Attachment Sites of Carbohydrate Moieties to Peptide Chain of Bovine κ -Casein from Normal Milk

Masao KANAMORI, Noboru KAWAGUCHI, Fumio IBUKI
and Hiroshi DOI

*Department of Agricultural Chemistry, Kyoto Prefectural University,
Shimogamo, Kyoto 606, Japan*

Received January 14, 1980

The attachment site of the carbohydrate moiety to the peptide chain of normal κ -casein was investigated with κ -casein component P-6 containing the most carbohydrates. Three short glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂ were prepared by cyanogen bromide cleavage and digestion of proteases (pronase P and thermolysin). Glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂ corresponded to residues 128~139 (Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val), residues 128~132 (or 127~131) and residues 135~139 of κ -casein A, respectively, and contained threonine and/or serine, but not asparagine. Glycopeptide 6-IB₁ was considered to have three carbohydrate chains because it contained three galactosamine residues. The results of alkali treatment of 6-IB₁ under reduction condition excluded serine residue as the binding site, and confirmed the existence of three binding sites in the carbohydrate moieties. The carbohydrate moiety was shown to attach to threonine residue No. 131 from analysis of 6-IB₂₋₁ and to threonine residue No. 135 (or 136) from analysis of 6-IB₂₋₂. It was concluded that the carbohydrate chains attached to threonine residues No. 131, 133 and 135 (or 136).

κ -Casein is a sole glycoprotein in casein components of cow milk. It is reported by many workers^{1~5)} that κ -casein is heterogeneous. In our previous paper,⁶⁾ the heterogeneity of κ -casein due to the carbohydrate moiety was confirmed and some properties of κ -casein components were investigated. κ -Casein plays a major role in the stabilization of casein micelles in the presence of calcium ion.⁷⁾ However, the function of the carbohydrate moiety of κ -casein is not clear. In order to make clear the role of the carbohydrate moiety, various experiments have been carried out in our laboratory^{8,9)} and others.^{2,10)}

Two major structures of the carbohydrate portion were indicated by Fournet *et al.*¹¹⁾ and one of the structures was confirmed by Saito *et al.*¹²⁾ The sugar chain of κ -casein was composed of *N*-acetylneuraminic acid, D-galactose and *N*-acetylgalactosamine.^{11~13)} Every carbohydrate structure reported had one mol of galactosamine in a molecule.^{11~13)} Wheelock and Sinkinson,¹⁴⁾ however, reported that man-

nose was contained in κ -casein. It is said that other carbohydrate chain exists in κ -casein.¹¹⁾ Therefore, the structure of carbohydrate chain from normal κ -casein is not established.

Also, the attachment site to the peptide chain of the carbohydrate chain is not clarified. Jollès and his coworkers have studied on the linkage between carbohydrate portion and peptide.^{15~19)} It was reported by the same group that polysaccharide linked to threonine residue No. 131 or 133.^{16,19)}

The κ -casein components containing much more carbohydrate have been obtained by DEAE-cellulose chromatography of κ -casein in our laboratory,⁶⁾ and this means the presence of much more residues as the binding site. κ -Casein component P-6 has each three mol of sialic acid and galactosamine.⁶⁾ This fact suggests that κ -casein component P-6 has three carbohydrate chains, considering the structure of carbohydrate portion supported by Fournet *et al.*¹¹⁾ In this paper, the attachment site of carbohydrate moiety is in-

vestigated using κ -casein component P-6.

MATERIALS AND METHODS

Materials. Pronase P was a product of Kaken Chemical Co. (Tokyo). Thermolysin (3 $\times$ cryst.) was purchased from Nakarai Chemical Co. (Kyoto). Carboxypeptidase P purified from *Penicillium janthinellum* was obtained from Protein Research Foundation (Osaka). Precoated thin layer plate (silica gel 60 F₂₅₄) was from Merck, Darmstadt, West Germany. All other reagents were of special or reagent grade.

Preparation of κ -casein component P-6. κ -Casein component P-6 was prepared from whole acid casein from an individual Holstein cow milk according to the procedure described in our previous paper.⁶⁾

Preparation of short glycopeptides from κ -casein component P-6. In order to obtain short glycopeptides from component P-6, the cleavage of peptide bond with cyanogen bromide and the digestion by pronase P and thermolysin were carried out. Sephadex G-50 (fine), Dowex 50W $\times$ 2 and Bio-Gel P-2 were used to fractionate glycopeptides (Scheme I). Aliquots were analyzed manually with ninhydrin reagent after alkaline hydrolysis²⁰⁾ and with phenol-sulfuric acid.²¹⁾

SCHEME I.

i) **Cleavage of κ -casein component P-6 with cyanogen bromide.** Component P-6 was dissolved in 70% formic acid at a concentration of 1 g per 100 ml. A 500-fold excess of crystalline cyanogen bromide to methionyl residue was added, the flask was tightly stoppered, and the reaction was allowed to proceed at 25°C for 20 hr. After the cyanogen bromide cleavage, the reaction mixture was diluted approximately 10-fold with distilled water, and lyophilized to complete dryness. Lyophilized sample (200 mg) was resolved in the eluent (formic acid-acetic acid-water = 25:87:888) and applied on a Sephadex G-50 (fine) column (4 $\times$ 93 cm), and then eluted with the same eluent.

ii) **Pronase P and thermolysin digestion of long glycopeptide 6-I obtained after CNBr cleavage of κ -casein component P-6.** Hydrolysis of long glycopeptide 6-I (120 mg) by pronase P and thermolysin was carried out in 4.5 ml of 20 mM Tris-HCl buffer, pH 8.0, at 37°C (pronase P-thermolysin-substrate = 1:1:100, w/w). After 24 hr, the same amounts of pronase P and thermolysin were added and hydrolysis was allowed to proceed for additional 24 hr. Digestion was stopped by the addition of 1 N HCl to pH 2, and all reaction mixture (5.0 ml) was applied on a column of Dowex 50W $\times$ 2 (2.2 $\times$ 40 cm) bufferized with IPH-DL (0.2 M sodium citrate-HCl buffer, pH 2.2, containing 0.17 M β -thiodiglycol and 1% BRIJ-35). Elution was carried out with each 150 ml of IPH-DL, IPH-1 (0.2 M sodium citrate-HCl buffer, pH 3.25, containing 0.04 M β -thiodiglycol, 1.7 M ethyl alcohol and 1% BRIJ-35), IPH-2 (0.2 M sodium citrate-HCl buffer, pH 4.25, containing 0.04 M β -thiodiglycol and 1% BRIJ-35) and IPH-4 (0.35 M sodium citrate-HCl buffer, pH 5.28, containing 0.046 M benzyl alcohol and 1% BRIJ-35), which were used in amino acid analysis. Ninhydrin and phenol-sulfuric acid reactions positive peaks were lyophilized and applied on a Bio-Gel P-2 column (2.0 $\times$ 150 cm) for desalination.

Chemical analyses. Amino acid composition was determined after acid hydrolysis in 6 N HCl for 22 hr at 110°C in sealed tube under vacuum. The determination of amino sugar was performed with a Hitachi amino acid analyzer, model KLA-5, after acid hydrolysis in 2 N HCl for 3 hr at 100°C.

Subsequential release of amino acids by carboxypeptidase P. Ten mg of glycopeptide from which the sugar moiety was removed by acid hydrolysis (2 N HCl, 100°C, 1 hr) was incubated with carboxypeptidase P in 0.6 ml of 50 mM sodium acetate buffer, pH 5.2, containing norleucine as the internal standard at 37°C. After 0.5, 1, 2 and 4 hr, 0.1 ml of the mixture was taken out. One tenth ml of 3 N NaOH was added to stop the reaction and solution was allowed to stand for 30 min. This mixture was adjusted to pH 2.2 with 3.5 N HCl and diluted with IPH-DL. Detection of amino acids released by carboxypeptidase P was performed with an amino acid analyzer.

Alkali treatment of glycopeptide in the presence of NaBH₄. Four mg of glycopeptide were dissolved in 0.6 ml of 0.1 N NaOH/1 M NaBH₄ and the mixture was incubated at 37°C. After 0, 4, 8 and 18 hr, 0.1 ml of aliquots was taken out and neutralized with 1 M acetic acid. Amino acid determination of these samples was carried out according to the procedure described in *chemical analyses*.

Thin layer chromatography. In order to monitor the purity of glycopeptide, thin layer chromatography was performed in the solvent ethylacetate-pyridine-water-acetic acid (5:5:3:1, v/v).²²⁾ Glycopeptide was detected

with ninhydrin reagent.

RESULTS

Preparation of short glycopeptides from κ -casein component P-6

Acidic conditions are necessary for the cleavage reaction of methionyl peptide bonds in protein with cyanogen bromide.²³⁾ Depending on the conditions of the reaction, cyanogen bromide may cleave essentially all the methionyl peptide bonds.²⁴⁾ Three different peptides are expected from component P-6 treated with cyanogen bromide, because each κ -casein component contains two methionyl residues in a molecule.⁶⁾ The Sephadex G-50 (fine) gel filtration pattern of component P-6 cleaved with cyanogen bromide is shown in Fig. 1. Three ninhydrin reaction positive peaks occurred as expected, while one major peak eluted first was detected only by phenol-sulfuric acid reaction. Glycopeptide 6-I might form a large molecule because of self-association. It is considered that almost all methionyl bonds were cleaved by cyanogen bromide. The first peak was pooled and

FIG. 1. Sephadex G-50 (fine) Gel Filtration Pattern of κ -Casein Component P-6 Cleaved with Cyanogen Bromide.

Two hundred mg of κ -casein component P-6 cleaved with cyanogen bromide were dissolved in 5 ml of the eluent and applied to a Sephadex G-50 (fine) column (4 $\times$ 93 cm). A flow rate was maintained at 38 ml/hr. One tube contained 8.5 ml.

FIG. 2. Dowex 50W $\times$ 2 Ion Exchange Chromatogram of Long Glycopeptide 6-I Hydrolyzed by Pronase P and Thermolysin.

Five ml of reaction mixture containing 120 mg of long glycopeptide 6-I hydrolyzed by pronase P and thermolysin were applied to a Dowex 50W $\times$ 2 column (2.2 $\times$ 40 cm). A flow rate was maintained at 54 ml/hr. One tube contained 5 ml.

lyophilized. A hundred and twenty mg of long glycopeptide 6-I were obtained from 200 mg of component P-6 by this gel filtration chromatography.

After glycopeptide 6-I was digested by pronase P and thermolysin in order to shorten the peptide chain, the reaction mixture was applied on a Dowex 50W $\times$ 2 column. Figure 2 shows the Dowex 50W $\times$ 2 ion exchange chromatogram of glycopeptide 6-I hydrolyzed by pronase P and thermolysin. The phenol-sulfuric acid reaction positive peaks 6-ID₁ and 6-ID₂ were pooled and lyophilized. Short glycopeptides 6-ID₁ and 6-ID₂ were dissolved in a small amount of water and were applied on a column of Bio-Gel P-2, individually. The gel filtration profiles were automatically monitored by absorbance at 220 nm with a flow cell attached to a spectrophotometer. Figures 3 and 4 show the profiles of 6-ID₁ and 6-ID₂, respectively.

Glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂

FIG. 3. Bio-Gel P-2 Gel Filtration Pattern of Glycopeptide 6-ID₁.

Glycopeptide 6-ID₁ fraction was concentrated to 3 ml and applied to a Bio-Gel P-2 column (2 × 150 cm). Water was used as the eluent. A flow rate was 12 ml/hr. One tube contained 4 ml.

FIG. 4. Bio-Gel P-2 Filtration Pattern of Glycopeptide 6-ID₂.

Experiment was carried out as described in Fig. 3.

were obtained by the gel filtration of 6-ID₁ and 6-ID₂. These glycopeptides were subjected to thin layer chromatography individually. Only one spot was recognized by the ninhydrin reagent in each glycopeptide. Therefore, glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂ were considered to be homogeneous.

Identification of glycopeptides from κ -casein component P-6

In order to obtain the information on the location of glycopeptides in κ -casein, amino

TABLE I. AMINO ACID AND AMINO SUGAR COMPOSITIONS OF GLYCOPEPTIDES FROM COMPONENT P-6

Constituent	6-IB ₁	6-IB ₂₋₁	6-IB ₂₋₂
Thr	4.2	1.1	1.7
Ser	1.3	1.1	—
Glu	2.1	0.7	0.8
Pro	2.0	0.7	—
Gly	1	1	—
Ala	1.2	—	1
Val	0.8	—	0.9
GalNH ₂	3.0	0.9	0.9

The number of residue was calculated based on one residue of glycine or alanine.

acid and amino sugar analyses, Edman degradation and carboxypeptidase P treatment of each glycopeptide were carried out.

Table I shows the amino acid composition and amino sugar contents of glycopeptides from component P-6. The primary structure of κ -casein has been established by Mercier *et al.*²⁵⁾ Considering the amino acid composition, it was concluded that these glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂ corresponded to the residues 128 ~ 139 (Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val), residues 128 ~ 132 (Gly-Glu-Pro-Thr-Ser) [or 127 ~ 131 (Ser-Gly-Glu-Pro-Thr)] and residues 135 ~ 139 (Thr-Thr-Glu-Ala-Val) of κ -casein A, respectively. There is no other fragment corresponding to these amino acid compositions of glycopeptides 6-IB₂₋₁ and 6-IB₂₋₂ in κ -casein A. In order to find the N- and C-termini of glycopeptide 6-IB₁, this glycopeptide was subjected to Edman degradation²⁶⁾ and carboxypeptidase P treatment. The N-terminus of glycopeptide 6-IB₁ was proved to be glycine residue by Edman degradation. Figure 5 shows the release of amino acids by the treatment with carboxypeptidase P from glycopeptide 6-IB₁ from which the sugar moiety was removed. No amino acid was released from untreated glycopeptide 6-IB₁ by the treatment with carboxypeptidase P. However, by removing the sugar part by

FIG. 5. Release of Amino Acids by Treatment with Carboxypeptidase P from Glycopeptide 6-IB₁ from which Sugar Moiety was Removed.

Experiment was carried out as described in MATERIALS AND METHODS.

limiting acid hydrolysis (2 N HCl, 100°C, 1 hr), valine, alanine, glutamic acid and threonine were sequentially released from C-terminus corresponding to the residues 128~139 of κ -casein A.

Glycopeptide 6-IB₁ contained three molecules of galactosamine (Table I). On the contrary, only one molecule of amino sugar was contained in glycopeptide 6-IB₂₋₁ or 6-IB₂₋₂. These facts suggest that glycopeptide 6-IB₁ has three sugar chains in a molecule, and that glycopeptides 6-IB₂₋₁ and 6-IB₂₋₂ have one carbohydrate chain, considering from the carbohydrate composition indicated by Fournet *et al.*,¹¹⁾ Saito *et al.*¹²⁾ and Tran and Baker.¹³⁾

Alkali treatment of glycopeptide 6-IB₁ in the presence of NaBH₄

In order to determine the amino acids bound to carbohydrate chain, glycopeptide 6-IB₁ was treated with alkali. The amino acid bound O-glycosidically to carbohydrate chain is converted to keto acid accompanied by the release of sugar chain under the alkaline condition.²⁷⁾ In the case of serine and threonine, these amino acids are converted to alanine and α -aminobutyric acid by the reduction of keto acid in the presence of sodium borohydride, respectively. The glycopeptide

FIG. 6. Effect of Alkali Treatment under Reducing Condition on Glycopeptide 6-IB₁.

Experiment was carried out as described in MATERIALS AND METHODS.

●—●, Thr; □—□, Ala; △—△, Ser; ●—●, Gly; ○—○, α -aminobutyric acid.

was incubated in 0.1 N NaOH/1 M NaBH₄ at 37°C for several hours. Figure 6 shows the effect of alkali treatment. Before this treatment, the glycopeptide contained 4 residues of threonine and one residue of serine, but aspartic acid was not contained. The number of serine and alanine residues was not changed after the treatment for 18 hr. On the other hand, the number of threonine decreased in proportion to the passage of time in alkaline solution, and only one residue of threonine remained after 18 hr. After 8 hr, 0.8 residue of α -aminobutyric acid was recovered in a molecule, and 1.4 residues were after 18 hr. Forty six % of decreased threonine were recovered as α -aminobutyric acid. This low recovery might be caused by incomplete reduction.²⁸⁾ The results of alkali treatment suggested that the amino acid bound to sugar chain was only threonine, and that three sugar chains existed in the glycopeptide 6-IB₁.

DISCUSSION

In the case of glycoprotein, it is important to know its carbohydrate structure and attachment site for the investigation on its function

and biosynthesis. Now, there are many studies on the carbohydrate structure,^{29,30)} but that on the attachment position is a few compared with the studies on the sugar part and the peptide chain. In general, it is found that O-³¹⁾ and N-glycosidic³²⁾ linkages are present as the linkage between carbohydrate and peptide chain in glycoprotein.

Studies on the binding site of sugar chain of κ -casein have been reported by Jollès and his coworkers.¹⁵⁻¹⁹⁾ First, it is reported by Fiat *et al.*¹⁵⁾ that an O-glycosidic linkage was characterized between a residue of threonine and N-acetylgalactosamine. The amino acid sequence of glycopeptide indicated in their report¹⁵⁾ was in conflict with the primary structure established by Mercier *et al.*²⁵⁾ Then, the report was presented that carbohydrate chain linked to threonine residue No. 131 or 133,^{16,19)} and that threonine residue No. 131 seemed to be most probable residue involved the sugar linkage.¹⁷⁾ On the other hand, threonine residue No. 133 was considered to be the attachment site of polysaccharide in their report proposed the structure of the carbohydrate portion.¹¹⁾ Thus, the location and structure of the carbohydrate moiety have not been established precisely.

N-Glycosidic linkage is stable against alkali treatment. On the contrary, O-glycosidic linkage is labile in alkaline solution, and carbohydrate moiety releases from peptide chain. Threonine and serine residues bound to carbohydrate chain are converted to α -amino-butyric acid and alanine under the reducing condition, respectively. It is reported by Tran and Baker that carbohydrate moiety attached to the peptide chain through the OH group of serine or threonine, or both.¹³⁾ Jollès *et al.*¹⁵⁾ ruled out that carbohydrate moiety bound to serine in κ -casein from the increase of absorbance at 241 nm in alkaline solution. But it is not sufficient to exclude the bond of carbohydrate chain with serine residue from this result.

In order to clarify the binding site of carbohydrate chain, various short glycopeptides 6-IB₁, 6-IB₂₋₁ and 6-IB₂₋₂ were prepared from κ -casein component P-6, which

contained most carbohydrate moiety. As glycopeptide 6-IB₁ did not contain asparagine residue, N-glycosidic linkage did not exist in the glycopeptide. It was confirmed from our result of alkali treatment under the reducing condition that all carbohydrate moieties attached to threonine residues (Fig. 6).

The results of amino acid and amino sugar analyses (Table I), Edman degradation, carboxypeptidase P treatment (Fig. 5) and alkali treatment (Fig. 6) of glycopeptide 6-IB₁ indicated that three carbohydrate chains linked to three threonine residues out of four residues No. 131, 133, 135 and 136 of κ -casein A. Glycopeptides 6-IB₂₋₁ and 6-IB₂₋₂ corresponded to the residues 128~132 (or 127~131) and the residues 135~139 of κ -casein A. Glycopeptide 6-IB₂₋₁ contained each one residue of galactosamine and threonine. This shows that one carbohydrate chain binds to threonine residue No. 131. Glycopeptide 6-IB₂₋₂ had one residue of galactosamine and two residues of threonine in a molecule. It is considered from these facts that one carbohydrate chain attached to threonine residue No. 135 or 136. Considering from the informations regarding glycopeptides 6-IB₂₋₁ and 6-IB₂₋₂, the other carbohydrate chain binds to threonine residue No. 133. These are summarized in Fig. 7.

FIG. 7. Glycopeptides from κ -Casein Component P-6.

Jollès *et al.*¹⁶⁾ reported that the attachment site of polysaccharide could not be the threonine residue No. 135. However, plural attachment site must exist in κ -casein, considering from the fact that the heterogeneity of κ -casein is due to the carbohydrate moiety.³³⁾ Jollès *et al.* might suggest only one threonine residue No. 131 or 133 as the binding site of polysaccharide chain, because they used sugar

poor κ -casein. It is concluded from our results that carbohydrate chains of κ -casein attach threonine residues No. 131, 133 and 135 (or 136). Genetic variant B of κ -casein has plural carbohydrate chains.^{3,33)} The possibility may be small that threonine residue No. 136 is the binding site of sugar chain, because residue No. 136 of κ -casein B is isoleucine.

REFERENCES

- 1) A. G. Mackinlay and R. G. Wake, *Biochim. Biophys. Acta*, **93**, 378 (1964).
- 2) A. G. Mackinlay and R. G. Wake, *ibid.*, **104**, 167 (1965).
- 3) J. H. Woychik, E. B. Kalan and M. E. Noelken, *Biochemistry*, **5**, 2276 (1966).
- 4) J. Pujolle, B. Ribadeau-Dumas, J. Garnier and P. Pion, *Biochem. Biophys. Res. Comm.*, **25**, 285 (1966).
- 5) H. J. Vreeman, P. Both, J. A. Brinkhuis and Corry van der Spek, *Biochim. Biophys. Acta*, **491**, 93 (1977).
- 6) H. Doi, F. Ibuki and M. Kanamori, *J. Dairy Sci.*, **62**, 195 (1979).
- 7) D. F. Waugh and P. H. von Hippel, *J. Am. Chem. Soc.*, **78**, 4576 (1956).
- 8) H. Doi, N. Kawaguchi, F. Ibuki and M. Kanamori, *J. Nutr. Sci. Vitaminol.*, **25**, 33 (1979).
- 9) H. Doi, F. Ibuki and M. Kanamori, *Agric. Biol. Chem.*, **43**, 1301 (1979).
- 10) L. K. Creamer, J. V. Wheelock and D. Samuel, *Biochim. Biophys. Acta*, **317**, 202 (1973).
- 11) B. Fournet, A.-M. Fiat, C. Alais and P. Jollès, *ibid.*, **576**, 339 (1979).
- 12) T. Saito, T. Ito and S. Adachi, Abstracts of Papers, Annual Meeting of Agricultural Chemical Society of Japan, Tokyo, April, 1979, p. 276.
- 13) V. D. Tran and B. E. Baker, *J. Dairy Sci.*, **53**, 1009 (1970).
- 14) J. V. Wheelock and G. Sinkinson, *J. Dairy Res.*, **40**, 413 (1973).
- 15) A.-M. Fiat, C. Alais and P. Jollès, *Eur. J. Biochem.*, **27**, 408 (1972).
- 16) J. Jollès, F. Schoentgen, C. Alais, A.-M. Fiat and P. Jollès, *Helv. Chim. Acta*, **55**, 2872 (1972).
- 17) J. Jollès, A.-M. Fiat, C. Alais and P. Jollès, *FEBS Lett.*, **30**, 173 (1973).
- 18) P. Jollès, *Mol. Cell. Biochem.*, **7**, 73 (1975).
- 19) B. Fournet, A.-M. Fiat, J. Montreuil and P. Jollès, *Biochimie*, **57**, 161 (1975).
- 20) C. H. W. Hirs, *Method Enzymol.*, **11**, 325 (1967).
- 21) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 22) J. Humphries, Y. F. Wan, G. Fischer and K. Falters, *Biochem. Biophys. Res. Comm.*, **57**, 675 (1974).
- 23) E. Gross and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).
- 24) H. J. Chahnmann, Ruth Arnon and Michael Sela, *J. Biol. Chem.*, **241**, 3247 (1966).
- 25) J.-C. Mercier, G. Brignon and B. Rebadeau-Dumas, *Eur. J. Biochem.*, **35**, 222 (1973).
- 26) S. Iwanaga, P. Wallen, N. J. Gröndahl, A. Henschen and B. Blombäck, *ibid.*, **8**, 189 (1969).
- 27) A. Gottschalk, "Glycoproteins," ed. by A. Gottschalk, Elsevier, Amsterdam, 1972, p. 470.
- 28) B. Anderson, M. Seno, P. Sampson, J. G. Riley, P. Hoffman and K. Meyer, *J. Biol. Chem.*, **239**, PC2716 (1964).
- 29) M. D. G. Oates, A. C. Rosbottom and J. Schrager, *Carbohydr. Res.*, **34**, 115 (1974).
- 30) J. F. Codington, K. B. Linsley, R. W. Jeanloz, T. Irimura and T. Osawa, *ibid.*, **40**, 171 (1975).
- 31) H. D. Hill, Jr., M. Schwyzler, H. M. Steinman and R. L. Hill, *J. Biol. Chem.*, **252**, 3799 (1977).
- 32) T. Yamauchi, M. Makino and I. Yamashina, *J. Biochem. (Tokyo)*, **64**, 683 (1968).
- 33) H. Swaisgood, *J. Dairy Sci.*, **58**, 583 (1975).