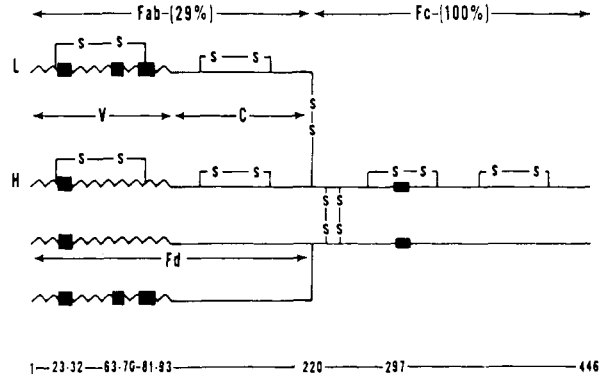


FIGURE 2: Schematic model of the γG-immunoglobulin molecule showing localization of the carbohydrate on heavy (H) and light (L) polypeptide chains; 29% of 76 myeloma proteins tested contained carbohydrate on the Fab fragment, whereas 100% contained carbohydrate on the Fc fragment (Abel *et al.*, 1968). Solid bars on the light chain, Fd and Fc fragments represent areas of attachment of carbohydrate. Numbering of the amino acid residues proceeds from the N terminus. V-variable portion and C-constant portion of the light chain and Fd fragment.

ningham *et al.*, 1969). The carbohydrate was most likely attached to the Asx residue in position 30. In the case of the protein Wil the amino acid sequence of the tryptic glycopeptide most resembled that of the Fd fragment Vin between positions 26 and 34. It has recently been shown that the Fd fragment of protein Vin belongs to a third Fd subtype (Kohler *et al.*, 1970; Pink and Milstein, 1970), but sequence data for proteins in this subtype other than Vin have thus far only been obtained for the first 20 N-terminal amino acid residues. The carbohydrate was most likely attached in the Fd fragment Wil to the Asx residue localized between positions 28 and 30.

Discussion

The localization of carbohydrate on human γG myeloma proteins based on the present and previous investigations (Abel *et al.*, 1968; Edelman *et al.*, 1969; Franklin, 1960; Grey and Abel, 1970) is shown in Figure 2. Whereas all γG myeloma proteins studied contained carbohydrate on the Fc fragment, only 29% contained additional carbohydrate on the Fab fragment. The carbohydrate of the Fab fragment was attached to the variable region of either the light chain or the Fd fragment and in a few cases to both. As shown in the present study, the point of attachment varied from protein to protein. However, it appeared to be restricted to three areas: one near the N-terminal cysteine residue of the heavy or light chains and the other two, either near the residue 70 of the light chains or close to the second cysteine at position 87. In patients forming both γG myeloma proteins and Bence Jones proteins, the carbohydrate content of the Bence Jones protein was similar to that of the corresponding light chains, except for the sialic acid content. The Bence Jones proteins contained three times more sialic acid than the light chains and whereas individual molecules in the light-chain preparations were homogeneous in their sialic acid content, individual molecules of the Bence Jones proteins varied in this respect.

Although no complete amino acid sequence could be performed on the light chains and Fd fragments in order to definitely localize the point of attachment of the carbohydrate, the striking homology between the glycopeptides and certain areas of the light chains and Fd fragments of known sequence made it possible to position the glycopeptides with a high degree of assurance in most cases. Two characteristics of the glycopeptides greatly facilitated their positioning in specific areas of the proteins of known sequence. First, the presence of relatively rarely occurring amino acids such as cysteine, methionine, and aromatic amino acids, and second, the relatively minor variability of the amino acid sequence near these residues (especially the cysteines) in different proteins of known sequence (Edelman and Gall, 1969). The glycopeptides of the three λ chains all contained one cysteine residue. The amino acid composition of these peptides excluded the positioning of them to include one of the three cysteine residues in the constant portion of the λ chains. In contrast, there was a high degree of homology between two of the glycopeptides and the region around cysteine-22 and between the third glycopeptide and the region around cysteine-87 (Table II). Similarly, the amino acid composition of the glycopeptides obtained from the two κ chains and the two Fd fragments was incompatible with any area of the constant region of the subunits but showed a good homology to restricted areas of the variable region. The amino acid composition of the two κ chain glycopeptides was identical. Out of a total of ten residues, three were identified as serine and three as glycine and phenylalanine was found in both the N- and C-terminal position. From these characteristics it appears extremely likely that these glycopeptides are derived from positions 62 to 71 of the κ chain (Table III). The position of the Fd glycopeptides was somewhat more uncertain. Extensive sequence analysis of the variable region of the heavy chains have been reported for only a few proteins so that the patterns of homology are not as easy to establish. However, since the amino

acid sequence analysis of the Fd glycopeptide Bou demonstrated the presence of the uncommon amino acid methionine and a good homology of the whole peptide to the area 28 to 34 of two proteins of Fd subtype II (Cunningham *et al.*, 1969), the positioning of this glycopeptide could be fairly well ascertained (Table IV). The homology between the Fd glycopeptide Wil and the published amino acid sequences of Fd fragments is less impressive. The best homology found was between residues 26 and 33 with the Fd fragment Vin, which appears to belong to a third Fd subtype (Kohler *et al.*, 1970; Pink and Milstein, 1970) (Table IV). When more detailed sequence data become available for the variable region of the heavy chains, it will be possible to more critically evaluate the proper positioning of this Fd glycopeptide. The point of attachment of the carbohydrate of the recently reported human and murine light chains, also was restricted to one of the areas of the variable portion similar to that found in the present study. The carbohydrate was attached on either residue 81 or 84 in two λ human Bence Jones proteins described by Edmundson *et al.* (1968); to residues 28 and 65 or 70 in two κ chains and to residue 26 in one λ chain reported by Cox and Hood (1970). In two murine κ Bence Jones proteins, the carbohydrate was attached at residue 28 (Hood *et al.*, 1969; Melchers, 1969). No Fd fragment having carbohydrate linked to the C-terminal end of the variable portion has been found. In the two Fd fragments studied, the carbohydrate appeared to be localized in an area homologous to that in both κ and λ chains. Therefore, the possibility exists, that when more proteins become available for study, a further site of attachment of carbohydrate to the Fd fragment corresponding to the C-terminal end of the variable portion of the light chains might be detected.

The finding that the carbohydrate is attached to the variable portion of the light chains and Fd fragment was expected since in previous studies no correlation was demonstrated between light-chain type or heavy-chain subclass with the presence of carbohydrate in the Fab fragment (Abel *et al.*, 1968). If the carbohydrate were to have been attached to the constant portion of these proteins one would have expected carbohydrate to be present on all proteins of the same type or subclass. However, though the carbohydrate is attached to the variable portion, the attachment is clearly not a random process, since it has been localized to limited areas of the variable portion in both the light chains and Fd fragments. It has been postulated by Marshall (1967) that enzymes which attach carbohydrate to serum proteins recognize the sequence Asn-X-Ser or Thr, where X can be any amino acid and the carbohydrate is attached to the Asn residue. The limited amino acid sequence data of the present glycopeptides are compatible with the hypothesis. However, several of the Bence Jones proteins of which the full amino acid sequence has been reported also have the sequence Asn-X-Ser/Thr in the variable region but do not have carbohydrate attached to the asparagyl residue (Edelman and Gall, 1969). This finding suggests, that although the amino acid sequence Asn-X-Ser/Thr appears to be necessary for the carbohydrate attachment, additional factors may be required to promote the binding of the carbohydrate to the asparagine residue.

In patients forming both γG myeloma proteins and Bence Jones proteins the carbohydrate composition of the Bence Jones proteins was found to be similar to that of the light chains isolated from the corresponding myeloma proteins, except for the sialic acid content. Whereas the light chains contained 1 mole of sialic acid/mole of protein, the Bence Jones protein preparations consisted of molecules whose sialic acid content varied from 1 to 6 moles per mole of protein. The reason for this difference is unknown. *In vitro* recombination experiments revealed the heavy chains to have no preference for light chains over Bence Jones proteins even of the highest sialic acid content. It appears, therefore, unlikely that excess sialic acid present on the light chains can prevent them from combining with heavy chains to form γG molecules. It has been shown that the attachment of sialic acid to immunoglobulins occurs late in the process of immunoglobulin production and may actually occur on the cell membrane (Swenson and Kern, 1968). It might be, therefore, that intact γG molecules spend less time on the membrane than do free light chains and thus only a relatively small amount of sialic acid can become attached to the light chains during this period, as compared to that which can become attached to the Bence Jones protein. Alternatively, the presence of heavy chains in the intact γG molecule might sterically prevent the attachment of large amounts of sialic acid to the light chains. In contrast to the present findings, Edmundson and coworkers (1968) reported a patient who excreted Bence Jones protein having a carbohydrate moiety whereas the light chains of the corresponding myeloma protein lacked carbohydrate. The reason for the discrepancy between this observation and ours is not known.

The relevance of the present findings on the carbohydrate composition of normal γG-immunoglobulins cannot be answered at this time. It has indeed been reported by Dische and Franklin (1963) that a minor population of Fab fragments of normal γG-immunoglobulin contains a carbohydrate moiety. This carbohydrate is most likely also attached to the variable portion of either the light chain or Fd fragment. However, the total carbohydrate of normal Fab fragments were lower than one would have expected if the incidence of carbohydrate of Fab fragments of normal γG was the same as that found on Fab fragments of myeloma proteins (Abel *et al.*, 1968). The possibility exists, therefore, that the presence of carbohydrate on the Fab fragment might be a unique feature of γG myeloma proteins.

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An Assay for Adenosine 3′,5′-Cyclic Monophosphate Based on the Association of the Nucleotide with a Partially Purified Binding Protein*

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ABSTRACT: The binding properties of a cyclic AMP receptor protein are utilized to provide a sensitive and rather specific assay for this nucleotide in biological tissues. A fivefold purification of the cyclic AMP binding protein from beef adrenal glands provides a preparation which allows the determination of as little as 20 pmoles of cyclic AMP isolated from tissue extracts. The assay is based upon the isotopic dilution of cyclic [^{3}H]AMP by the cyclic AMP which is being measured and the subsequent attachment of the cyclic nucleotide to the receptor protein. The rapid separation of free ligand from that bound to the protein is accomplished by passing the mixture through a membrane filter with a demonstrated affinity for the protein–ligand complex. At levels of cyclic AMP determined by the assay, a high specificity for the cyclic nucleotide was observed in the presence of similar concentrations of other nucleotides. The assay was used to determine the levels of cyclic AMP isolated from various tissues of the rat.

The binding of adenosine 3′,5′-cyclic monophosphate[1] to a protein fraction from beef adrenal cortex has been previously reported by this laboratory (Gill and Garren, 1969). Utilizing the equilibrium dialysis technique, the binding activity was found to be present in both the soluble and microsomal fractions of the adrenal cortex. Although the role of this protein in metabolism is as yet unknown, an association with a cyclic AMP dependent protein kinase activity has been suggested through partial purification of the two activities from

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[1] Cyclic AMP, adenosine 3′,5′-cyclic monophosphate; cyclic IMP, inosine 3′,5′-cyclic monophosphate; cyclic GMP, guanosine 3′,5′-cyclic monophosphate; cyclic CMP, cytidine 3′,5′-cyclic monophosphate; AMP, adenosine 5′-phosphate; ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate.