Studies on Human α_s - and κ -Casein Fractions and Human Caseinoglycomacropeptide

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1. Fractions have been obtained from human whole casein closely resembling the α_s - and κ -fractions of cow casein. 2. The α_s -fraction (human α_s -casein) is calcium-sensitive, heterogeneous in zone analysis and inert towards rennin. 3. The κ -fraction (human κ -casein) is calcium-insensitive, heterogeneous in zone analysis, and forms a soluble glycopeptide when acted upon by rennin. 4. Human κ -casein stabilizes human α_s -casein in the presence of Ca^{2+} ions. 5. The glycopeptides released by rennin from human casein and from cow casein have been compared. There are important differences in both the peptide and non-peptide structures of the two compounds. 6. In both human and bovine glycopeptides some of the carbohydrate residues are joined to the peptide by *O*-glycosidic links with threonine, and possibly with serine.

It is well established that the rennin-sensitive fraction of cow casein referred to as κ -casein (Waugh & Hippel, 1956), has the important function of stabilizing the casein micelle in the presence of Ca^{2+} ions and so preventing micelle precipitation. More specifically it is the calcium-sensitive, or α_s -fraction of the micelle (Waugh, 1961), that is involved with κ -casein in this stabilizing action (for problems of nomenclature see Lindqvist, 1963). The stabilizing power is lost when glycopeptide is split from the κ -fraction in the micelle by rennin action. It seems probable that the rennin-sensitive part of human casein serves a similar basic role in maintaining micelle stability in human milk, but the nature of the human-casein micelle is less well understood and hitherto no satisfactory fractionation of human casein has been reported to enable the properties of its different components to be studied.

In addition to its rennin-sensitivity and its stabilizing function, cow κ -casein has other distinguishing properties which by analogy might be reproduced in its human equivalent: the glycopeptide which is split from it by the action of rennin is identical with the glycopeptide prepared by the action of rennin on whole cow casein in its amino acid composition, though differences were noted in the quantitative carbohydrate analyses of the two compounds (Jollès, Alais & Jollès, 1961; Alais & Jollès, 1961); unlike other casein subfractions it is not precipitated by Ca^{2+} ions, but flocculates after the addition of rennin even in the absence of calcium (Payens, 1961); it is relatively richer in neutral

sugar, hexosamine and sialic acid than whole casein or other subfractions of the casein aggregate (Alais & Jollès, 1961) and shows weakly staining and very diffuse properties in electrophoretic analysis (Wake & Baldwin, 1961; Woychik, 1965).

A fraction has been obtained from whole human casein which resembles κ -casein in all these respects: it is stable in the presence of calcium, but shows an increasing turbidity, and ultimate precipitation, when acted upon by rennin in the absence of Ca^{2+} ; it has a higher carbohydrate content than whole human casein, and is not associated with any of the stronger bands that are characteristic of human casein subjected to electrophoresis (Malpress & Hytten, 1964). A glycopeptide is formed from it which is indistinguishable in its amino acid content from the glycopeptide made by the action of rennin upon human whole casein; and most important of all from the point of view of definition it has the power of stabilizing the calcium-sensitive fraction of the human casein micelle. This calcium-sensitive fraction of human casein has also been isolated and shows properties in general accord with cow α_s -casein.

The present paper reports on the preparation and properties of human α_s - and κ -casein fractions, and presents some comparative data on the caseinoglycopeptides released by the action of rennin from human and bovine casein.

METHODS

Preparation of casein. Human casein was prepared by the method of Malpress & Hytten (1964); the precipitation of casein from the skim milk was improved by diluting the milk

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with twice its volume of water before adjusting to pH 4.7; the milks were then immediately warmed to 25°, without any preliminary overnight cooling. In the present paper 'casein' refers to samples prepared from milk from a fully established lactation, giving the characteristic six-band pattern in starch-gel electrophoresis. Samples which do not come within this definition will be specifically noted as 'immature'.

Cow casein was prepared from fresh skim milk by adding N-HCl with stirring to pH 4.7. The precipitated casein was centrifuged and washed twice with 0.01 M-acetate adjusted to pH 4.7 with N-NaOH. The washed precipitate was dissolved in water with the addition of minimum amounts of 0.1 N-NaOH, the pH at all stages being kept below 10, and reprecipitated at pH 4.7 from a volume three times as great as the original milk volume; the final casein precipitate was washed twice with ethanol-ether (1:1, v/v), twice with ether, and dried in air.

Fractionation of human casein in urea solutions. Human casein (1 g.) was dissolved in 10 ml. of 0.1 N-NaOH containing 4 M-urea and the solution was brought to pH 5 with N-acetic acid. An equal volume of acetic acid-sodium acetate buffer (pH 5.0, 10:2) containing 4 M-urea was added and the solution (approx. 30 ml.) applied to a column (40 cm. $\times$ 2.5 cm.) of CM-Sephadex C-50 equilibrated in acetate buffer (pH 5.0, 10:1) containing 4 M-urea. Fractions were eluted from the column by the successive use of acetic acid-sodium acetate buffer (pH 5.0), phosphate buffer (KH_2PO_4 - Na_2HPO_4 ; pH 7.35) and tris-HCl buffer (pH 8.2), all with ionic strength equal to 0.1 and containing 4 M-urea. Protein was detected by measuring $E_{280 \text{ m}\mu}$. Fraction A2, which was the largest obtained from mature milk, precipitated readily when dialysed against several changes of dilute acetate buffer (0.01 M; pH 4.7). The precipitate was collected, washed twice with ethanol-ether (1:1, v/v), twice with ether, and dried in air. A further precipitate, obtained when the dialysed solution was left at 0° for some days, was added to the main sample. Fractions A1 and B were dialysed against water, pervaporated in the dialysis sac in a current of air to a volume of 40 ml. and freeze-dried.

Preparation of glycomacropeptides. Crystalline rennin (Sigma Chemical Co., St Louis, Mo., U.S.A.) was added to an unbuffered solution of casein (2-4%, pH 6.7), or human casein fraction B (1%), to give a final enzyme concentration 1-2 mg./100 ml. After incubation for 30 min. at 37° enzyme activity was stopped by the addition of an equal volume of 24% trichloroacetic acid. The solution was centrifuged at 35000 g at 10° for 15 min. and the clear supernatant extracted three times with an equal volume of ether. The aqueous layer was then neutralized and the residual ether was removed by aeration at room temperature; the solution was dialysed for 24 hr. against several changes of distilled water, and the volume was reduced to about 10% of the volume of the initial casein solution by pervaporation.

The concentrated solution was applied to a column (24 cm. $\times$ 1.5 cm.) of DEAE-Sephadex A-25 (medium grade) equilibrated in tris-HCl buffer (pH 7.8, 10:01) containing 0.1 M-NaCl. Elution was carried out in the same buffer by stepwise increments in the concentration of NaCl (0.1-0.4 M). Fractions absorbing at 215 m μ were collected, samples within the same peak being bulked, dialysed against distilled water and pervaporated to about 50 ml. before freeze-drying.

Hydrolysis with trypsin. Human glycopeptide (200 mg.), or 100 mg. of cow glycopeptide, dissolved in 10 ml. of

0.01 M-ammonium carbonate (pH 8.5) was incubated at 37° for periods varying from 2 to 10 hr. with 2 mg. of crystalline trypsin (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.), which had been initially dialysed against mN-HCl at 0° and subsequently freeze-dried. The incubated solution was immediately applied to a column (24 cm. $\times$ 1.5 cm.) of DEAE-Sephadex A-25 (medium grade) prepared in water and eluted with ammonium carbonate solutions of increasing concentration (0.025-0.25 M; pH approx. 9.2). Fractions containing material absorbing at 230 m μ were collected, and samples within the same peak were bulked and dried *in vacuo* over phosphorus pentoxide. Water was added to the residues and the drying was repeated several times until all ammonium carbonate had been removed. The lysine-containing fractions obtained in this way proved to be mixtures of peptides and were treated further in one, or both, of the following ways: (a) they were fractionated again on DEAE-Sephadex columns by elution with a more finely graded range of ammonium carbonate molarities from 0.01 to 0.1 M; (b) they were applied to columns of CM-Sephadex C-50, equilibrated in acetic acid-sodium acetate buffer (pH 3.6; 10:05), and eluted with acetate buffers (pH range 3.6-4.0; 10:05). In the latter case peptide material was identified by the ninhydrin reaction (Moore & Stein, 1954) applied directly to samples to which 4 N-sodium acetate buffer (pH 5.5) had been added to ensure an initial approx. pH 5.5.

Turbidimetry. Human casein, or human casein fractions, were dissolved in water with the aid of minimal amounts of 0.1 N-NaOH where necessary. The pH was adjusted to 5.65 with 0.1 N-acetic acid and the solution diluted to give a concentration of 2 mg. of casein or casein fraction/ml. An equal volume of acetic acid-sodium acetate buffer (pH 5.65; 10:1) was added to give a final protein concentration 1 mg./ml. To 3 ml. of this solution in a cuvette 0.1 ml. of 0.01% crystalline rennin solution was added at room temperature and the solution was mixed immediately by inversion. The increase in turbidity was measured at 390 m μ over a 10 min. period with a Unicam SP.600 absorption spectrophotometer; this period gave maximum change under our conditions. Rennin was adsorbed on to the surface of the cells and each cell was cleaned in a chromic acid cleaning mixture before re-use.

Stabilization in the presence of calcium ions. The calcium-insensitive fraction B of human casein can prevent the precipitation by Ca^{2+} of the calcium-sensitive fraction A2, a process called stabilization. Solutions of both fractions (6 mg./ml.) were prepared in the KCl-imidazole buffer system of Noble & Waugh (1965). Varying amounts (0-0.4 ml.) of the calcium-insensitive fraction were mixed in a test tube (10 cm. $\times$ 1 cm.) with a constant volume (0.5 ml.) of the calcium-sensitive fraction. All solutions were then brought to a volume of 0.9 ml. by the addition of buffer. A period of 10 min. at 37° was allowed for equilibration before the addition of 0.1 ml. of CaCl_2 solution (0.05-0.2 M). The Ca^{2+} ion concentration was constant for any given experiment. Control tubes in which the two fractions were incubated separately with or without Ca^{2+} were included in each experiment. After incubation for 60 min. at 37°, with shaking, the tubes were centrifuged at 400 g for 1 min. The supernatants were decanted, diluted with 0.05 M-sodium citrate to 3 ml., and the extinction values were read at 280 m μ .

None of the calcium-sensitive fractions used in these

experiments was completely precipitated by Ca^{2+} (see Fig. 4). A purified preparation was made by treating a solution of fraction A2 with excess of CaCl_2 (final concn. 0.1M). The precipitate obtained was dissolved in 0.05M-sodium citrate and freed from calcium by dialysis. After freeze-drying it showed a solubility in the presence of 0.01M- Ca^{2+} , under the conditions of the stabilization test, of 5%, compared with 30% for the original fraction (see Fig. 4). In calculating stabilization ratios (the ratio by weight of the calcium-sensitive fraction stabilized by unit weight of the calcium-insensitive fraction) the amount of the calcium-sensitive fraction was first corrected for any soluble content. Percentage stabilization is defined as the ratio, expressed as a percentage, of the E_{280} value of any test supernatant to the E_{280} value given by solutions in the same series of experiments in which complete stabilization was achieved and in which no precipitate was formed; both values were corrected for (a) the E_{280} absorption due to the soluble portion of the calcium-sensitive fraction, which was a constant factor in any particular experiment, (b) the E_{280} absorption of the calcium-insensitive fraction, which varied from tube to tube in any experimental series.

Amino acid analysis. Separation and quantitative analysis of amino acids was carried out by an Amino-acid Analyzer (Technicon Instruments Co. Ltd.) after hydrolysis of peptides for 16–20 hr. in 6N-HCl at 110° in N_2 . A three-column version of the instrument was used, with the buffer system recommended by Thomson & Miles (1964) for 5½ hr. chromatograms, modified for three columns according to the recommendations of Pate (1966). A standard correction of 10% was made to threonine and serine values.

Relative hexosamine values were obtained for human glycopeptide from those charts in which the glucosamine peak, which showed a rather variable point of elution, did not overlap the elution of valine.

End-group analysis. The *N*-terminal sequence of amino acids in the glycopeptides was investigated by (a) the phenylthiohydantoin method (Edman, 1950), with the paper-strip modification outlined by Fraenkel-Conrat, Harris & Levy (1955) and the recommendations given by Harris & Roos (1959) for removing excess of pyridine. The solvents developed by Sjöquist (1953) and by Edman & Sjöquist (1956) were used for the identification of phenylthiohydantoin compounds by paper chromatography; (b) the acrylonitrile method (Fletcher, 1966) with the subtractive identification of the *N*-terminal amino acid after hydrolysis and amino acid analysis (see preceding section); (c) leucine aminopeptidase: the enzyme, dissolved in tris-HCl buffer (1 mg./ml.; pH 8.5) and activated with 0.01M- MgCl_2 and 0.01M- MnCl_2 , was added in equal volume to 0.5–5 mg. of peptide dissolved in 1 ml. of tris-HCl buffer (pH 8.5). The solution was incubated at 37°. Samples (0.5 ml.) were removed at intervals up to 2 hr. and the reaction stopped by the addition of 2 ml. of ethanol. The solutions were centrifuged and the supernatants dried *in vacuo* over P_2O_5 and redissolved in 1 ml. of HCl-sucrose solution for amino acid analysis in the automatic Analyzer. *C*-terminal residues were determined by the use of carboxypeptidase A-DFP (Sigma Chemical Co.) and carboxypeptidase B (Sigma Chemical Co.). Glycopeptide (5 mg.) was dissolved in 5 ml. of water and the pH adjusted to 7.5 with 0.05N-ammonium carbonate. Carboxypeptidase A (0.5 mg.), or carboxypeptidase B (0.1 mg.), was added and the solution was incubated at 37°. Samples (1 ml.) were

removed at intervals up to 2 hr. and treated in the same way as samples from the leucine-aminopeptidase incubation.

Action of alkali on glycopeptide. Samples (1 mg.) of glycopeptide, or of fractions prepared from glycopeptide, were dissolved in 3 ml. of 0.5N-NaOH. The change in extinction at 241 m μ , at room temperature, was followed for periods up to 20 hr. in a Unicam SP.500 spectrophotometer.

Further analytical methods. Starch-gel electrophoresis was carried out by the discontinuous tris-citrate-borate buffer system of Poulik (1957) in the presence of 7M-urea as described by Wake & Baldwin (1961). Carboxymethyl-*S* derivatives of casein fractions were prepared by the method of Crestfield, Moore & Stein (1963). Neutral sugars and hexosamines were identified by paper chromatography after hydrolysing glycopeptide materials in N-HCl for varying periods and drying the solutions over NaOH pellets *in vacuo*. The solvent systems used were: ethyl acetate-pyridine-water (12:5:4, by vol.) and propan-1-ol-ethyl acetate-water (7:1:2, by vol.). Reducing sugars were detected with alkaline AgNO_3 (Trevelyan, Procter & Harrison, 1950); hexosamines by the Elson-Morgan reagent (Smith, 1958). Neutral sugars were estimated by the orcinol method (Winzler, 1955). It was found that fucose gave positive values when readings were made at 540 m μ and results have been corrected for this where necessary. Fucose was measured by the $\text{C}_4\text{R}10$ method of Dische & Shettles (1948). Hexosamine was determined by the procedure of Randle & Morgan (1955) after hydrolysis of the peptide material in 2N-HCl in sealed tubes at 100° for 3 hr. The differentiation of glucosamine and galactosamine was made by the method of Stoffyn & Jeanloz (1954). Sialic acid was determined by the thiobarbiturate method (Warren, 1959).

All dialyses were carried out in Visking tubing (24/32 in.) used without pretreatment.

RESULTS

Fractionation of human casein in urea solution. Figs. 1(a) and 1(b) show fractionation patterns of immature and mature whole human casein in 4M-urea solution and varying buffer systems on columns of CM-Sephadex C-50. The immature sample was prepared from milk collected in the first 10 days after parturition. Differences in the quantitative distribution of the peaks confirm differences in the patterns of immature and mature milks in starch-gel electrophoresis, in which the former show a very incomplete band structure (Malpress & Hytten, 1964); this corresponds to the relatively small A2 peak in the urea-fractionation pattern. Fraction A1 gave only an intensification of the boundary in starch-gel analysis. Other properties of this fraction were that it was not precipitated by calcium, gave no turbidity reaction with rennin, and had a high carbohydrate content (18.3% in the preparation from mature milk). It is not considered further in this study.

Starch-gel analysis of fraction A2 showed the characteristic band pattern of whole human casein, lacking only the 'boundary smudge' of the complete unfractionated protein (Fig. 2). In most of its

Fig. 1. Fractionation of human casein on CM-Sephadex C-50 by a three-stage buffer sequence incorporating 4 M-urea: (a) immature casein; (b) mature casein. Change of pH is indicated by the dotted line. Buffers: 1, acetate-urea; 2, phosphate-urea; 3, tris-urea.

properties this fraction resembled the α_s -fraction of cow casein: thus, like α_s -cow casein (Waugh *et al.* 1962), it contained no carbohydrate residues and no sialic acid; it was readily precipitated by calcium, but showed no increase in turbidity when treated with rennin. Fraction B, in contrast, was not precipitated by calcium, but gave a strongly positive turbidity reaction with rennin. The total carbohydrate content of different preparations of fraction B varied from 4.4 to 8.8% (hexose 2.6–4.3%; hexosamine 1.0–2.9%; fucose 0–1.0%; sialic acid 0.36–0.70%); these values, which were minimal (see the Discussion section), were of the same order as those for the whole casein from which the preparations were made (hexose 2.8%; hexos-

Fig. 2. Starch-gel electrophoresis of human casein fractions A2 and B in tris-citrate-borate buffer (pH 8.6) in the presence of 7 M-urea.

Fig. 3. Starch-gel electrophoresis in tris-citrate-borate buffer (pH 8.6) in the presence of 7 M-urea of human casein fraction B and its carboxymethyl-S derivative, before and after the action of rennin. a, Fraction B; b, fraction B after rennin; c, carboxymethyl-S B; d, carboxymethyl-S B after rennin. Band 6, normally absent, was present as a contaminant in this preparation of fraction B.

amine 1.6%; fucose 0.7%; sialic acid 0.3%). In all these respects fraction B closely resembled cow κ -casein, and this likeness was reinforced by its behaviour on starch-gel electrophoresis (Fig. 2).

The feebly staining and almost featureless electrophoretogram which it gave was changed to a

pattern of fine bands when the analysis was run in the presence of 2-mercaptoethanol; similar changes have been shown for reduced cow κ -casein (Schmidt, 1964; Woychik, 1965). This change to a discrete band structure was best observed after first converting the reduced B fraction into its carboxymethyl- S derivatives. In the most favourable runs nine bands could then be differentiated, all migrating towards the anode and all moving as fast, or faster than, band 1 of the whole casein pattern. The bands are shown in Fig. 3 together with the changed pattern given after rennin action upon the carboxymethylated B fraction, and the corresponding patterns of untreated fraction B before and after rennin action. In no case were there any bands moving towards the cathode.

Stabilization of the calcium-sensitive fraction A2. Fig. 5 shows the percentage stabilization of A2 by the calcium-insensitive fraction B, plotted against the reciprocal of the stabilization ratio, for three concentrations of Ca^{2+} ion. At each of these concentrations A2 showed some solubility (Fig. 4) and this has been taken into account in calculating the stabilization ratios. The effectiveness of fraction B decreased as the calcium concentration increased from 0.005 to 0.02M, the stabilization ratios at the point of 100% stabilization falling from 2.6 to 1.2. With different preparations of fraction A2 with solubilities in the presence of 0.01M- Ca^{2+} of 30% (Fig. 5) and 5% (Fig. 6) stabilization ratios of 1.8 and 2.5 were obtained. The efficiency of stabilization was increased by lengthening the period of equilibration before adding Ca^{2+} ; or by first converting fraction B into its reduced form

with 2-mercaptoethanol, with or without subsequent formation of the carboxymethyl- S derivative (Fig. 6). In our experiments the highest stabilization ratio obtained at the point of 100% stabilization was 6.6, using the carboxymethyl- S form. Reduced and alkylated derivatives of cow κ -casein have previously been shown to retain quantitatively the ability to stabilize cow α_1 -casein (Mackinlay & Wake, 1965; Woychik, 1965).

Fractionation of glycomacropeptides released by rennin from whole casein. It was found that peptide material absorbing at 215m μ was present in control solutions in which casein had been precipitated by trichloroacetic acid under conditions identical to those used in the glycopeptide preparation, but without the use of rennin. Satisfactory separation of the glycopeptides from the contaminating material was given by the use of anion-exchange columns, as outlined in the Methods section (Fig. 7a, b). The results showed a marked difference in the behaviour of the human and the cow materials: the former (human GlyMP; human glycomacropeptide) was eluted from the column in a single sharp peak when the concentration of sodium chloride was 0.1M; the latter was eluted at 0.1, 0.2 and 0.4M-sodium chloride, with more than one peak in each zone. The largest fraction was eluted by 0.2M-sodium chloride and is referred to in this paper as cow glycomacropeptide (cow GlyMP). An analysis of these cow fractions showed that the main difference was in their sialic acid content, which increased

Fig. 4. Solubility of human casein fraction A2 (3 mg./ml.) in the presence of increasing Ca^{2+} concentrations. ●, A2 as eluted from CM-Sephadex C-50 column. ○, A2 after purification by Ca^{2+} precipitation (see the Methods section).

Fig. 5. Stabilization of the calcium-sensitive human casein fraction A2 (unpurified, see Fig. 4; 3 mg./ml.), by the calcium-insensitive fraction B, at three Ca^{2+} concentrations: ○, 0.005M; ×, 0.01M; ●, 0.02M. The stabilization ratio for each curve at the point of 100% stabilization is shown in brackets (see the Methods section for definition).

from 2.4% in the fraction eluted by 0.1M-sodium chloride to 5.9% and 8.7% in the succeeding fractions; for the fraction eluted by 0.1M-sodium chloride there were significant differences in the amino acid composition as well. The yield of cow GlyMP in three preparations was 0.8–0.9% of the weight of casein used; in three preparations of human GlyMP from whole human casein the yield was 3.1–3.2%.

Fig. 6. Stabilization of the calcium-sensitive human casein fraction A2 (purified, see Fig. 4; 3 mg./ml.), by the calcium-insensitive fraction B, in native or reduced form. O, Native form, after 10 min. equilibration at 37°; ●, native form, after 16 hr. equilibration at room temp.; Δ, reduced form, after 16 hr. equilibration at room temp. in KCl-imidazole buffer containing 4% (v/v); 2-mercaptoethanol ▲, reduced carboxymethyl-S form, after 16 hr. equilibration at room temp. The stabilization ratio for each curve at the point of 100% stabilization is shown in brackets.

Human GlyMP, whether prepared from fraction B (Fig. 1b) or from whole casein, had the same qualitative properties. Analyses of human GlyMP and cow GlyMP are shown in Tables 1 and 2. The most notable points of difference in the amino acid composition of human GlyMP compared with cow GlyMP are the lower relative contents of aspartic acid, glutamic acid, serine and lysine, and the absence of glycine, leucine and methionine. The most extensive differences, however, are in the non-peptide part of the two glycopeptides; this forms more than half of the human GlyMP molecule when prepared from whole casein but only one-tenth of cow GlyMP. This quantitative disparity is accompanied by two qualitative differences: the occurrence of fucose and of glucosamine (as well as

Fig. 7. Fractionation of caseinoglycomacropeptides on DEAE-Sephadex by tris buffer (pH 7.8) incorporating increasing NaCl concentrations. (a) Human GlyMP; (b) cow GlyMP. -----, Control solutions (see text).

Table 1. Analysis of the non-peptide fraction of human and cow caseinoglycomacropeptides

The samples analysed were prepared as described in the text from whole human casein (three specimens) or from human fraction B (one specimen), and from whole cow casein (three specimens). Figures represent the amount of each component as a percentage of the dry weight of glycomacropeptide. The range for multiple estimations is given in parentheses.

	Human GlyMP from		Cow GlyMP from whole casein
	Fraction B	Whole casein	
Sialic acid	0.8	4.5 (4.0–5.3)	6.3 (5.6–7.4)
Hexose	6.2	25.6* (24.5–26.9)	3.1* (2.9–3.3)
Fucose	1.5	5.8 (5.0–7.3)	0
Hexosamine	3.9	19.3† (17.8–22.0)	2.2‡ (1.7–2.7)
Total non-peptide fraction	12.4	55.2 (52.2–56.9)	11.6 (10.5–12.6)

* Galactose.

† Glucosamine + galactosamine.

‡ Galactosamine.

Table 2. *Amino acid and amino sugar analysis of human and cow caseinoglycomacropeptides*

The samples analysed were the same as in Table 1. Figures represent molar ratios given to the nearest whole number.

	Human GlyMP from		Cow GlyMP from
	Fraction B	Whole casein	whole casein
Asp	2	2	5
Thr	9-10	10	11
Ser	3	3	5-6
Glu	4	4	9
Pro	8-9	9	8-9
Gly	0	0	1
Ala	4-5	5	5
Val	+	4	5
Met	0	0	1
Ile	6	5-6	5
Leu	0	0	1
Phe	0	0-1	0
Lys	2	2	3
Glucosamine	+	12	0
Galactosamine	+	6	2

Fig. 8. Fractionation of tryptic peptides prepared from human caseinoglycomacropeptide on DEAE-Sephadex eluted with ammonium carbonate (pH 9.2).

galactose and galactosamine) in the human GlyMP molecule. The glucosamine:galactosamine ratio was 2:1. The much smaller carbohydrate content found in human GlyMP prepared from fraction B (Table 1) is considered further in the Discussion.

Action of trypsin. The two lysyl peptides expected on theoretical grounds by the action of trypsin on human GlyMP were eluted from DEAE-Sephadex by ammonium carbonate in the same peak (HT1; Fig. 8). Refractionation of this mixture (see the Methods section) gave one peptide with the composition: (Pro₂Ala,Ile₂)-Lys; the second lysyl peptide was not isolated. Peaks HT2 and HT3 (Fig. 8) gave non-lysyl peptides having an identical amino acid composition: (Asp,Thr,Ser₂Glu₂Pro₄Ala₃Val₃Ile₂). The reason for the separation of these peaks is not certain, but it seems probable that small variations in the amount of carbohydrate material associated with the peptide may be responsible (HT2: hexose 33.1%, hexosamine 19.2%, sialic acid 5.9%; HT3: hexose 28.7%, hexosamine 17.6%, sialic acid 8.5%). An equivalent of eight residues of glucosamine and four residues of galactosamine were combined with the peptide, HT2.

From tryptic digests of cow GlyMP two lysyl peptides have been isolated: (Pro₂Ala,Met,Ile)-Lys and (Asp₂Glu)-Lys. These findings confirm the earlier reports of Jollès *et al.* (1961) and of Delfour, Jollès, Alais & Jollès (1965). A third lysyl peptide having the composition (Asp₂Thr,Glu,Pro,Ile,Lys) is regarded, in the light of the recent sequence studies carried out by Delfour, Alais & Jollès (1966), as a non-specific 'overlap' peptide in which the lysyl

residue does not occupy a terminal position. A large non-lysyl peptide with 39 residues was also recovered. Galactose 5.2%, galactosamine 4.6% and sialic acid 9.0% were associated with this peptide.

End-group analysis. The *N*-terminal amino acid of human GlyMP was identified by the acrylonitrile subtractive method as isoleucine; the Edman procedure failed to give unequivocal confirmation of this, but established alanine as the second residue in the *N*-terminal sequence. Leucine aminopeptidase gave no release of amino acids from human GlyMP under conditions in which isoleucine and alanine were readily released in that order from the peptide (Pro₂Ala,Ile₂)-Lys reported in the previous section. The reason why leucine aminopeptidase failed to act upon the whole glycopeptide is obscure, but the very high carbohydrate content of the whole peptide may well be an important factor. A similar explanation might be given for the failure of carboxypeptidase A to release the calculated amounts of *C*-terminal residues from human GlyMP. Only very small amounts of alanine, followed by traces of serine, were obtained. Under identical experimental conditions the *C*-terminal residue sequence for cow GlyMP was determined as -Ser-Thr-Ala-Val followed by (Val,Thr), in close agreement with the results of Jollès, Alais & Jollès (1962). The *N*-terminal residues of cow GlyMP were established as Met-Ala- by both the Edman and acrylonitrile methods, confirming the work of Delfour *et al.* (1965, 1966).

Action of alkali on human GlyMP, cow GlyMP and their tryptic peptides. The action of alkali has been

used to detect the presence of *O*-glycosidic linkages to serine and threonine residues (Tanaka, Bertolini & Pigman, 1964; Anderson *et al.* 1964; Carubelli, Bhavanandan & Gottschalk, 1965). Under these conditions acrylic acid and crotonic acid derivatives, which absorb strongly at 241m μ , are formed by

β -elimination. In the present experiments (Fig. 9*a*, *b*) the effect was greatest in the non-lysyl peptides, which contained an enhanced carbohydrate content relative to the parent glycopeptide; it was not found in the three lysyl peptides isolated, in which carbohydrate was absent. Amino acid analysis of the alkali-treated compounds showed the destruction of three threonine residues and one lysine residue in human GlyMP and of three threonine residues in its non-lysyl peptide; two threonine residues were destroyed in the non-lysyl peptide from cow GlyMP. There was also evidence of some loss of serine in the non-lysyl peptides from both sources, but in neither case was the loss sufficient to account for the complete destruction of one residue. The action of alkali upon HT1 (Figs. 8, 9*a*), which contained a mixture of lysyl peptides, suggests that the lysyl peptide which has not yet been isolated from human GlyMP may contain an *O*-glycosidic link.

DISCUSSION

The fractionation of human casein in this study has given two preparations, A2 and B, which in their most significant properties are analogous to the α_s - and κ -fractions of cow casein. In contrast with the typical starch-gel pattern of cow casein, which shows two main staining areas referred to as the α - and β -casein bands, the human pattern under the same conditions has a multi-band form. It is difficult therefore to link human and cow casein fractions by a common terminology, however desirable this might be. Fraction A2 includes all the calcium-sensitive material in human casein and in this respect is equivalent to the calcium-sensitive, or α_s - part of cow α -casein, plus the β -fraction of cow casein which is also calcium-sensitive and shows stabilizing reactions with κ -casein (Payens, 1961; Zittle & Walter, 1963). It also contains variable amounts of a form not directly precipitated by calcium (Fig. 4). There seems to be no reason at present for differentiating the human bands, all of which represent calcium-sensitive material, into α_s - and β -types; on the other hand the resemblance between the bands of fraction A2 and the seven bands of fractionated cow α_s -casein (Waugh *et al.* 1962) offers a surprisingly close parallel. We propose therefore to refer to fraction A2 as human α -casein, and to adopt the nomenclature of Waugh *et al.* (1962) for its calcium-sensitive subfractions, which will be called human α_s -caseins.

Fraction B we shall refer to as human κ -casein. Like cow κ -casein its behaviour on starch-gels suggests that it is a mixture of several forms which together make the human κ -complex. The resolution of its diffuse staining into a discrete band pattern when electrophoresis is carried out in the presence of 2-mercaptoethanol, or when the

Fig. 9. Increase in E_{241} absorption produced by the action of 0.5N-NaOH at room temp. on caseinoglycomacropeptides and products of their tryptic hydrolysis. (a) Human: A, non-lysyl tryptic peptide; B, human GlyMP; C, lysyl-peptide mixture (see text). (b) Cow: A, non-lysyl tryptic peptide; B, cow GlyMP; C, lysyl peptides.

carboxymethyl-*S* derivatives are used may indicate that the heterogeneity of human κ -casein, like that of cow κ -casein is due to the random formation of S-S bonds from a relatively few fundamental components (Mackinlay & Wake, 1964, 1965).

The state of oxidation or reduction of the cystine residues of human κ -casein appreciably affected the stabilization of α_s -casein by the κ -form. Other factors were also involved in this action, notably a mass-action relationship between κ -casein and Ca^{2+} , and the length of the equilibration period before the addition of Ca^{2+} to α_s -casein/ κ -casein mixtures. The intricacy of the kinetic relationships in stabilization experiments, and in the formation of casein micelles, has been amply demonstrated by Noble & Waugh (1965) and by Waugh & Noble (1965) for the analogous cow fractions.

The single glycopeptide fraction (human GlyMP), obtained by the action of rennin on human whole casein (Fig. 7a), gave consistent analyses for four separate preparations, though the analyses did not agree with figures reported earlier (Alais & Jollès, 1962). Qualitative, as well as quantitative, differences were found between human GlyMP and cow GlyMP in both their amino acid and carbohydrate contents (Tables 1 and 2). Alais & Jollès (1961) gave a value of 21% for the non-peptide fraction of cow GlyMP compared with 11.6% found in this study; the figure for human GlyMP was much higher, ranging from 52 to 57%. An influence of the preparative method on the carbohydrate content of human GlyMP was indicated by the lower value (12.4%) for the single sample obtained from human κ -casein. The disparity offers some justification for criticisms, put forward with reference to cow κ -casein by Beeby & Nitschmann (1963) and by Hill (1963), that the use of urea alters the κ -casein complex radically, forming a product resembling *para*- κ -casein. The studies reported here suggest that for human κ -casein the effect of urea is far less drastic: we have detected no loss of sensitivity towards rennin and no solubility changes that could suggest the conversion of our material into the *para* form. The only change has been in the amount of carbohydrate associated with the fraction; it appears that much of this can be readily removed by the urea used in the fractionation procedure without changing the general physical properties of the fraction. Our experience is therefore closer to that of Noble & Waugh (1965), who found similar micelle-forming properties and starch-gel patterns for cow κ -casein preparations made by different methods, in one of which a urea fractionation was used, and in the others avoided. The higher carbohydrate figure for human GlyMP is therefore considered by us to represent more accurately the content associated with the glycopeptide as it exists in whole casein.

The presence of fucose and glucosamine in human GlyMP, neither of which is present in cow GlyMP, is of special interest in relation to the occurrence of fucose and *N*-acetylglucosamine in various oligosaccharides which have been isolated from human milk (Kuhn, 1958). In both glycopeptides some carbohydrate is attached to the peptide chain by *O*-glycosidic links with threonine; similar links with serine may be implicated to a smaller degree.

Our failure to isolate two lysyl peptides from tryptic hydrolysates of human GlyMP prevents a definitive statement about the *N*-terminal amino acid sequence of the glycopeptide beyond the first two residues: Ile-Ala-. However, since these are the same as the *N*-terminal residues of the single lysyl peptide which we have isolated, it seems probable that this peptide forms the terminal hexapeptide in human GlyMP. The likelihood is increased by the near similarity between this hexapeptide and the *N*-terminal lysyl peptide of cow GlyMP. These compounds differ in one residue only, isoleucine for methionine, and the sequences are: human, Ile-Ala-(Pro₂Ile)-Lys; cow, Met-Ala-Ile-Pro-Pro-Lys (Delfour *et al.* 1966). This terminal peptide has special importance due to its proximity to the site of rennin action, and Delfour *et al.* (1966) in their elucidation of the *N*-terminal octadecapeptide sequence of cow GlyMP have noted an identical sequence in the corresponding sheep octadecapeptide. It is evident, however, from a consideration of the octadecapeptide sequence that, apart from the difference in the actual *N*-terminal residues themselves, the reduced number of lysine residues in human GlyMP must preclude any *N*-terminal sequence similarity of human and cow glycomacropptides beyond the decapeptide stage.

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