The Carbohydrate Moiety of Bovine Colostrum Whey M-1 Glycoprotein and its Relationship to Casein

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Streptococcal hydrolase degradation of the 275 mμ-positive and negative bovine colostrum whey glycoproteins showed that their carbohydrate moieties were identical. The carbohydrate moiety of the crude whey M-1 glycoprotein fraction was removed by the β-elimination reaction and was purified by gel filtration and chromatography on Dowex 50 resins. The isolated material contained 98% carbohydrate and accounted for 18% of the carbohydrate present in the starting M-1 glycoprotein fraction. The molecular weight of the isolated material was determined by gel filtration, and it was concluded that the carbohydrate moieties of the 275 mμ-positive and negative proteins had a molecular weight of 1038–1330 and apparently contained 2 galactose, 1 to 2 sialic acid, 1 glucosamine, and 1 galactosamine residues per carbohydrate chain. The detection of galactosaminitol in the isolated carbohydrate preparation and of α-aminobutyric acid in the nondiffusible residue following the β-elimination reaction of the M-1 glycoprotein suggested that the carbohydrate moiety of this protein was linked to its polypeptide chain via an O-glycoside linkage involving galactosamine and threonine. Assuming that the isolated carbohydrate material is representative of all carbohydrate present in the 275 mμ-positive and negative proteins, the former would then contain two carbohydrate chains, whereas the latter would possess 3 to 4 such chains. A component was found in the acid casein fraction of bovine colostrum, whose carbohydrate moiety closely resembled that of the whey M-1 glycoprotein.

Previous work in this laboratory (Bezkorovainy, 1967) demonstrated the presence of low-molecular weight glycoproteins (termed the M-1 glycoprotein fraction) in bovine colostrum whey. This glycoprotein fraction had variable carbohydrate composition and multiple amino acid N-terminal residues. The monosaccharide ratio was, however, constant from one preparation to another with galactose:hexosamine:sialic acid = 1:1:0.7.

The M-1 glycoprotein fraction of bovine colostrum whey was separable into two components on the basis of size. The heavier component had no absorption maximum in the 240 to 300 mμ range and was, therefore, termed the 275 mμ-negative protein. The lighter component contained tyrosine and showed an absorption maximum at 275 mμ. It was termed the 275 mμ-positive protein. The heavier protein had a slightly higher carbohydrate content than the lighter material (Bezkorovainy and Grohlich, 1969).

The present communication reports the results of an investigation on the size of the carbohydrate moieties of the two glycoproteins and the mode of their attachment to the polypeptide backbone. Evidence is also presented to the effect that the carbohydrate chains of the two glycoproteins are identical, and that a portion of the carbohydrate present in the acid casein fraction of bovine colostrum has some properties similar to those of the carbohydrate moiety of the whey glycoprotein.

MATERIALS AND METHODS

Colostrum. Bovine colostrum was collected from individual Holstein animals in the presence of thimerosal (final concentration 1 to 10,000) and represented the material obtained from the first milking following parturition. It was defatted and separated into the whey and casein fractions essentially by the method of McKenzie (1967a). Defatted colostrum was heated to 30° C in a water bath and the pH was adjusted to 4.6 with HCl. No precipitation of casein was observed at this point. The acidified colostrum was then dialyzed against distilled water in the cold for 4 days with daily water changes, and at the end of this period the coagulation of casein took place. It was separated from the whey by centrifugation and washed three times with distilled water (10 volumes each time). The yield was 60 g of casein per liter of colostrum. Unpasteurized milk, under similar circumstances, yielded 25 g of acid casein per liter.

Fractionation of Whey. The M-1 glycoprotein from colostrum whey was isolated by the method of consecutive chromatography on DEAE-cellulose, CM-cellulose, and Sephadex G-100 as previously described (Bezkorovainy, 1967). It was further separated into the 275 mμ-positive and negative fractions according to Bezkorovainy and Grohlich (1969).

Enzymes and Their Use in the Study of Glycoproteins. Extracellular streptococcal glycosidase complex (source: SL-1 cariogenic oral streptococcus), containing neuraminidase, fucosidase, β-galactosidase, and hexosaminidase activities (Pinter *et al.*, 1969) was used to remove carbohydrate components from the glycoproteins as described by Pinter *et al.* (1970). The enzymatic reaction was continued for 16 hr at 37° C, and was then analyzed for free sugars: hexose by the β-galactose dehydrogenase method of Wallenfels and Kurz (1962), sialic acid by the thiobarbituric method of Warren (1959), hexosamines by the method of Reissig *et al.* (1955), and fucose was not determined, because the whey M-1 glycoprotein fraction did not contain this sugar (Bezkorovainy, 1965).

Materials. Sodium borohydride used in this work was purchased from Metal Hydrides, Inc. It was 98% pure. All other chemicals were of reagent grade purity and came from Fisher Laboratories. Sephadex gel filtration media were obtained from Pharmacia Corporation, and DEAE- and CM-cellulose ion exchangers were purchased from Biorad Laboratories. The capacities of the two exchangers were 0.9 and 0.7 meq per g, respectively, and both were of the coarse grade.

Analytical Procedures. High voltage electrophoresis was done in a refrigerated Brinkmann instrument using Whatman

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No. 3 paper as supporting medium at 1000 to 1500 volts for 1.5 hr. The buffer consisted of 100 ml of glacial acetic acid and 12 ml of pyridine diluted to 1 liter with water to give a final pH of 3.0. Carbohydrates were visualized on the pherograms by the periodate-permanganate spray reagent (Lemieux and Bauer, 1954), and peptides by the ninhydrin spray (0.9 g ninhydrin in 100 ml acetone).

Identification of hexoses and hexosamines by paper chromatography following acid hydrolysis of the glycoproteins was done as previously described (Bezkorovainy and Grohlich, 1969). Quantitative estimation of bound hexoses, hexosamines, sialic acid, and "protein" was done by the orcinol, the Elson-Morgan, the Warren (1959), and the Lowry procedures, respectively (Bezkorovainy, 1965).

Amino acid analysis was done on a Spinco Model 120C apparatus by the method of Moore *et al.* (1958). Glucosamine and galactosamine were resolved either on the short column or the long column using a 240-min elution time. Galactosaminitol was estimated from its elution from the short column. Hydrolysates were prepared for the amino acid-hexosamine analysis by heating 2 mg of the proteins in 2 ml of 5.7 M HCl for 24 hr at 110° C under nitrogen, followed by repeated lyophilization.

Molecular weights of carbohydrate preparations were determined by gel filtration, essentially by the method of Bhatti and Clamp (1968). Sephadex G-50 (medium grade) was used in a column having the dimensions of 1.2 × 90 cm and with 0.15 M NaCl as the solvent. The rate of flow was 13 ml per hr, and fractions of 1.7 ml each were collected. Carbohydrate was detected in these fractions by the phenol-H_2SO_4 reaction (Dubois *et al.*, 1956). The void volume (V_0, blue dextran) of this column was 49.0 ml, and the V_{100} (glucose) was 126.5 ml. Each sample applied to the column contained 0.2 mg each of blue dextran, glucose, and the unknown carbohydrate in a total volume of 0.3 ml.

RESULTS AND DISCUSSION

Digestion with Streptococcal Glycosidase. Previous work on the 275- mμ-positive and negative glycoproteins (Bezkorovainy and Grohlich, 1969) demonstrated the following with respect to their carbohydrate moieties. The galactose:hexosamine:sialic acid ratios were practically identical (1:1:0.7); periodate destroyed galactose only following the removal of sialic acid by neuraminidase from both proteins. The galactose remained intact in both proteins following their oxidation with periodate without prior removal of sialic acid; alkali-borohydride treatment of both proteins resulted in the splitting of their carbohydrate chains from their polypeptide moieties with quantitative recovery of galactose and sialic acid, and a 50% recovery of hexosamine.

These studies suggested to us that the carbohydrate moieties of both proteins may be identical. To further test this hypothesis, both proteins were treated with a mixture of glycosidases, called streptococcal glycosidase, and the sugars released were quantitated at the end of the reaction. The results are shown in Table I. It may be observed that the action of the hydrolases on the two proteins was very similar if not identical. Surprisingly little galactose was released by the enzymes from either protein. These results support our previous suggestion that the carbohydrate chains of the two proteins are identical.

Isolation of the Carbohydrate from Casein and the Whey M-1 Glycoprotein. A detailed study of a glycoprotein must necessarily involve the separation of the carbohydrate from its polypeptide moiety. The lability of the carbohydrate-peptide linkage to alkali-borohydride in the two colostrum glycoproteins (Bezkorovainy and Grohlich, 1969) provided a convenient way of affecting such a separation. In addition, a comprehensive study of the carbohydrate moiety of a glycoprotein requires relatively large amounts of material, and the tedious and lengthy procedures of separation of the 275 mμ-positive and negative proteins precluded the possibility of accumulating enough protein for a reasonably thorough study of their carbohydrate moieties. However, in view of the apparent identity of the carbohydrate components of the proteins, it was decided to isolate the carbohydrate material of the crude M-1 glycoprotein fraction without its separation into the 275 mμ-positive and negative components.

Referring to Figure 1, curve A of the work of Bezkorovainy and Grohlich (1969), the material purified on CM-cellulose was passed once through a column of Sephadex G-75 to remove any high-molecular weight proteins, and the retarded fractions (a mixture of the 275 mμ-positive and negative proteins) were pooled and used for the degradation studies.

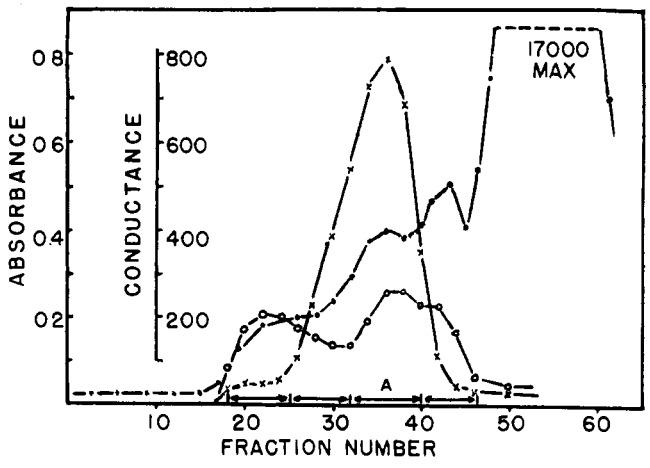

Figure 1. Gel filtration on Sephadex G-25 of the diffusible material following alkali-borohydride treatment of the colostrum whey M-1 glycoprotein fraction

Column dimensions: 1.5 × 90 cm. solvent water; rate of flow: 3 ml per hr, 1.5 ml fractions collected. Open circles: Lowry reaction-positive materials; closed circles: the conductance; crosses: orcinol-positive material (hexose)

Table I. Action of Streptococcal Hydrolase on the 275 mμ-Negative and Positive Colostrum Whey Glycoproteins

	275 mμ-negative protein	275 mμ-positive protein
Sialic acid (% of total present in protein)	9.1	10.0
released (% of total sialic acid present)	102	107
Galactose (% of total present in protein)	9.0	8.0
released (% of total galactose present)	20	16
Hexosamine (% of total present in protein)	12.5	10.0
released (% of total hexosamine present)	53	53

The stepwise degradation procedure of the crude whey M-1 glycoprotein fraction was as follows:

1. Three hundred and sixty mg (38% carbohydrate) of the protein were dissolved in 5 ml water, and the pH was adjusted to 8. This was followed by the addition of 0.1 ml of 5 N NaOH and 57 mg $NaBH_4$. The solution was flushed with nitrogen and kept for 43 hr at 37° C in a sealed container. The $NaBH_4$ was then destroyed by acidification to pH 4.6 with 6 N HCl, and the reaction mixture was placed in a dialysis tubing.
2. The dialysis was performed twice each against 40 volumes of distilled water for 24 hr each time, the two portions of the diffusate were combined and lyophilized. The dialysis residue was also lyophilized yielding 165 mg glycoprotein material with 17.1% total carbohydrate.
3. The lyophilized diffusate (henceforth termed the whey diffusate) was desalted and partially resolved on a 1.5 × 90 cm Sephadex G-25 column with water as the eluant. The elution diagram is given in Figure 1. The orcinol-positive material eluted from the column accounted for some 75% of the hexose present in the starting material; however, only the fraction designated by A in Figure 1 was used for further work. The yield of this fraction was 48 mg and it contained 75% carbohydrate. The rest of fraction A (25%) was accounted for by peptide material.
4. Fraction A was dissolved in water and adjusted to pH 2.1 with HCl. It was then passed through a column of Dowex 50 × 8 in the hydrogen form. The carbohydrate-containing material was eluted in the void volume, whereas the peptide material was retained by the column. The yield was 25 mg of material containing 98% carbohydrate. This represented some 18% of the carbohydrate originally present in the crude M-1 glycoprotein preparation.

Colostrum acid casein was also subjected to a degradation with alkali-borohydride. The procedure used was as follows:

1. Two 20-g quantities of casein (3.3% carbohydrate) were each suspended in 100 ml water, and their pH was adjusted to 8.0 with 5 N NaOH to affect their dissolution. One of the acid casein solutions (test sample) was adjusted to pH 10 with 5N NaOH, then 2 ml of 5N NaOH were added to give a final NaOH concentration of 0.1 N. This was followed by the addition of 1.14 g of $NaBH_4$ to give a final $NaBH_4$ concentration of 0.3 M. Both the test sample and the casein solution that had remained at pH 8 (control sample) were incubated under nitrogen at 37° C for 43 hr, after which the pH was adjusted to 4.6. The precipitates that were formed were removed by centrifugation, and the supernatants were both placed in dialysis tubing.
2. Each of the two supernatants was dialyzed against 1 l. of distilled water for 24 hr, the dialysis was repeated against fresh portions of water, and the corresponding diffusates (henceforth called the casein diffusates) were combined and lyophilized. The dialysis residues were discarded.
3. Both casein diffusates (test sample and control) were desalted on a column of Sephadex G-25 as described for the whey diffusate. The entire carbohydrate-containing material eluted from the column with the control sample accounted for only 2% of the carbohydrate present in the original 20 g of casein. On the other hand, all material eluted from the column containing the test sample accounted for 21.6% of the carbohydrate originally present in the acid casein. Its carbohydrate content was, however, only 6%, the rest being peptide material.

3a. A fraction corresponding to fraction A of the whey diffusate in Figure 1 was subjected to chromatography on a carboxymethyl-cellulose column equilibrated with a 0.03 M acetate buffer at pH 4.1 (Bezkorovainy, 1965). The carbohydrate-containing material emerged with the void volume. After desalting on a Sephadex G-25 column, some 65 mg of material was obtained, whose carbohydrate content was 75%, the rest being accounted for by peptide material.
4. The CM cellulose purified fraction was then chromatographed on a column of Dowex 50 × 8 resin as described for the whey diffusate. The yield was 35 mg of material containing 96% carbohydrate. The carbohydrate thus isolated accounted for 5% of the carbohydrate present in the starting 20 grams of acid casein.

Composition of the Purified Carbohydrate Materials. The amino acid and carbohydrate composition of the colostrum acid casein, the M-1 glycoprotein fraction of whey, their carbohydrate moieties (as isolated in step 4 of the degradation procedures), and the nondiffusible fraction of alkali-borohydride treated whey M-1 glycoprotein fraction (step 2 of the degradation procedure) are given in Table II. The carbohydrate isolated from the whey M-1 glycoprotein fraction contained a total of 9.4 mg hexose, or 23% of the hexose present in the starting material. The casein oligosaccharide accounted for a total of 12 mg hexose or 4% of the hexose present in the original 20 g of casein.

α-Aminobutyric acid was present in the nondiffusible portion of the alkali-borohydride treated whey M-1 glycoprotein fraction, a total of 25 μmoles being recovered in this fraction. α-Aminobutyric acid is a degradation product of O-threonyl glycosides; however, it is well-known that the recovery of α-aminobutyric acid following the β-elimination reaction is only 30% of the expected value (Gottschalk, 1967). We recovered approximately 1 μmole of α-aminobutyric acid per 1 μmole of protein (assuming a molecular weight of 12,000 as in Bezkorovainy, 1967) Taking into account the 70% loss of α-aminobutyric acid, it is probable that some 3–4 threonyl residues are involved in the formation of glycoside bonds in the M-1 glycoprotein fraction. The recovery of all amino acids following the β-elimination reaction of the whey M-1 glycoprotein fraction was, with the exception of serine and threonine, nearly quantitative. This included alanine, the degradation product of O-seryl glycosides. The overall recovery of serine and threonine was 76 and 59%, respectively. These low recoveries of serine and threonine were probably due to the destruction of these amino acids by alkali and, in case of threonine, due to the β-elimination reaction.

The hexose:hexosamine ratios were 2:1 in both the casein and whey carbohydrate preparations. Compared to the native M-1 glycoprotein, this represented a loss of one hexosamine residue for every two hexose residues. Galactosaminitol was present in both carbohydrate preparations; however, it accounted for only 40% of the hexosamine lost in the whey M-1 glycoprotein. The hexosamine remaining in the two carbohydrate preparations following the β-elimination reaction was identified by its degradation to pentose and paper chromatography. In both carbohydrate preparations, glucosamine was the only type of hexosamine present. The galactosamine present in the original whey M-1 glycoprotein appears to be completely lost following the β-elimination reaction.

The fact that the β-elimination reaction of the whey M-1 glycoprotein fraction results in the loss of galactosamine and the appearance of galactosaminitol and α-amino butyric

Table II. Amino Acid and Carbohydrate Composition of Bovine Colostrum Glycoproteins and Their Degradation Products

All values are in g-residue per 100 g

Component	Colostrum acid casein	Crude whey M-1 glycoprotein fraction	Casein carbohydrate	Whey carbohydrate	Nondiffusible fraction of whey M-1 glycoprotein after β-elimination reaction
Lys	8.0	4.1	0.2	0.3	4.4
His	2.6	0.2	0.0	0.0	0.3
Arg	3.9	0.3	0.0	0.0	0.5
Asp	7.0	4.9	1.0	0.1	7.0
Thr	4.3	9.6	0.3	0.2	9.2
Ser	5.9	4.5	0.3	0.3	5.2
Glu	20.7	12.5	0.5	0.5	18.4
Pro	8.3	7.6	0.2	0.0	10.8
Gly	1.8	0.7	0.1	0.1	0.0
Ala	2.9	3.7	0.2	0.2	5.9
Cys	trace	0.0	0.0	0.0	0.0
α-ABA[a]	0.0	0.0	0.0	0.0	1.3
Val	5.4	4.0	0.4	0.3	5.9
Met	2.0	0.5	0.0	0.0	1.2
Ile	4.2	6.6	0.1	0.1	9.4
Leu	8.1	1.7	0.4	0.1	2.7
Tyr	5.5	0.8	0.0	0.0	0.3
Phe	4.8	0.6	0.0	0.0	0.4
Hexose	1.5	11.5	34.5	37.4	5.1
N-AHA[b]	1.1	12.0	22.0	24.8	6.1
SA[c]	0.7	14.4	33.9	26.7	5.9
GA[d]	0.0	0.0	6.3	8.6	trace

[a] α-Aminobutyric acid. [b] N-acetylhexosamine. [c] Sialic acid. [d] Galactosaminitol.

acid strongly suggests that the carbohydrate chain(s) of this protein complex are linked to the polypeptide moiety *via* the O-threonyl galactosaminide bond. The same statement appears to apply to the carbohydrate moiety of casein herein described, although the participation of threonine has not been established in this case. Finally, the low amino acid content of the casein and M-1 glycoprotein carbohydrate preparations (4 and 2%, respectively) precludes the existence of glycopeptides in the materials isolated. The peptide material observed in our final preparations probably represents contaminants not removed by the purification procedures used.

Physical Properties of the Casein and Whey M-1 Glycoprotein Carbohydrate Preparations and Their Correlation with Composition Data. High voltage electrophoresis of the casein and whey M-1 glycoprotein preparations prior to their chromatography on the Dowex 50 columns (following steps 3 and 3a of the degradation procedures) is shown in Figure 2. It may be seen that four carbohydrate-positive bands (A, B, C, and D) were observed, one of them (band A) being identifiable as sialic acid. Hydrolysis of these preparations at pH 3 for 1 hr at 100° C abolished bands B and C, leaving bands A and D only. Such hydrolysis procedure was previously shown to remove sialic acid from the intact whey M-1 glycoprotein fraction without the release of free hexose, hexosamine, or amino acids. It may be concluded that the apparent heterogeneity of the two preparations was due to different amounts of sialic acid present in the otherwise identical molecules. Band D may thus be identified as the carbohydrate chain containing no sialic acid.

High voltage electrophoresis of the final carbohydrate products (following step 4 of the degradation procedures) revealed a pattern consisting of bands A and D only in both the casein and M-1 glycoprotein carbohydrate preparations.

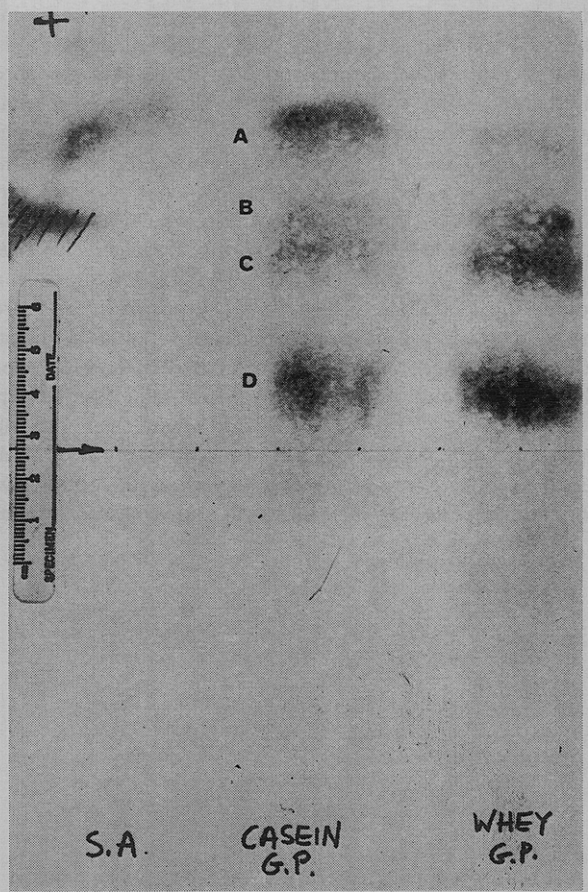

Figure 2. High voltage electrophoresis of the casein (casein GP) and whey M-1 glycoprotein (whey GP) carbohydrate preparations before their final purification on Dowex 50 columns

Two mg material was used in each case

The Dowex 50 procedure had apparently resulted in a loss of the sialic acid from both preparations. In view of the low pH value (pH = 2) at which this procedure was performed, this result in not surprising, since the sialic acid links are very susceptible to weak acid hydrolysis. We may conclude from such studies that the bond linking sialic acid to the rest of the carbohydrate chain is, as expected, a very weak one, which is easily broken under acid conditions or during isolation procedures. The monosaccharide ratios of the native whey M-1 glycoprotein fraction may very well be hexose:hexosamine:sialic acid = 1:1:1, where part of the sialic acid is lost during the isolation procedure to give a ratio of 1:1:0.7 in the purified M-1 glycoprotein fraction, and a ratio of 2:1:0.8 in the final carbohydrate preparation that has gone through the β-elimination reaction, lyophilization, desalting, and the various column chromatography steps.

Molecular weights of the two carbohydrate preparations were estimated from gel-filtration columns. The $(V_e - V_0)/(V_{100} - V_0) \times 100$ values for stachyose (mol wt 660) and neuraminlactose (mol wt 634) were 92 and 88 ml respectively (V_e, V_{100}, and V_0 are elution volumes of the unknown, glucose, and blue dextran, respectively). This gave apparent molecular weights of 600 and 650 for these standard sugars, respectively. Both the casein and the whey M-1 glycoprotein carbohydrate preparations gave single peaks in these gel filtration experiments with $(V_e - V_0)/(V_{100} - V_0) \times 100$ values of 84.0, and hence a molecular weight of 750. Since the high voltage experiments above suggested that these preparations contained no bound sialic acid, one then is left with the hexose, glucosamine, and galactosaminitol (or a derivative thereof). The structure of such a compound is best approximated by 2 hexose (galactose), 1 glucosamine, and 1 galactosaminitol residues with a theoretical molecular weight of 746. If we assume that the native M-1 glycoprotain contains either 1 or 2 sialic acid residues per each galactose group, then each carbohydrate chain would have a molecular weight of 1038 or 1330, depending on whether it contained 1 or 2 sialic acid residues.

The 275 mμ-negative and positive whey glycoproteins contained 39 and 28% carbohydrate, respectively (Bezkorovainy and Grohlich, 1969). The carbohydrate thus accounts for 4700 Daltons and 2000 Daltons in the two proteins, respectively. If we assume that the carbohydrate chains of the two glycoproteins are identical and that each carbohydrate chain contains one or two sialic acid residues and has a molecular weight of 1038–1330, then each 275 mμ-negative protein would contain 3–4 carbohydrate chains, whereas each 275 mμ-positive protein would contain two such chains. The 275 mμ-negative and positive proteins studied in this work (Table I) would contain three and two such chains, respectively. This is in agreement with the number of α-aminobutyric acid residues recovered following the β-elimination reaction of the M-1 glycoprotein fraction.

The variation in carbohydrate content of individually isolated whey M-1 glycoprotein preparations (Bezkorovainy, 1965, 1967) may now be explained on the basis of differences in the average number of carbohydrate chains per protein molecule in these preparations. The O-glycoside bond linking the oligosaccharide chain to the polypeptide chain cannot be considered to be a very strong one, compared to the carbohydrate-polypeptide linkage in such proteins as orosomucoid, and it is conceivable that many such chains are split from the native glycoprotein during handling procedures or by bacterial or intrinsic glycosidases.

It is clear from our study that a component similar to the whey M-1 glycoprotein exists in the acid casein fraction fo colostrum. This component may arise in casein via specific or nonspecific absorption of the whey M-1 glycoprotein, or it may be an intrinsic component of the colostrum casein complex. McKenzie (1967b) has raised the question of relationship between the whey and casein sialic acids in connection with the rather large variation of sialic acid content of individually prepared acid casein or κ-casein samples. Our work provides a partial answer to this question.

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