STUDIES ON CASEIN

A Thesis

By

Michael Edward Quinton Pilson

Submitted to the Faculty of Graduate
Studies and Research in partial
fulfilment of the requirements for
the degree of Master of Science.

McGill University
September, 1958.

ACKNOWLEDGMENTS

The author wishes to thank Professor B. E. Baker for his advice and encouragement during the course of these researches and the preparation of this report.

Mr. G. O. Henneberry provided the starting material from which the several casein fractions were isolated, and also a sample of gamma casein. For this the author is most grateful.

The author is indebted to Mr. Lincoln Reynolds for performing the carbohydrate analyses reported herain.

Thanks are also due to Dr. R. P. Harpur of the Institute of Parasitology who allowed the author to use his fraction collector.

The financial support of the National Research Council of Canada is gratefully acknowledged.

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GENERAL INTRODUCTION

Casein was first recognized as a component of milk by Scheele in 1780. For many years casein was regarded as a chemical individual remarkable among proteins for its ease of isolation in a relatively pure state, and for this reason has probably been the subject of more investigations than any other protein.

Acid precipitation of the casein in milk yields a product which contains three electrophoretically distinct components, designated alpha, beta and gamma, in order of decreasing mobility. The fourth known component, termed kappa casein, has an electrophoretic mobility close to that of alpha casein, and is not ordinarily distinguishable by free electrophoresis. Kappa casein interacts strongly with alpha casein, forming molecular aggregates. This interaction seems to be important in the stabilization of the micelles of calcium caseinate in milk. The strong interaction between alpha casein and kappa casein also makes their separation by chemical means difficult. As far as the writer is aware, no published reports have appeared describing the isolation of pure kappa casein, or any of its properties after isolation.

All the components of the casein complex contain phosphorus. The amount of phosphorus in the various components varies from 0.11 to about 1.00 per cent. There is evidence that the phosphorus is linked to the protein molecule in different ways, and that some of the phosphorus may form cross-linkages between the peptide chains.

This report deals with (a) the removal of the phosphorus from alpha casein by means of alkaline intestinal phosphatase, and the separation of the resulting mixture of long chain peptides, and (b) the separation of kappa casein from alpha casein prepared chemically, and the properties of the products of this separation.

HISTORICAL INTRODUCTION

1. Isolation and General Properties of Casein

Casein has been known since ancient times in the form of curd and cheese. In 1780, Scheele, who was the first to investigate milk systematically, recognized casein as one of the constituents of milk (1). Subsequent investigators showed that casein is the main nitrogenous constituent of milk, amounting to about 3 per cent of the weight of cows' milk and about 80 per cent of the nitrogen. The whiteness and opacity of milk is due to large colloidal aggregates of molecules of calcium caseinate. These micelles also contain a small amount of insoluble calcium phosphate.

As casein consists of several components, its composition may vary with the method used in its preparation. This situation leads to some ambiguity in the use of the term casein. It would probably be desirable to use the term casein to denote that mixture of proteins which constitutes the micelles of skim milk. Unfortunately it is not known at present whether all the commonly accepted fractions of the casein complex do actually form a part of the micelle structure. The term casein, as will be used in this report, refers to that mixture of proteins which is obtained by the method of acid precipitation. Casein prepared by other methods will be so designated, where necessary.

The method of Hammarsten is the best known of the earlier methods for the laboratory preparation of casein (2). Skim milk was diluted with four times its volume of water and the casein was precipitated by the addition of dilute acetic acid. The product was then purified by a series of reprecipitations in which the smallest possible amounts of acid and alkali were employed. After the last precipitation the casein was washed thoroughly with water. Traces of fat were removed by washing the casein with alcohol and with ether, and it was then dried over a desiccating agent. Brown (1) suggests that casein prepared by the above method may vary from batch to batch, since there is no specific recommendation made for the control of the hydrogen ion concentration. Brown also pointed out that the alcohol extraction of the wet casein may dissolve some of the alcohol soluble material (3) in casein.

Van Slyke and Bosworth (4) modified the Hammarsten method by the substitution of ammonium hydroxide for sodium hydroxide. They considered that the risk of alkali denaturation would be lessened by this procedure. An excess of ammonium hydroxide and ammonium oxalate was added to precipitate traces of calcium as the oxalate. The casein was finally precipitated with HCl, washed with alcohol and ether, and dried over concentrated sulphuric acid. Van Slyke and Baker introduced (5) a procedure in which casein was suspended in water and was dissolved by the addition of 0.1 N sodium hydroxide to the suspension, care being taken that the pH not rise above 7.0. They claimed that at this point the remaining traces of calcium and magnesium phosphates were still insoluble and in suspension, and could be removed by centrifugation. After centrifugation, acetic acid was added to the supernatant until pH 4.7 was attained, and the last traces of inorganic phosphate were removed by electrodialysis. The precipitate was then recovered by centrifugation and treated with hot neutral 70 to 80 per cent ethanol, anhydrous ethanol, anhydrous ethyl ether, and petroleum ether. This method has also been criticized by Brown (1), who questioned the wisdom of discarding all material insoluble at pH 7.0.

Brown (1) has recommended that the grain curd method for the industrial manufacture of casein be adapted for laboratory preparation by lowering the temperature of precipitation. A single precipitation from skim milk is carried out with dilute HCl, with constant stirring during the addition of the acid. The addition of acid is stopped at pH 4.6. The precipitate is washed thoroughly with water acidified to the isoelectric point of casein. The product has a low ash content, but if it is desired to further reduce the ash content, the casein is suspended in water and dissolved with the aid of dilute alkali, care being taken that the pH be kept below 7.5. The casein is reprecipitated by the addition of acid to the solution, and the precipitate washed with water. Brown claims that this procedure yields a product which is reasonably free of contaminants, and which has undergone little destructive treatment.

Rennet casein is a product which has been precipitated from milk by the action of the enzyme rennin. In the usual procedure for the preparation of this material (6), fresh skim milk is heated to 37°C. and rennet, an extract from the lining of the fourth stomach of the calf, is added. The rennin acts upon the casein in such a way that the casein is no longer soluble in the presence of calcium ions. It coagulates and precipitates. The curd is allowed to settle, washed several times with water, and dried with alcohol and ether. The product can be purified by one of the methods used for the purification of acid-precipitated casein.

Von Hippel and Waugh (7) have recently developed a method for the preparation of casein which they claim must closely resemble native casein in composition. Raw, pooled skim milk was cooled to 5°C. and centrifuged for 30 minutes at 900 g. to remove suspended matter. Sixty milliliters of 2.0 M. calcium chloride solution was added per liter of milk, and the milk was then centrifuged for 90 minutes at 45,000 g. The precipitate was washed by suspending it in dilute calcium chloride solution and was recovered by centrifugation. The calcium was removed by precipitation with potassium oxalate. The protein-containing supernatant was dialysed against water and then shell frozen in liquid nitrogen.

Casein, prepared by acid precipitation (1), is a white or yellow powdery material, practically insoluble in water, soluble with ease in dilute alkali and with difficulty in dilute acids. The isoelectric point is close to pH 4.6. The phosphorus content of 0.88 per cent places casein in the catagory of phosphoproteins. All the common amino acids, with the exception of cysteine, are present in casein, and indeed several were first discovered in hydrolysates of this protein.

The presence of a carbohydrate group in casein has been debated (8). Tillmans and Philippi (9) and Sorensen and Haugaard (10) concluded, on the basis of colorimetric methods, that casein contains a carbohydrate residue. Sorensen and Haugaard identified it as galactose, and reported the quantity as 0.31 per cent in Hammarsten casein. Tillmans and Philippi reported that the carbohydrate content dropped from 0.89 per cent after three precipitations, to 0.36 per cent after a fourth reprecipitation. Gortner (8) questioned whether this carbohydrate was an integral part of the casein molecule and suggested that more reprecipitations would further reduce the amount present.

Masamune and Maki (11) isolated, from a barium hydroxide hydrolysate of casein, a carbohydrate which they reported to be electrophoretically homogeneous. They reported that this material contained hexosamine, mannose, galactose, and seven amino acids. Stary and Cindi (12) demonstrated the presence of carbohydrate in casein samples prepared by seven different methods. The amount of carbohydrate varied from 0.3 to 2.6 per cent. Their analyses indicated that it consists exclusively of hexoses, mainly galactose, and they claimed that no hexosamine was present.

Nitschmann et al (13) isolated a glycomacropeptide which was liberated during the curdling of milk by rennet. This peptide had a molecular weight of 6,000 to 8,000, (determined in the ultracentrifuge), and contained 15.2 per cent galactose and 4.3 per cent glucosamine.

The molecular weight of casein has been the subject of much investigation (14). Svedberg et al (15) found micelle weights of 75,000 to 375,000, the weights found depending on the conditions of the determination and the method of preparation of the casein. All preparations were polydisperse. Pedersen (16) found that casein prepared by the dialysis of skim milk against phosphate buffer at pH 6.8 and with no other treatment, could be separated by ultracentrifugation into at least seven bands, which he designated $\delta, \epsilon, \zeta, \eta, \theta$ and ι. The sedimentation velocities of these bands ranged from 10.4 to about 30 x 10^{-13}, which indicated very high micelle weights. The pattern which was obtained was markedly dependant upon the conditions of ultracentrifugation employed.

Osmotic pressure measurements on solutions of casein in phenol (17) indicated a molecular weight of 25,000, while measurements made on solutions containing 6,66 M. urea (18) led to the value of 33,600.

2. Separation and Properties of the Fractions of the Casein Complex

(a) Heterogeneity of Casein.

Osborn and Wakeman (3) in 1918 extracted acid-precipitated casein with alcohol, and isolated from the extract a proteinaceous material of low phosphorus content. This was the first indication of the heterogeneity of casein. The phosphorus content of various samples ranged from 0.03 to 0.28 per cent. They also claimed to have isolated the same protein from the milk serum remaining after the casein was precipitated by acids, indicating that this protein was more soluble in acidified water than was casein. Osborn and Wakeman considered this material to be a contaminant of casein.

In 1929 Linderstrom-Lang and Kodama (19,20) published the results of an extensive series of solubility studies on casein, and concluded that it was a heterogeneous material. They prepared seven fractions differing in phosphorus content, solubility, and other properties. They did not believe, however, that their fractions were pure substances.

Many other attempts were made prior to 1939 to separate the components of casein. The methods which were used included extractions with neutral salts or sodium benzenesulphonate, the addition of acids to ammonium hydroxide solutions of casein in 70 per cent ethanol, the alternate addition of acid and acetone to alkaline ammonium chloride solutions of casein (14), and the stepwise addition of anhydrous ethanol to solutions of casein in anhydrous phenol at 70°C. or in 40 per cent aqueous urea (21). Due to the difficulty in ascertaining the degree of fractionation accomplished by each step, these attempts were unsuccessful in producing clearly definable components of the casein complex. They did, however, conclusively demonstrate that casein is a mixture of proteins.

(b) Electrophoretic Separation of the Components of Casein:

The electrophoretic method which was developed by Tiselius (22,23) in 1937 provided a sensitive and convenient method for establishing the homogeneity of a protein preparation. Mellander (24) employed this method in an examination of cows' milk and reported the presence of three components, which he designated alpha, beta and gamma in order of decreasing mobility. The alpha casein was present in the highest, and the gamma casein in the lowest concentration. He isolated the alpha and gamma casein fractions in the electrophoresis apparatus and, with the assumption that casein had 15.6 per cent nitrogen, the phosphorus contents were calculated to be 0.96 and 0.05 per cent respectively.

Warner (25) also separated casein, in a Tiselius apparatus (employing a phosphate buffer at pH 6.96), into alpha and beta caseins, but was unable to show the presence of gamma casein. He reported the values of 80.7 and 19.3 per cent respectively for the alpha and beta contentsof casein. Krejci et al (26) studied the differences in the patterns of the ascending and descending boundaries in a Tiselius apparatus, and concluded that there was complex formation between alpha and beta casein. Warner (25) confirmed this result by the determination of the mobilities of the alpha and beta fractions in both phosphate and veronal buffers, in which the relative concentrations of the two fractions were varied. He found that the mobility of the alpha casein was reduced by increasing the concentration of the beta casein, and attributed this fact to complex formation between the two fractions.

More recently, Hipp et al (27) determined the relative amounts of alpha, beta and gamma casein in the casein complex by the Tiselius method, and reported values of 75, 22 and 3 per cent respectively.

Kutacek and Kratochvil (28)and MacRae and Baker (29) have separated the three casein fractions by paper electrophoresis. Quantitative evaluation by densitometry of the stained papers gave results in agreement with those obtained in the Tiselius apparatus, if no correction was made for differences in dye-binding capacity. MacRae and Baker, however, found that the three fractions differed in the amount of dye (naphthalene black) bound by each fraction. If corrections for these differences were made, the values were 59.3, 31.1 and 9.6 per cent for the alpha, beta and gamma components respectively. These values were in agreement with Kjeldahl nitrogen determinations of the nitrogen in each band on the paper strips.

(c) Chemical Separation of the Components of Casein:

The first of the modern methods for the separation of the caseins on a preparative scale was reported (25) by Warner in 1944. He obtained, from acid-precipitated casein, two fractions which he named alpha casein and beta casein, because upon electrophoresis each gave one of the major bands found by Mellander. No gamma casein was isolated. Warner's procedure, which is frequently termed the water solubility method, is based upon the higher solubility of beta casein in water at pH 4.4 and at a temperature of 2°C. The procedure is described in detail later in this report under "Materials".

Hipp *et al* (30) reported the isolation of gamma casein. The casein in skim milk was precipitated at pH 4.5 by the addition of dilute HCl. The casein was redissolved in dilute NaOH to form a 10 per cent solution at pH 7.0. An equal volume of absolute ethanol was added slowly to the solution, after which 1.0 N. HCl was added until pH 5.7 was attained. The precipitate, which was mostly alpha and beta casein, was removed by filtration. The filtrate was cooled to 2°C. and a precipitate was formed which was rich in gamma casein. This precipitate was recovered by centrifugation and was combined with a similar precipitate obtained by a further extraction of the alpha and beta casein. The combined precipitates were dissolved in dilute alkali and the solution adjusted to a concentration of about five per cent; and the alpha and beta caseins were precipitated as before. The gamma casein recovered from the filtrate was still contaminated with some alpha and beta casein, and these were removed by isoelectric precipitation in water at pH 4.7 and 2°C. The gamma casein was recovered by precipitation at pH 5.8 and 30°C. and was electrophoretically pure.

Hipp et al (31) have reported two procedures for the preparation of the three fractions of casein which are claimed to be less tedious than Warner's procedure. The first of these is termed the alcohol solubility method. This method involves partial precipitation of the casein with ethanol and the purification of the resulting fractions by isoelectric precipitation. The solutions were never made more alkaline than pH 7.0 and ammonium hydroxide was used instead of sodium hydroxide. The second procedure is known as the urea solubility method. It is based on the high solubility of whole casein in 6.66 M. urea solution. The alpha casein was precipitated from this solution by the addition of water until the concentration of the urea was reduced to 4.6 M. Beta casein was then precipitated by further reduction of the urea concentration to 1.7 M. Gamma casein was precipitated by the addition of ammonium sulphate to the supermatant from the precipitation of beta casein. Details of this procedure are given in the section entitled "Materials" of this report.

Cherbuliez and Baudet (32) claim to have fractionated the alpha casein obtained by the procedure of Warner. They added ammonium sulphate to a solution of alpha casein and this precipitated the alpha casein. To the supernatant from this precipitation they added trichloroacetic acid; this precipitated a protein which they termed gamma casein. After removing the gamma casein they added ammonium molybdate to the supermatant and this precipitated a protein they termed delta casein. Cherbuliez and Baudet considered that delta casein was identical to the proteose of Hammarsten. They also reported that the alpha casein obtained by the ammonium sulphate precipitation could be fractionated to produce α_1 and α_2 components. Henneberry (33), however, has repeated their procedures, and he found that the subfractions were mixtures of the alpha and beta caseins.

Waugh and von Hippel (34) have recently adapted their ultracentrifugation method for the preparation of casein, (described earlier in this report), to the separation of the components of casein. A solution of the whole casein (prepared by the ultracentrifugation method), from which the calcium had been removed, was adjusted to pH 7.0 and 37° C. Enough calcium chloride solution was quickly added to make the solution 0.25 M. with respect to calcium ion. A coarse precipitate was formed. The supernatant was saved and the precipitate was treated with potassium oxalate to remove the calcium, and was then dialysed against distilled water. The electrophoretic and ultracentrifugation patterns which were obtained with this material indicated that both alpha and beta caseins were present in the precipitate. A solution of this material, however, did not form the white micelles characteristic of calcium caseinate when a small amount of calcium ion was added to the solution, but instead a precipitate formed immediately.

Examination of the supernatant saved from the precipitation of the casein with 0.25 M. calcium ion disclosed the presence of beta casein, and also of another component, which migrated at about the same rate as alpha casein in the electrophoresis apparatus, but which in the ultracentrifuge produced a pattern indicative of a fairly high degree of polymerization. The addition of solutions of this component, named kappa casein by Waugh and von Hippel, to solutions of the previously prepared alpha and beta caseins, produced a mixture in which stable micelles were formed when low concentrations (below 0.1 M.) of calcium chloride were added. High concentrations of calcium ion (about 0.25 M.) precipitated the alpha and beta caseins as before. Waugh and von Hippel believed that an interaction between the kappa casein and the alpha casein was the essential factor in the stabilization of the calcium caseinate micelles. They suggested that the original proportions of the proteins in their whole casein were 55 per cent alpha, 30 per cent beta, and 15 per cent kappa casein. They did not report the presence of gamma casein in the whole casein prepared by the ultracentrifugation method.

Waugh and von Hippel also investigated a commercial sample of acid-precipitated casein, and their results indicated that the procedure of acid precipitation gives high molecular weight complexes of marked stability. These complexes could be broken down by treatment with dilute alkali (pH 12) for a short time. When the reaction of the solution was adjusted to pH 7.0 the ultracentrifuge pattern obtained was similar to that of their whole casein. They suggested that alpha casein, as had been prepared previously by other workers, might contain kappa casein.

(d) Properties of the Casein Fractions:

Those parts of the following discussion which refer to the chemical composition of alpha casein all refer to a product which probably contained an unknown quantity of kappa casein. As far as the author is aware, no reports have appeared concerning the chemical composition of either purified alpha casein or purified kappa casein.

The three fractions of casein (Table 1) differ markedly in their content of phosphorus and several amino acids (35,36,37). The most striking differences are observed in their valine, proline, cystine, tryptophane, and tyrosine contents. Alpha casein is the only fraction containing cystine. Cysteine does not occur in casein (35,38). From a consideration of the relative amounts of a number of the constituents common to both alpha and beta caseins, Gordon, et al (35) found an average value of 69:31 for the ratio of alpha to beta casein in whole casein prepared by acid precipitation. Warner (25) had found from classical electrophoresis methods a ratio of 80:20, while Hipp, et al (30) reported a ratio of 75:22:3 for the alpha, beta and gamma caseins. The value of 3 per cent for the gamma casein content of acid-precipitated casein was consistent with data which were obtained from electrophoresis investigations.

The physical properties (Table 2) of the caseins can be correlated fairly well with their amino acid compositions (30,39,36). Alpha casein contains 291 ionic groups per 10^5 grams, compared with 219 and 168 for beta and gamma casein respectively. These differences account for the relative electrophoretic mobilities of the three preparations. The presence of a large number of phosphoric ester groups in alpha casein is noteworthy in this connection, because of their contribution to the mobility of the protein under alkaline conditions. However, the total number of non-polar groups in beta and gamma caseins is higher than in alpha casein, and this could account for the greater solubility of these two proteins in 50 per cent ethanol.

Table 1

CHEMICAL COMPOSITION OF THE CASEINS

	Alpha casein	Beta casein	Gamma casein
Nitrogen - per cent	15.53	15.33	15.40
Phosphorus - per cent	0.99	0.61	0.11
Phosphorus - moles/10^5 gm.	31.9	19.7	3.55
Amino Nitrogen - per cent	0.99	0.72	0.67
Amide Nitrogen - per cent	1.6	1.6	1.6
Amino Acids - moles/10^5 gm.			
Glycine	30.2	20.8	19.8
Alanine	42.9	22.4	25.8
Valine	53.9	14.5	89.6
Leucine	60.4	88.6	91.6
Isoleucine	41.0	41.9	33.6
Proline	65.2	130.1	140.8
Phenylalanine	27.7	35.2	35.2
Cystine	1.77	0	0
Methionine	16.8	22.9	27.4
Tryptophane	7.75	3.10	5.87
Arginine	24.9	19.7	11.0
Histidine	18.7	20.0	23.9
Lysine	61.0	44.5	42.6
Aspartic Acid	61.0	35.8	29.0
Glutamic Acid	153.0	158.0	156.0
Serine	60.0	64.8	52.6
Threonine	41.3	42.9	37.1
Tyrosine	44.8	17.7	20.3

Table 2

PHYSICAL PROPERTIES OF THE CASEINS

	Alpha casein	Beta casein	Gamma casein
Isoelectric Point - pH	4.7	4.9	5.8-6.0
Specific Rotation	-90.5°	-125.2°	-131.9°
Electrophoretic mobility (Veronal buffer-pH 8.4, μ= 0.1)	-6.75	-3.05	-2.01
Sedimentation constant (S)			
pH 12 at 4° C.	1.18	1.31	-
pH 7 at 4° C.	4.4	1.30	-
Polar Groups / 10^5 gm.	548.	465.	421.
Anionic Groups /10^5 gm.	176.	128.	(total) ( )
Cationic Groups /10^5 gm.	115.	91.	(=168)
Non-polar Groups /10^5 gm.	341.	442.	444.
Specific Volume -			
- calculated	0.725	0.743	0.749
- experimental	0.728	0.741	0.750

McMeekin, et al (39) demonstrated that values for the apparent specific volumes of alpha and beta caseins, calculated from the specific volumes of the amino acid residues, were in close agreement with values calculated from density determinations. Gordon, et al (36) reported similar agreement in the values for gamma casein.

Alpha and beta casein are both precipitated from water as coarse white flocculant materials, which can be fairly readily dried. Hipp et al (30) reported that gamma casein, however, precipitates from water in a viscous, oily state, which is not readily dehydrated. Dehydration by freeze drying produced a hard glassy product. They were able to obtain a fine white precipitate of gamma casein by dissolving the oily material in 50 per cent ethanol at room temperature and then cooling the solution to 2° C. The precipitate which formed was dehydrated with acetone and ether. Hipp et al suggest that this behaviour could be due in part to the large number of non-polar groups and the relatively small number of ionic groups which are present in gamma casein.

Striking differences in the effects of temperature on the solubilities of the three fractions in water and in 50 per cent ethanol have been reported by Hipp et al (30), from which the data in Table 3 have been taken.

Table 3

SOLUBILITY BEHAVIOUR OF THE CASEINS

Solubility as mgm. of N./100 ml.

	In H_2O			In 50% Ethanol		
Casein	pH	25°C.	2.5° C.	pH	25° C.	2.5° C.
Alpha	4.7	0.05	-	5.6	0.135	0.120
Beta	4.7	0.41	8.46	5.6	1.90	0.175
Gamma	5.95	1.0	15.6	6.2	14.5	0.88
Gamma	5.3	2.83	-			

All the values in Table 3 are approximate points of minimum solubility. Gamma casein is in all cases more soluble than alpha or beta casein. Both alpha and beta casein are more soluble in water near the freezing point than at room temperature. The reverse is true in solution in 50 per cent ethanol.

Mellon et al (40) determined the end groups of alpha and beta casein by means of the fluorodinitrobenzene technique. Hydrolysates of both the labeled casein components contained both the mono- and the di-dinitrophenyl derivatives of lysine, and the dinitrophenyl derivative of arginine. Alpha casein contained 9.3 moles of arginine and 1.3 moles of lysine end groups per 100,000 gm. of derivative (87 per cent protein). Beta casein contained 4.7 and 2.1 moles of arginine and lysine end groups respectively per 100,000 gm. of derivative (89 per cent protein). There were large losses of these amino acid derivatives during the hydrolysis of the proteins. These losses amounted to 27 per cent for dinitrophenylarginine and 82 per cent for di-dinitrophenyllysine. The large correction factors required to make allowance for these losses make the absolute quantities of the end group determinations somewhat doubtful. As far as the writer is aware, the end groups of gamma casein have not been reported.

Seno et al (41) claim to have isolated the N-terminal lysyl peptides of alpha casein and to have established the following sequence: lys.leu.val.ala.glu.asp.

MacRae et al (42) have found carbohydrate material in the alpha, beta and gamma components of the casein complex. They stained, for carbohydrate, paper electropherograms of whole casein prepared by acid precipitation, and also electropherograms of the three chemically purified fractions, and found stain on the alpha band only. When the three chemically purified fractions were analysed for the presence of carbohydrate by the anthrone reaction, however, it was found that the alpha, beta and gamma caseins contained 1.30, 0.66 and 1.59 mg. of carbohydrate per gram of protein respectively. Paper chromatography of hydrolysates made from the proteins showed the presence of galactose, glucose, and mannose in all three proteins. They also determined the quantity of hexosamine present in all three fractions, and these values are shown in Table 4.

Table 4

SUGAR AND HEXOSAMINE CONTENTS OF CASEIN SAMPLES

Casein Samples	Sugar - mg./gm.	Hexosamine - mg./gm.
Whole casein	3.38	1.98
Alpha casein	1.30	1.79
Beta casein	0.66	0.99
Gamma casein	1.59	2.06

Von Hippel and Waugh (7) investigated the molecular weights of alpha and beta casein by the method of ultracentrifugation. They adjusted a solution of the two proteins to pH 12, and then dialysed to lower the pH values. They analysed the sedimentation patterns obtained at pH 12 and during successive stages of the dialysis procedure, and found that casein is dissociated at pH 12 into particles with an average molecular weight of approximately 15,000, and that it associates progressively into larger aggregates as the pH is lowered. They considered that the weights of the monomers of alpha casein and beta casein were 13,000 to 15,000, and 15,000 to 25,000 respectively.

Later work by Waugh and von Hippel (34) showed the presence of kappa casein combined with alpha casein. Their studies with the ultracentrifuge indicated that kappa casein occurs in the ratio of approximately four alpha to one kappa. At high pH values the alpha-kappa complex was completely dissociated into monomers with a sedimentation constant $S_{20} = 1.3S$, and a molecular weight of approximately 15,000. As the pH was lowered, aggregates with a sedimentation constant $S_{20} = 7.5S$ were formed. They believed that these aggregates probably consisted of 4 molecules of alpha casein combined with 1 molecule of kappa casein. When no kappa casein was present in the solution of alpha and beta casein, the alpha casein was partially aggregated at pH 7.0 and the aggregates had a sedimentation constant $S_{20} = 4.5S$. As the pH was raised these aggregates dissociated into monomers.

Waugh and von Hippel found that at a low temperature (2^{o} C.) and at neutral pH values beta casein occurred as monomers with a sedimentation constant $S_{20} = 1.3S$. If calcium was present in the solution there was a gradual association into colloidal aggregates as the temperature was raised. Although there was some association of the alpha with the beta casein, this was not so pronounced as that of alpha casein with kappa casein.

Waugh and von Hippel found that ionic calcium had no effect on the sedimentation patterns formed by kappa casein. However, alpha casein was completely precipitated by calcium ion. The aggregate of 4 alpha to 1 kappa did not precipitate in the presence of calcium ion concentrations up to 0.2 M.; it did, however, form large micelles under these conditions. Beta casein did not appear to be necessary for the formation of these micelles.

Perlmann (43) studied the nature of the phosphorus linkages in alpha and beta caseins. She treated these two components of casein with various specific phosphatase enzymes, and, from the proportions of the total phosphorus removed by each enzyme, she calculated the amounts of the different types of phosphorus linkages which were present in the fractions. The following enzymes were used in her studies on casein:

Prostate phosphatase - specific for monoesters of phosphorus with the -O-P linkage

Yeast pyrophosphatase - specific for pyrophosphate linkages -O-P-O-P-O-

Snake venom diesterase - hydrolyses one bond of a diester linkage -O-P-O-

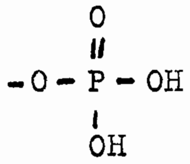
Intestinal phosphatase - at pH 5.6 hydrolyses only monoesters of phosphorus with the linkage -N-P

Treatment of alpha casein with prostate phosphatase removed 39.6 per cent of the phosphorus. Electrophoresis of the reaction products at various stages of the hydrolysis showed that the alpha band was gradually reduced, and a series of 5 other bands appeared with lower mobilities than the alpha band.

Treatment of alpha casein with phosphodiesterase or with pyrophosphatase did not remove any phosphorus from the protein; but there was a slight drop in pH in the solution containing pyrophosphatase, which indicated a release of acid groups. Subsequent treatment, of the phosphodiesterase-treated material, with prostate phosphatase resulted in no increase in phosphorus above the amount released by prostate phosphatase alone. Treatment of the pyrophosphatase-pretreated alpha casein with prostate phosphatase resulted in the release of 59.5 per cent of the phosphorus. Treatment of the phosphodiesterase-treated material with intestinal phosphatase, at pH 6.0, resulted in the release of 78 per cent of the phosphorus.

Perlmann concluded from these results that phosphorus occurs in alpha casein in the following forms:

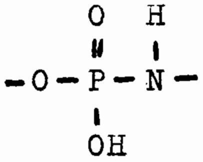
Monoester



$$-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OH$$

40 per cent

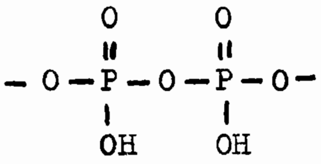
Diester



$$-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-\overset{\overset{H}{|}}{N}-$$

40 per cent

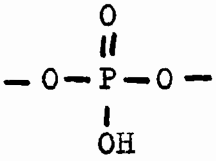
Pyrophosphate diester



$$-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-O-$$

20 per cent

In a less detailed investigation, Perlmann treated beta casein at pH 5.8 with snake venom diesterase, and found no release of phosphorus. When this material was subsequently treated with prostate phosphatase, 72 per cent of the phosphorus was released. Perlmann concluded that the beta casein contains diester bonds of the following type:



$$-\mathrm{O}-\underset{\underset{\mathrm{OH}}{|}}{\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}}-\mathrm{O}-$$

Perlmann (43a) has also found that alkaline intestinal phosphatase would release all the phosphorus from alpha casein, and that the products of the complete liberation of phosphorus were not precipitated by 10 per cent trichloroacetic acid.

Perlmann concluded from these results that the phosphorus present in diester and pyrophosphate linkages may cross-link the peptide chains.

3. Methods of Investigating the Structure of Proteins

No attempt will be made here to review this subject thoroughly. Springall (44), and Anfinsen and Redfield (45) have reviewed the large amount of literature which is available concerning these methods.

The advent of partition chromatography, and particularly the introduction of paper chromatography by Consdon, Gordon and Martin (46) in 1944, made possible the modern attack upon the problems of protein structure determination. Consdon *et al* were able to separate and identify all the amino acids from as little as one or two hundred micrograms of protein, or from a smaller quantity of low molecular weight peptides. Block *et al* (47) have reviewed the large number of papers which have appeared since 1944 reporting improvements and adaptations of the paper chromatographic method.

Ion exchange chromatography is another technique very useful to the protein chemist. Samuelson (48) has described many uses of this method of separation. Moore and Stein (49,50) have developed the most refined of the ion exchange methods of separation of the amino acids and peptides. The procedure of Moore and Stein makes it possible to separate, identify and quantitatively estimate all the common amino acids, as well as several other nitrogenous substances, with a single passage of these materials through an ion exchange column.

Paralleling the development of these sensitive and versatile methods for the separation of complex mixtures has been the development of chemical procedures for the determination of the serial order of amino acids in a peptide chain. The most important of these is the fluorodinitrobenzene method reported by Sanger (51). Fluorodinitrobenzene is added to a solution of a protein or peptide in a mildly alkaline buffer. Free amino groups react with the fluorodinitrobenzene to form the N-dinitrophenyl derivatives. Most of these derivatives are stable enough to survive acid hydrolysis. Thus the labeled protein or peptide may be hydrolysed, and the dinitrophenyl derivatives of all the amino acids, except arginine and the mono derivative of lysine, may be extracted into ether. After evaporation of the ether the derivatives, which are coloured, are separated (51, 52) either by column or paper partition chromatography. In this way the amino-terminal end group may be identified, and by the application of quantitative techniques (52, 40) the average chain length can be determined. By combining the results from the study of a large number of peptides obtained by partial hydrolysis of insulin with acid and enzymes, Sanger and his co-workers (53, 54) were able to deduce the complete structure of the insulin molecule.

The application of the many recently developed methods for the elucidation of protein structure has resulted in the publication of the complete chemical structures of vasopressin, oxytocin, glucagon, corticotropin from several species, and insulin from three species. In addition, considerable insight has been gained into the structures of egg white lysozyme, the collagens, hypophyseal growth hormone, silk fibroin, pepsin, and ovalbumin. The study of ribonuclease, an enzyme containing about 124 amino acids, is well advanced (45, 55).

EXPERIMENTAL

1. Materials

(a) Preparation of Alpha Casein:

Two separate lots of alpha casein were prepared. The first of these was purified by the method of Warner (25). Mr. Henneberry of this department had precipitated casein from fresh skim milk with HCl and washed this several times. About 150 gm. of this material was obtained from Mr. Henneberry and treated in the following manner. The casein was suspended in 8 liters of water and dissolved by the addition of 0.05 N. NaOH until the pH was 6.5. HCl (0.05 N.) was quickly added to the resulting solution to bring the pH to 3.4. After the precipitate dissolved, the solution was chilled to 3°C. and diluted to 48 liters with prechilled water. NaOH (0.01 N.) was added slowly and the solution stirred continuously. The casein precipitated when pH 4.3 was reached. The product was allowed to settle and the supernatant was decanted until the volume of the precipitate was reduced to 2 liters. The casein was dissolved as before, diluted to 48 liters and reprecipitated three additional times. During the last precipitation step the flocculation occurred between pH 4.1 and pH 4.2. The final precipitate was dried with alcohol and ether, and the yield of alpha casein was 50 grams. The phosphorus content, based on the weight after drying in a vacuum oven at 100°C. for 5 hours, was 1.06 per cent. Electrophoresis of the product showed that the casein contained no detectable quantity of either beta or gamma casein.

The second lot of casein was prepared by the urea fractionation method (31). The starting material for this preparation was also obtained from Mr. Henneberry. He had precipitated a large quantity of crude casein from 50 per cent ethanol in the separation of gamma casein. About 5,500 gm. (1,000 gm. dry weight) of this precipitate was washed to remove the alcohol and was then dissolved in 10 liters of 6.6 M. urea solution in water. The solution was centrifuged to remove a small amount of insoluble material, and was then diluted by the slow addition of 4.4 liters of water with continuous stirring. The precipitate, which was slimy and very viscous, was removed by centrifugation (15 minutes at 1,000 g.), and both precipitate and supernatant (Solution 1) were saved. It was noticed that during the centrifugation a small yellow scum, which seemed to be fatty in nature, formed at the top of the bottles, and this was removed. The precipitate was dissolved by the addition of 40 gm. of NaCl and sufficient 6.6 M. urea solution to give a final volume of 6½ liters of 5.8 M. urea. The alpha casein was reprecipitated in the same manner twice again. After the last precipitation the precipitate was suspended in 4 liters of water and recovered by centrifugation. The precipitate was then suspended in 8 liters of water and dissolved by the addition of 0.2 N. NaOH. HCl (0.2 N.) was quickly added until pH 3.0 was attained, and after the precipitate was completely dissolved the solution was diluted to 40 liters and cooled to 2°C. NaOH was added to precipitate the casein at pH 4.2. The flocculant precipitate was washed by decantation once with 20 liters and again with 5 liters of water, dried with alcohol and ether, and then air dried. The yield was 336 gm.

Beta casein was recovered from Solution 1. Eight liters (approximately 4/5 of the total) of this solution was diluted with water to give a total volume of 22 liters (1.7 M. urea), and 0.2 M. HCl was added until pH 4.7 was attained. The precipitate which formed was recovered by centrifugation and then dissolved by the addition of sufficient water and urea to make 4 liters of solution in which the urea concentration was 4.6 M. Water was added to bring the solution to 3.3 M. with respect to urea, and the heavy precipitate which formed was discarded. Water was slowly added to the supernatant until the urea concentration was reduced to 1.7 M. The precipitate which formed was washed by suspension in 1 liter of water and recovered by centrifugation. This precipitate was suspended in 3½ liters of water and NaOH added until pH 8.0 was attained. The protein dissolved, with some difficulty. Beta casein was precipitated from this solution by the slow addition of 0.2 M. HCl. It was found that precipitation was complete at pH 5.2. This precipitate was washed twice by decantation with 2 liters of water, and then dried with alcohol and ether. The yield was 56.9 gm. of beta casein.

Electrophoresis of the fractions prepared by urea separation showed that the alpha casein was electrophoretically pure. The beta casein, however, showed a faint trace of another component which migrated at a rate intermediate between alpha and beta casein. This component was probably alpha casein, as a great excess of beta casein can cause a lowering of the mobility of alpha casein (25).

(b) Phosphatase Preparations:

Four commercial intestinal alkaline phosphatase preparations (Armour, Nutritional Biochemicals, Pentex Inc. - Technical grade and Purified) were tested for potency. Armour's product was superior to that supplied by Nutritional Biochemicals, but was not as potent as the Purified grade supplied by Pentex, Inc., which assayed approximately 115 Morton Units (52) per milligram. The Purified grade (designated Enzyme 1) was used in the following work.

(c) Preparation of Dowex 50 Resin:

The procedure recommended by Moore and Stein (49, 50, 57) was employed for the preparation of the resin. One half pound of Dowex 50 (2 per cent cross-linked, Lot 4177-42, 200-400 mesh) was placed in a sintered glass Buchner funnel. It was washed with 2½ liters of 4.0 N. HCl, and then with distilled water until the reaction of the effluent was pH 5.5. The resin was treated, during a period of nine hours with occasional stirring, with 5 changes of twice its volume of 2.0 N. NaOH. The NaOH was then replaced with 5 changes of 0.2 N. sodium acetate buffer (pH 5.1), and the resin was stored in this buffer.

(d) Preparation of Hyndrindantin (58, 59):

Ninhydrin (7 gm., Analar Reagent) was dissolved in 175 ml. of distilled water and the solution heated to 90° C. A solution of 7.0 grams of ascorbic acid in 35 ml. of water at a temperature of 45° C. was added. The crystallization of hyndrindantin started immediately. After being allowed to cool to room temperature the product was recovered by filtration with suction through a sintered glass funnel. It was washed 5 times with water and then dried in a vacuum desiccator. The dry powder was stored in a dark brown bottle away from the light.

(e) Preparation of Ninhydrin Reagents:

All solutions were prepared just before use.

Ninhydrin Reagent 1 (60) - Ninhydrin (0.20 gm.) was dissolved in 100 ml. of redistilled acetone.

Ninhydrin Reagent 2 - Four milliliters of glacial acetic acid was added to ninhydrin reagent 1.

Ninhydrin Reagent 3 (58, 59) - Two grams of ninhydrin and 0.3 gm. of hyndrindantin were dissolved, with gentle stirring, in 75 ml. of Methyl Cellosolve (ethylene glycol monomethyl ether). When all the hyndrindantin had dissolved, 25 ml. of 4.0 N. sodium acetate buffer, adjusted to pH 5.5 with acetic acid, was added, and the reagent immediately poured into the burette from which it was to be dispensed. The Methyl Cellosolve was occasionally tested for the presence of peroxides by adding 2 ml. of the solvent to 1 ml. of 5 per cent aqueous KI. There was never any evidence of yellow colour.

(f) Preparation of DNP Derivatives of Amino Acids:

1-Fluoro, 2,4-dinitrobenzene (Nutritional Biochemicals, Inc.) was dissolved in absolute ethanol to make a 6 per cent solution. This solution was used for all reactions involving FDNB.

Derivatives of arginine and lysine were prepared, as well as mixed derivatives of the 18 amino acids.

Dinitrophenyl arginine was prepared as follows. Ten milligrams of L-arginine was dissolved in 1 ml. of 1 M. sodium bicarbonate buffer. Two hundred microliters of the FDNB solution was added, and the mixture was shaken intermittantly for 3 hours. The solution was then extracted several times with 2 ml. portions of ethyl ether. After extraction the solution was acidified, dried, and the dry residue was extracted with slightly acidified butanone. The butanone extract was evaporated and the residue of DNP-arginine was dissolved in 50 ml. of hot water. The water solution was evaporated to a volume of 10 ml., and cooled. The crystals were recovered by centrifugation, and were then washed with cold water and with alcohol and ether.

Di-dinitrophenyl lysine was prepared as follows. Ten milligrams of L-lysine hydrochloride was dissolved in 2 ml. of 1 M. bicarbonate buffer, and the solution adjusted to pH 10 with sodium carbonate. Two hundred microliters of the FDNB solution was added, and the tube shaken intermittantly for 3 hours. The alkaline solution was extracted with ether, and the extract was discarded. The solution was acidified and was again extracted with ether. The acid extract was evaporated to dryness, and the residue of di-DNP-lysine was dissolved in boiling methanol-water, and then cooled to -10° C. A stiff oil precipitated, which did not crystallize after several weeks. A drop of this oil was placed on a glass cover slip and smeared with the end of a spatula. This caused a slow crystallization of the oil. The melting point of this material was 162-163° C.

Dinitrophenyl derivatives of 18 amino acids were prepared as follows. One milliliter of the aqueous solution of 18 amino acids was placed in a small flask, diluted with 1 ml. of water, and adjusted to pH 10 with sodium carbonate. Three hundred microliters of the FDNB solution was added and the flask shaken intermittantly for 3 hours. The alkaline solution was extracted with ether, and the extract, which should have contained all the ether-extractable amino acids, was diluted to 25 ml.

2. Methods

(a) Nitrogen Analysis:

Analyses for nitrogen were carried out by the micro-Kjeldahl method (61).

(b) Phosphorus Analysis:

The method of Dickman and Bray (62) was employed for the determination of the phosphorus which was enzymatically released from sodium β-glycerophosphate during the procedure of enzyme assay. This method employs a molybdate hydrochloric acid reagent, and colorimetric determination of the phosphomolybdate blue after reduction by stannous chloride.

The Dickman and Bray method could not be employed for the estimation of the phosphorus released by the enzymatic treatment of casein, as the protein degradation products precipitated the molybdate and caused turbidity. For these analyses the more inconvenient and slightly less precise method of Martin and Doty (63) was employed. This involves precipitation of the proteinaceous materials with silicotungstic acid, and, without filtration, extraction of the unreduced phosphomolybdate from the water with a 1:1 mixture of isobutanol and benzene. The colour is developed in the organic phase with stannous chloride, and is stable for at least 24 hours. This method was also used for the determination of the phosphorus in casein samples which had been digested with a 1:10 mixture of perchloric and nitric acids.

(c) Enzyme Assay - Phosphatase Activity:

The method developed by Morton (56) was employed for the determination of phosphatase activity. This method involves the hydrolysis of sodium β-glycerophosphate in ethanolamine buffer, in the presence of magnesium chloride, and the determination of the released phosphorus.

(d) Enzyme Assay - Proteolytic Activity:

The method of Anson (64) was employed for the analysis of the phosphatase preparations for the presence of proteolytic activity. Hemoglobin (2.2 gm., "Hemoglobin Substrate Powder" - Worthington Biochemical Corp.) and 36 gm. of urea were suspended in 50 ml. of water. Then 8 ml. of 1.0 N. NaOH was added, the volume adjusted to 100 ml., and the solids dissolved by slow swirling. The flask was left to stand at room temperature for one hour, after which the contents were poured into 10 ml. of 1.0 M. KH_2PO_4 in which was dissolved 4 gm. of urea. This gave a solution with a final pH value of 7.5. Two milliliters of a 0.1 per cent solution of Merthiolate (Sodium ethylmercurithiosalicylate - Bios Laboratories, Inc.) was added as a preservative, and the solution was stored at 5° C.

The assay was carried out as follows. Five milliliters of hemoglobin substrate solution was placed in a test tube in a constant temperature bath (37° C.) and then 1 ml. of the enzyme solution was added. The mixture was incubated for a period of 10 minutes when the activity was high, and for periods of not more than 5 hours when the activity was very low. At the end of the incubation period 10 ml. of 5 per cent trichloracetic acid was added and the mixture was allowed to stand for 30 minutes before filtration (Whatman #40). Five milliliters of the filtrate was placed in a 50 ml. Erlenmyer flask, and 10 ml. of 0.5 N. NaOH and 3 ml. of the Folin and Ciocalteu reagent (65) diluted with 2 parts of water were added. After 5 minutes the colour intensity was measured at 660 mμ. The standard curve was made by using a series of dilutions of a tyrosine solution containing 1.6 x 10^{-7} equivalents per ml. in 0.2 N. HCl.

The degree of proteolytic activity may be compared by using the microequivalents of tyrosine released. The values which are obtained are not, however, linear with either concentration or time, so these were corrected to trypsin units by the use of the graph prepared by Anson (66).

(e) Electrophoresis of Casein:

The electrophoresis apparatus manufactured by Spinco (Durrum type) was employed in the separation of casein and casein degredation products. The following conditions (29) were used unless otherwise noted:

Veronal Buffer - pH 8.6
- ionic strength 0.05

Time of Running - 16 hours

Current Strength - 7½ milliamperes per cell

Staining - Bromphenol Blue (Spinco procedure)

Quantity of Protein applied to each strip - 0.5 mg.

(f) Analysis of Effluent from Ion Exchange Column:

The effluent was normally collected in 4 to 6 ml. fractions. One milliliter of each fraction was pipetted into a 13 x 150 mm. test tube, 1 ml. of Ninhydrin Reagent 3 was added, and the tubes, in lots of 48, were heated in a boiling water bath for 15 minutes.

When the tubes had cooled to about 40° C., 5 ml. of 50 per cent v/v ethanol - water was added as a diluent. The tubes were stoppered and shaken in a rack for 30 seconds. The optical densities (570 $m\mu$) were measured with an Evelyn Colorimeter.

When the effluent was alkaline (above pH 8) it was neutralized to pH 6 by the addition of concentrated acetic acid before the addition of the ninhydrin reagent.

(g) Chromatographic Methods:

Butanone (Eastman Kodak Co.) was distilled prior to use and the first 20 per cent, and all that distilled above 80° C., was discarded.

Phenol and pyridine (British Drug House) were distilled under vacuum with zinc dust.

Table 5 contains a list of the solvent systems used in connection with this report. The aqueous phase of each two phase system was used to provide a suitable atmosphere in the chromatography cabinets.

Table 5

LIST OF CHROMATOGRAPHIC SOLVENT SYSTEMS

(All proportions are given by volume.)

Solvent 1 - Phenol, water; 4:1. Stored in the dark. Beaker with 0.5 N. aqueous NH_3 placed in cabinet. Small (10 ml.) beaker with solid NaCN placed in cabinet.

Solvent 2 - n-Butanol, glacial acetic acid, water; 4:1:5.

Solvent 3 - Toluene, chloroethanol, pyridine, 0.8 M. aqueous NH_3; 10:6:3:6.

Solvent 4 - Phosphate Buffer, 1.5 M., pH 6.2; 1 mole NaH_2PO_4, 0.5 mole Na_2HPO_4 per liter.

Solvent 5 - Butanone, peroxide-free ethyl ether, water; 66:34:66.

Note - Solvent 2 forms butyl acetate on standing, with a consequent lowering of the water content of the organic phase, and a lowering of the mobilities of the amino acids. This solvent, therefore, was used at a reasonably constant age.

i. Chromatography of the amino acids: Standard solutions (100 millimolar) of each amino acid which occurs in casein were made in water containing 10 per cent isopropanol. Where necessary, a few drops of concentrated HCl were added to aid in the solution of the amino acids. A solution containing the 18 amino acids was made by mixing 1 ml. of each of the above solutions and adjusting the volume of the mixture to 25 ml. Ten microliters of the mixture, and one microliter of the solutions of each amino acid, were sufficient to produce readily visible spots on the chromatograms.

An attempt was made to use the two dimensional procedure reported by Dent (67, 68), which employs phenol in the first direction and collidine in the second direction. This attempt was unsuccessful, probably because the isomer composition of the collidine may not have been the same as that used by Dent.

The procedure that was finally adopted employed a two dimensional system with phenol (46) in the first direction (Solvent 1) followed by the butanol-acetic acid-water solvent developed by Partridge (69) in the second direction (Solvent 2). The procedure was as follows. The solution was applied to a chromatographypaper (Whatman #1, 18" sq.) near the corner, 3½ inches from each side. Not more than 2 microliters was applied at each time, and the spots were dried between each application. Two applications of 2 microliters each of 30 per cent hydrogen peroxide were made to the point of application of the amino acids, and thoroughly dried before developing the chromatogram. The papers were equilibrated with the atmosphere of the cabinet for two hours before starting the development of the chromatograms. After development with Solvent 1 the phenol was removed by drying at room temperature overnight. The last traces of phenol were removed by drying over a hot plate in a fume cupboard arranged so that a constant stream of air passed over the paper. After development with Solvent 2, the paper was dried overnight at room temperature. It was then dipped quickly in Ninhydrin Reagent 1 and, after the acetone had evaporated, was passed back and forth over a hot plate until the colours of the spots could be seen. The colour was allowed to develop completely overnight in the dark. This procedure separated all the amino acids present in acid hydrolysates of casein except the leucines, which moved together.

If the addition of hydrogen peroxide was omitted, methionine ran very close to valine. The purpose of the hydrogen peroxide was to oxidize the methionine to methionine sulphone and methionine sulphoxide. These two oxidation products appeared in regions well separated from the other amino acids. Dent (68) stated that hydrogen peroxide would not oxidize methionine to the sulphoxide, but only to the sulphone. It was found, however, that the spot due to the sulphoxide, which was always present when the standard solution of methionine was run on chromatograms, was considerably intensified by the action of the peroxide.

The addition of the hydrogen peroxide also oxidized the cystine to cysteic acid, which is more stable under chromatographic conditions than is cystine.

ii. Chromatography of the dinitrophenyl amino acids: The paper chromatographic methods reported by Mellon et al (40), and Blackburn and Lowther (70) were tried, but suitable separations were not obtained. The ether-soluble DNP amino acids streaked badly, and the water soluble DNP amino acids could not be separated from two other coloured water-soluble materials present in hydrolysates of the DNP casein. Anfinsen and Redfield (45) recommended the two dimensional paper chromatography procedure, which employs the toluene and phosphate solvents (Solvents 3 and 4) developed by Levy (52). This procedure was used for the separation of the ether soluble amino acids.

It was not found possible to separate the water soluble DNP amino acids from the other two coloured materials in hydrolysates of DNP casein by the use of Levy's toluene solvent. These two materials ran so close to DNP arginine that they could not be distinguished from it. They did not, however, react to the Sakaguchi reagent (71) as does DNP arginine.

The silica gel column chromatography procedure for the separation of water soluble DNP amino acids developed by Bailey (72), and modified by Mellon *et al* (40), was employed, and this procedure gave a good separation of the DNP arginine from the other materials. Five grams of silica gel and 3 ml. of formaldehyde solution (1 part USP Formaldehyde Solution mixed with 9 parts of water) were mixed by grinding with a mortar and pestle. The silica gel mixture was then suspended in 40 ml. of Solvent 5. The columns were prepared in glass tubes with an internal diameter of either 0.3 cm. or 0.9 cm. They were constricted at one end and were plugged with glass wool. The silica gel suspension was poured into the tube until the packed gel reached a height of 10 cm. The material to be separated was applied to the top of the column and the column was then eluted with solvent 5. The progress of the elution could be followed easily, as the coloured bands could be seen moving down the column.

3. Hydrolysis of Alpha Casein with Intestinal Phosphatase

(a) Preliminary Experiments:

Alpha casein prepared by the urea solubility method (lot 2) was used throughout the investigations with phosphatase. Several experiments were carried out to find conditions most suitable for the removal of the phosphorus from the alpha casein with alkaline intestinal phosphatase. In a typical experiment, alpha casein was suspended in water and dissolved by the addition of dilute NaOH solution until pH 9.5 was attained. The solution was adjusted to a concentration of 5 per cent protein. Three milliliter portions of this solution were placed in each of a series of test tubes, and 1 ml. of 0.0025 M. $MgCl_2$ solution added to each tube. One milliliter of the required dilution of the enzyme solution in water was added to each tube, and the tubes were placed in a water bath at 37° C. After suitable time intervals the tubes were removed from the bath and the enzyme inactivated by placing the test tubes in a boiling water bath for 5 minutes. It is unlikely that this procedure caused any non-enzymatic release of phosphorus, as increasing the time to 10 minutes did not cause any increase in the phosphorus released.

It was found that much more enzyme was required to release a given amount of phosphorus from alpha casein than from sodium β-glycerophosphate. For instance, 70 units of the enzyme would hydrolyse in 8 hours all the phosphorus present in 5 ml. of a solution of sodium β-glycerophosphate containing 2700 micrograms of phosphorus. It required 230 units of phosphatase to hydrolyse all the phosphorus in 5 ml. of alpha casein solution containing 1350 micrograms of phosphorus (Figure I).

The products which were obtained by the treatment of alpha casein with 230 units of phosphatase for each 135 mgm. of alpha casein were subjected to paper electrophoresis. The method of electrophoresis was that described before, but the time of running was only 5 hours. A current of 5 milliamps per cell separated some bands which moved very quickly, while a current of 10 milliamps per cell was used for the separation of other bands which moved slowly.

An attempt was made to stain the papers with Bromphenol blue, according to the Spinco procedure. It was found that none of the products of the later stages of hydrolysis was stained. It was interesting to note, however, that the products from the early stages of

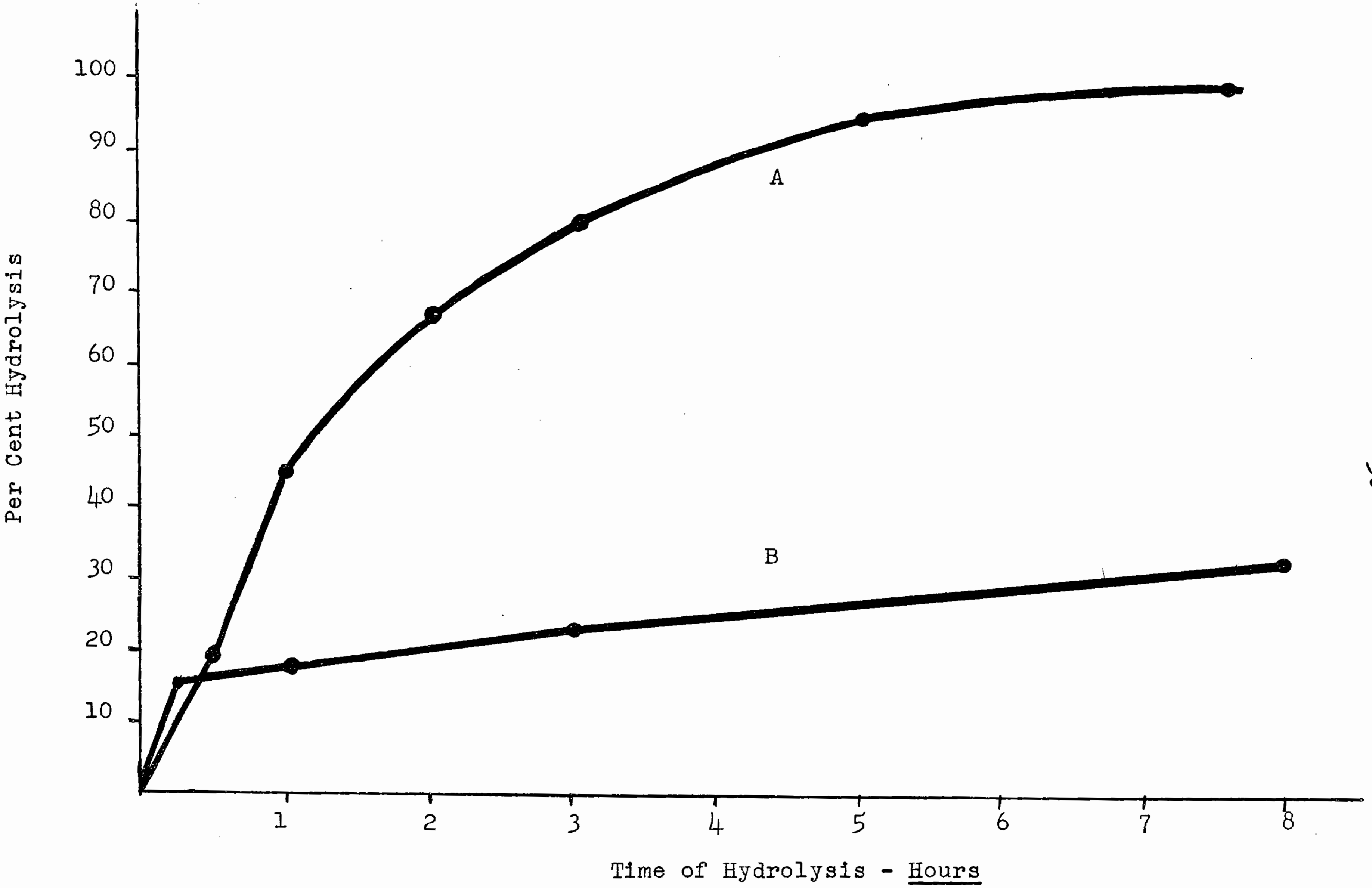


Figure I. A. - Hydrolysis of 135 Mgm. Alpha Casein with 230 Units of Alkaline Intestinal Phosphatase (Enzyme 1).

B. - Hydrolysis of 135 Mgm. Alpha Casein with 250 Units of Phosphatase after Purification (Fraction A).

hydrolysis ($\frac{1}{2}$ hour to 1 hour) were stained by Bromphenol Blue, and they showed a series of 4 bands which moved more slowly than alpha casein. One of these bands moved towards the cathode. These four bands might have been due to the serial removal of the monoester phosphoric acid groups, and they may correspond to the peaks found by Perlmann (43) in free electrophoresis of alpha casein hydrolysed by prostate phosphatase. Ninhydrin (Ninhydrin Reagent 2) stained all the bands observed with Bromphenol Blue, and, in addition, stained a number of other bands, some of which could also be detected by their fluorescence under ultraviolet light.

Twelve or thirteen bands were separable by paper electrophoresis after 8 hours hydrolysis with complete removal of the phosphorus. Many of these bands disappeared after longer periods of hydrolysis. It seemed, therefore, that considerable proteolysis had occurred, probably by the action of trypsin, which is the most likely contaminent (73) of intestinal phosphatase.

The phosphatase was assayed for the presence of proteolytic activity. This was found to be high, about 0.55 units of trypsin activity per gram.

(b) Purification of Intestinal Phosphatase:

Schmidt and Thannhauser (74) reported a method for the removal of trypsin as one step in the purification of intestinal phosphatase. This method involves the addition of kaolin at pH 8.8 to absorb the trypsin. They stated that three additions of 5 per cent kaolin will completely remove the trypsin from a solution of the phosphatase.

One gram of phosphatase (Enzyme 1) was dissolved in 40 ml. of 0.025 M. ammonium acetate buffer and the solution was dialysed at room temperature overnight against 2 liters of the same buffer at pH 8.8. Two grams of kaolin (acid washed at 100° C. with 5 per cent nitric acid to remove heavy metals) was added and the resulting slurry was stirred for about 5 minutes and then centrifuged. This kaolin precipitation was repeated twice, and each time the progress of the purification was followed by measuring the proteolytic and phosphatase activities of the solutions. The first precipitation removed about 25 per cent of the proteolytic activity, the second about 25 per cent of the remainder, and the third about 20 per cent of the remainder. There still remained about 0.35 units of activity expressed as trypsin. At the same time there was a loss of about 15 per cent of the phosphatase (Table 6).

Table 6

PURIFICATION OF INTESTINAL PHOSPHATASE

Procedure	Total Enzymatic Activity Remaining	
	Trypsin units	Phosphatase units
Start (Enzyme 1)	0.55	115,000
1st kaolin precipitation	0.39	103,000
2nd kaolin precipitation	0.26	-
3rd kaolin precipitation	0.21	97,000
3rd kaolin precipitation plus soybean trypsin inhibitor	0.09	-
1st acetone precipitation	0.11	80,000
2nd acetone precipitation (Fraction A)	0.0036	78,000
Fraction A plus soybean trypsin inhibitor	0.0015	-

Soybean trypsin inhibitor (Armour), when added to the phosphatase solutions in amounts ranging from 0.1 to 1.0 mg. per 2.5×10^{-4} units of proteolytic activity, resulted in a further reduction in the activity by 50 per cent.

Morton (73) has reported procedures for the purification of alkaline intestinal phosphatase. Stages 3, 4 and 5 of his procedure were carried out on the phosphatase remaining after the third kaolin precipitation. This solution amounted to 38 ml. and to it was added 14 ml. of n-butanol. The mixture was heated to 38° C. for 10 minutes, and was then centrifuged at 20° C. for 35 minutes at 2100 rpm. The water layer was removed by means of a pipette, filtered through celite, and was then assayed for phosphatase. The pH was adjusted to 8.5 with 0.5 N. ammonium hydroxide, and the solution was left overnight at 2° C. There was a small precipitate which was removed by centrifugation. the pH was adjusted to 6.4 with 0.5 N. acetic acid, 4 ml. of ether was added, and the solution was cooled to 0° C. Sixty milliliters of redistilled acetone prechilled to 0° C. was added slowly with stirring, and the solution was left at -10° C. overnight. The precipitate was removed by centrifugation at -5° C. and the supernatant was discarded. The residue was dissolved in 30 ml. of 0.05 M. veronal-HCl buffer at pH 6.4. (It may be noted, however, that veronal is not a good buffer at this low pH and is indeed very insoluble.) This solution was centrifuged to remove a very small precipitate, was dialysed for 2 days against 2 changes of 0.015 M. magnesium acetate buffer at pH 6.4, and was then filtered through celite. The filtrate was then cooled to 0° C., and cold acetone (42 per cent v/v) was added and the solution left at -10° C. overnight. The precipitate was removed by centrifugation at -5° C., the supernatant was discarded, and the precipitate was dissolved in veronal buffer at pH 6.4 and this solution dialysed against distilled water at 4° C. for 18 hours. Phosphatase activity was then determined. The solution was adjusted to pH 4.9 with 0.05 M. acetic acid, then rapidly heated to 48° C., and held there for 2 minutes. It was cooled rapidly to room temperature, and then 1.5 ml. of 0.5 M. magnesium acetate was added, and the pH adjusted to 6.4 with dilute ammonia. The solution was then cooled to 0° C. and acetone (37 per cent v/v) was added. The precipitate was removed by centrifugation and both precipitate and supernatant were saved. The precipitate was dissolved in 25 ml. of veronal buffer at pH 6.4 (Fraction A). Enough acetone was added to the supernatant to make it 50 per cent v/v acetone and the solution was allowed to stand overnight at -10° C. The precipitate which was formed was removed by centrifugation, and was dissolved in 25 ml. of veronal buffer (Fraction B). According to Morton the phosphatase should all have been found in Fraction B. This was not the case, however. Fraction B contained 1620 units of phosphatase activity, while Fraction A contained 78,000 units. Fraction B was discarded. Fraction A was tested for proteolytic activity. Only 0.0036 units of proteolytic activity remained, and one half of this could be inhibited by soybean trypsin inhibitor (Table 6). The proteolytic activity was now low enough that a hydrolysis time of several hours was required to produce sufficient proteolysis to be measured by the assay procedure used.

(c) Hydrolysis of Alpha Casein with Purified Phosphatase:

Alpha casein was incubated with the purified enzyme (Fraction A) using 250 units of phosphatase for each 135 mgm. of casein. Figure I shows that less than 50 per cent of the phosphorus was hydrolysed in 25 hours. An interesting observation was that there was a very fast removal of 15 per cent of the phosphorus, and a very slow removal of the next 35 per cent. At three hours there was a pronounced granular precipitate of crystalline form, but this precipitate disappeared after hydrolysis for 10 hours. Paper electrophoresis of the hydrolysate showed, even after 25 hours of incubation, only one main band which moved slightly more slowly than alpha casein, and two bands near the origin. The two bands near the origin stained faintly with Bromphenol Blue and were somewhat more strongly stained by ninhydrin. Bands similar to these two have been found in all electropherograms of products obtained by the treatment of alpha casein with phosphatase; one band moves slightly (½ cm.) towards the anode, and the other a short distance (1 cm.) towards the cathode.

Another hydrolysis of alpha casein was carried out, using this time 750 units of phosphatase and 0.1 mgm. of soybean trypsin inhibitor for each 135 mgm. of alpha casein. The concentration of magnesium chloride was raised to 0.006 M., and the pH was raised to 9.9. If a higher concentration of magnesium ion was used at this pH, the protein precipitated; and, if a lower pH was used at this concentration of magnesium ion, the protein also precipitated. Under these conditions, about 80 per cent of the phosphorus was hydrolysed in 96 hours (Figure II). In contrast to the products obtained from the hydrolysis

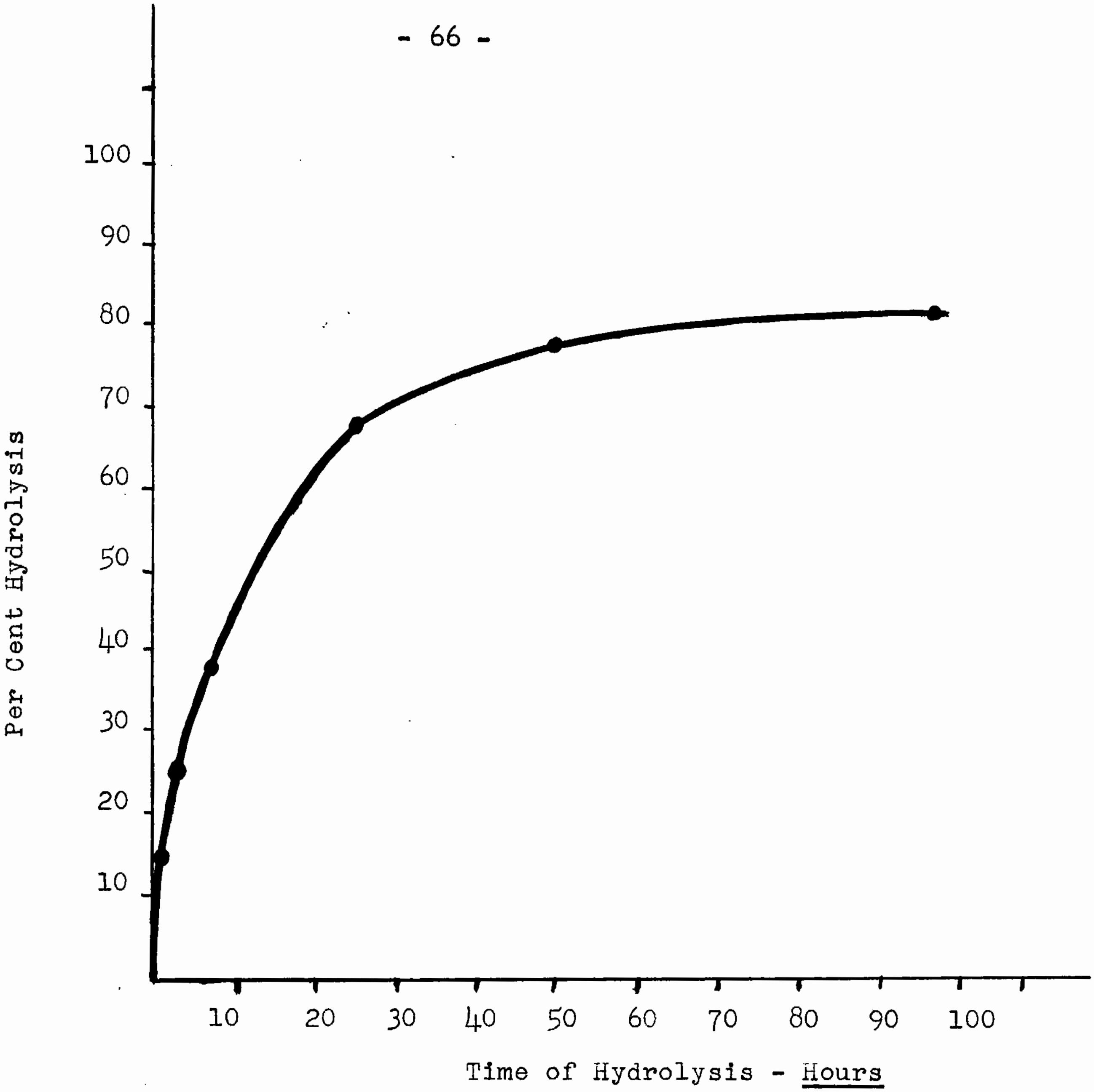


Figure II. Hydrolysis of Alpha Casein with 750 Units of Purified Phosphatase (Fraction A) for Each 135 Mgm. of Casein.

of alpha casein with impure enzyme (Enzyme 1), the products obtained by hydrolysis with the purified enzyme (Fraction A) gave a precipitate when treated with 2 per cent trichloroacetic acid.

Paper electropherograms of the 26 hour and the 96 hour hydrolysates were prepared. Both hydrolysates gave two bands which stained with Bromphenol Blue. The largest band moved somewhat more slowly than alpha casein and the other band, which was very small, showed a low mobility towards the cathode. When the electropherograms were stained with ninhydrin, at least 12 bands were discernable. The one which stained most strongly, as always, was the band which moved 1 cm. towards the cathode. Most of the bands were weakly stained, and they were probably due to materials present in low concentration.

Several attempts were made to fractionate chemically the 96 hour hydrolysate. If a portion of the solution was slightly acidified and 5 per cent by volume of 0.05 M. MgCl added, a small precipitate formed. Upon electrophoresis it gave only one band. This band corresponded to the band mentioned previously which stained with Bromphenol Blue and moved slightly more slowly than alpha casein. The remaining material gave no precipitate upon the addition of acid. Successive additions of acetone, and also of ethanol, at a neutral pH caused successive precipitates to appear. Electrophoresis of these precipitates showed that each consisted of several components, all of which stained with ninhydrin.

(d) Ion Exchange Chromatography of Casein Hydrolysates:

Ion exchange chromatography was carried out with the products obtained by treatment of 135 mg. of alpha casein with 750 units of phosphatase (Fraction A). A modification of the method developed by Moore and Stein (49, 50, 57, 58, 59) was followed.

A column with a height of 60 cm. and an internal diameter of 0.9 cm. was employed. The resin rested on a sintered glass disc sealed into the column, and the bottom of the column was closed with a stop cock. At the top of the column was a ball and socket joint, and this was connected with Tygon tubing to a 550 ml. mixing flask. The mixing flask, which was supported on a magnetic stirrer, was in turn connected to a 1000 ml. reservoir flask. All connections were sufficiently strong that a positive pressure of up to 30 cm. of mercury could be applied through the reservoir flask.

The resin suspension (purified as described in Section 1c) was poured into the column until the packed resin reached a height of 36 cm. It was washed with 150 ml. of 0.2 N. NaOH containing 0.5 per cent of BRIJ-35 solution. A 50 per cent solution of BRIJ-35 (a nonionic detergent, polyoxyethlyene lauryl alcohol, which increases the rate of elution) was made up in water and added to the eluting buffers. The column was then washed with 150 ml. of sodium citrate buffer (21 gm. citric acid, 8.4 gm. NaOH, 16 ml. concentrated HCl per liter, pH 2.2) containing 0.5 per cent of the BRIJ solution. The 96-hour alpha casein hydrolysate (1.7 ml., containing about 50 mgm. of protein) was acidified to pH 2.5 with HCl. This solution was applied to the column, and three 0.3 ml. portions of the sodium citrate buffer were added to wash the remaining traces of the sample onto the resin. Elution was then started with sodium acetate buffer at pH 3.1 (25 ml. glacial acetic acid, 11 ml. concentrated HCl, 27 gm. sodium acetate, 0.5 per cent of BRIJ solution). Both mixing flask and reservoir were filled with this buffer. The buffer solution passed through the column at a rate of 25 to 30 ml. per hour. The effluent, in fractions of 5 ml. each, was collected in a fraction collector equipped with an automatic siphon type volume control. After 550 ml. had passed through the column, the reservoir was emptied and then filled with sodium acetate buffer, pH 5.1 (136 ml. glacial acetic acid, 70 gm. NaOH, 0.5 per cent of BRIJ solution). Elution with this buffer was continued until a further 750 ml. had been collected. The reservoir was emptied and then filled with sodium acetate buffer, pH 6.5 (272 gm. sodium acetate per liter, adjusted to pH 6.5 with acetic acid, and 0.5 per cent of BRIJ solution added). Elution with this buffer continued until a further 400 ml. of effluent had been collected, at which time the mixing flask was detached and the column allowed to drain until the liquid level was almost to the top of the resin column. The column was then eluted with sodium acetate buffer at pH 9.5 (1.0 N. sodium acetate, 0.5 per cent of BRIJ solution) without passing this buffer through the mixing flask. A further 600 ml. of effluent was collected. The column was then washed with 1.0 N. NaOH, and the effluent from this washing was collected as before.

One milliliter of the effluent was removed from each tube and analysed by the ninhydrin method for the presence of amino groups. Leucine was used as a standard for the preparation of a standard curve.

A second one milliliter aliquot from each tube was placed in a test tube and 1 ml. of 2.5 N. NaOH was added. Alkaline hydrolysis was carried out for one hour in an autoclave at 120° C. It was found that a longer time of hydrolysis could not be used because too much silica was dissolved, and this interfered with the ninhydrin analysis. After cooling, the contents of the tubes were neutralized with 25 per cent acetic acid solution, and then were analysed for the presence of amino groups.

The results of these analyses were plotted as a graph, interesting portions of which are shown in Figure III. In those places in Figure III where only one line can be seen the colour yield after hydrolysis was the same as before hydrolysis. Where two lines can be seen the higher one represents the colour yield after hydrolysis. The increase in colour yield after hydrolysis indicates the presence of peptide materials in the effluent. A lack of increase in colour yield indicates the presence of amino acids only, or of peptide materials resistant to hydrolysis under the conditions employed.

Figure III shows that there was no significant quantity of peptide material eluted from the column by the buffers recommended by Mocre and Stein (those buffers

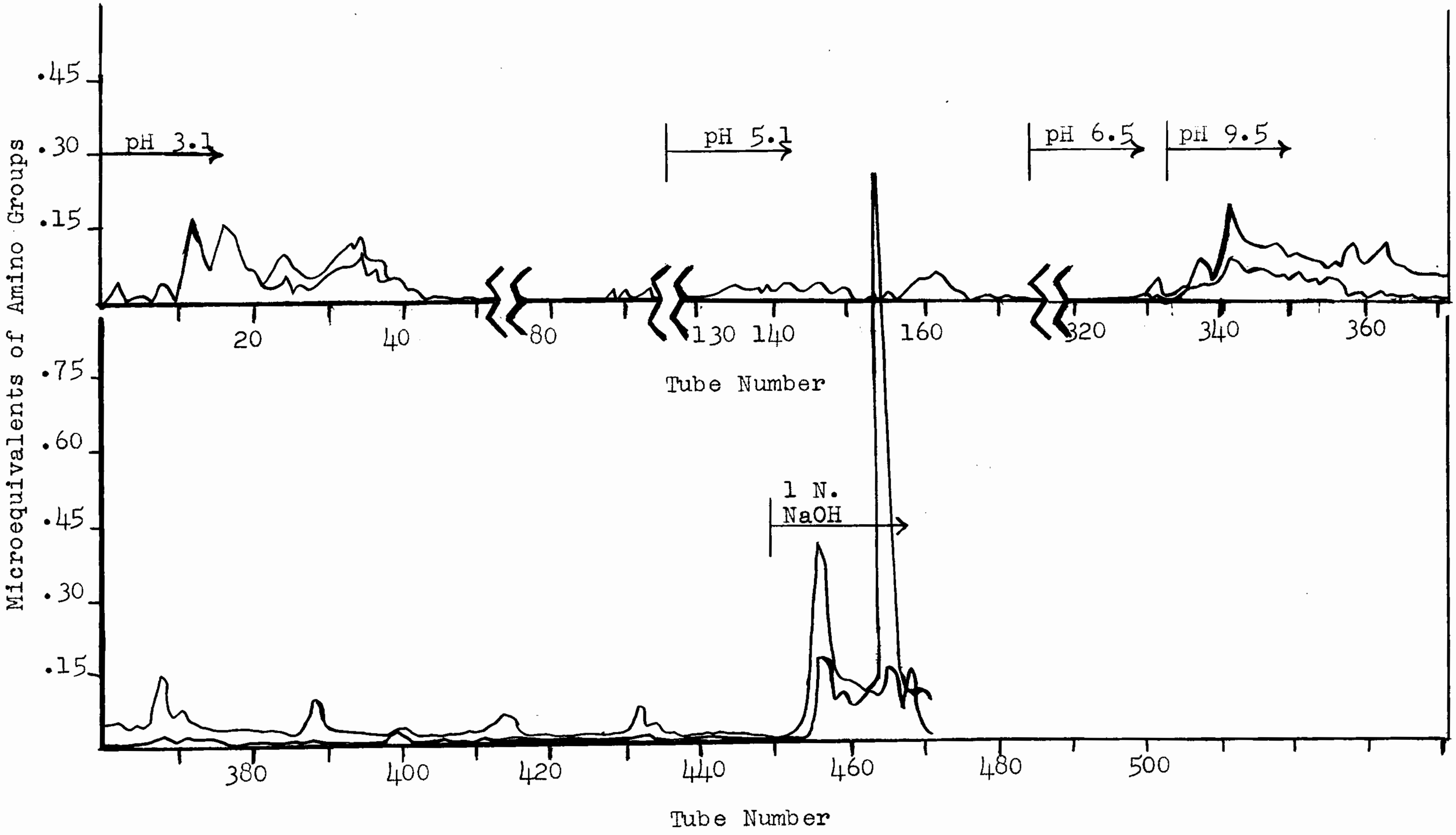


Figure III. Results of ninhydrin reaction, before and after Hydrolysis, with Eluate from the ion exchange separation of the 96-hour Hydrolysate of Alpha Casein.

with pH 6.5 or less). Peptide materials began to appear in the effluent when a buffer of pH 9.5 was passed through the column. Only small traces of material were eluted from the column, however, until it was washed with 1.0 N. NaOH. At this point at least two bands appeared, and the peptides present in these bands may well have undergone considerable degredation by the action of the NaOH.

Another separation of the casein hydrolysate was attempted, this time with the aim of increasing the rate of movement of the traces of amino acids at the start, and then of raising the pH more gradually at the end, to avoid possible degredation of the eluted products.

The resin column was prepared as before, but the height of the column was 50 cm. Four milliliters of the 26-hour hydrolysate of alpha casein (about 105 mgm. of protein) was applied to the top of the column. Elution this time was carried out with buffers which contained no BRIJ-35. The pressure applied to the top of the column was adjusted to produce a flow of about 30 ml. per hour. Elution was started with the acetate buffer, pH 3.1. The acetate buffer of pH 6.5 was substituted in the reservoir after 280 ml. had passed through the column. The sodium acetate buffer, pH 9.5, was placed in the reservoir after a further 840 ml. had gone through. After another 840 ml. had passed through, the reservoir was emptied and filled with 0.5 N. NaOH. Elution was continued until a total of 3200 ml. had been collected. Those fractions collected which were as alkaline as pH 10 or higher were neutralized by the addition of acetic acid as soon as possible after collection. The effluent was analysed for the presence of amino groups both before and after hydrolysis, and the results have been plotted in Figure IV. It can be seen from Figure IV that the substances appearing in the effluent in the first 160 tubes were probably all amino acids. Some of these were identified by paper chromatography. Fraction B13, for example, was due to arginine, while B7 was due to leucine. The amino acid bands are crowded together in contrast to the results published by Moore and Stein. This was probably due to the shorter column used in the present study, and to the higher pH of the buffers used for the elution. Three, or perhaps four, major bands were composed of high molecular weight materials. These bands were not present in high concentration. Band number B14 appeared when the pH of the effluent was about 10, but after tube number 530 the pH rose rapidly, and the bands which followed appeared

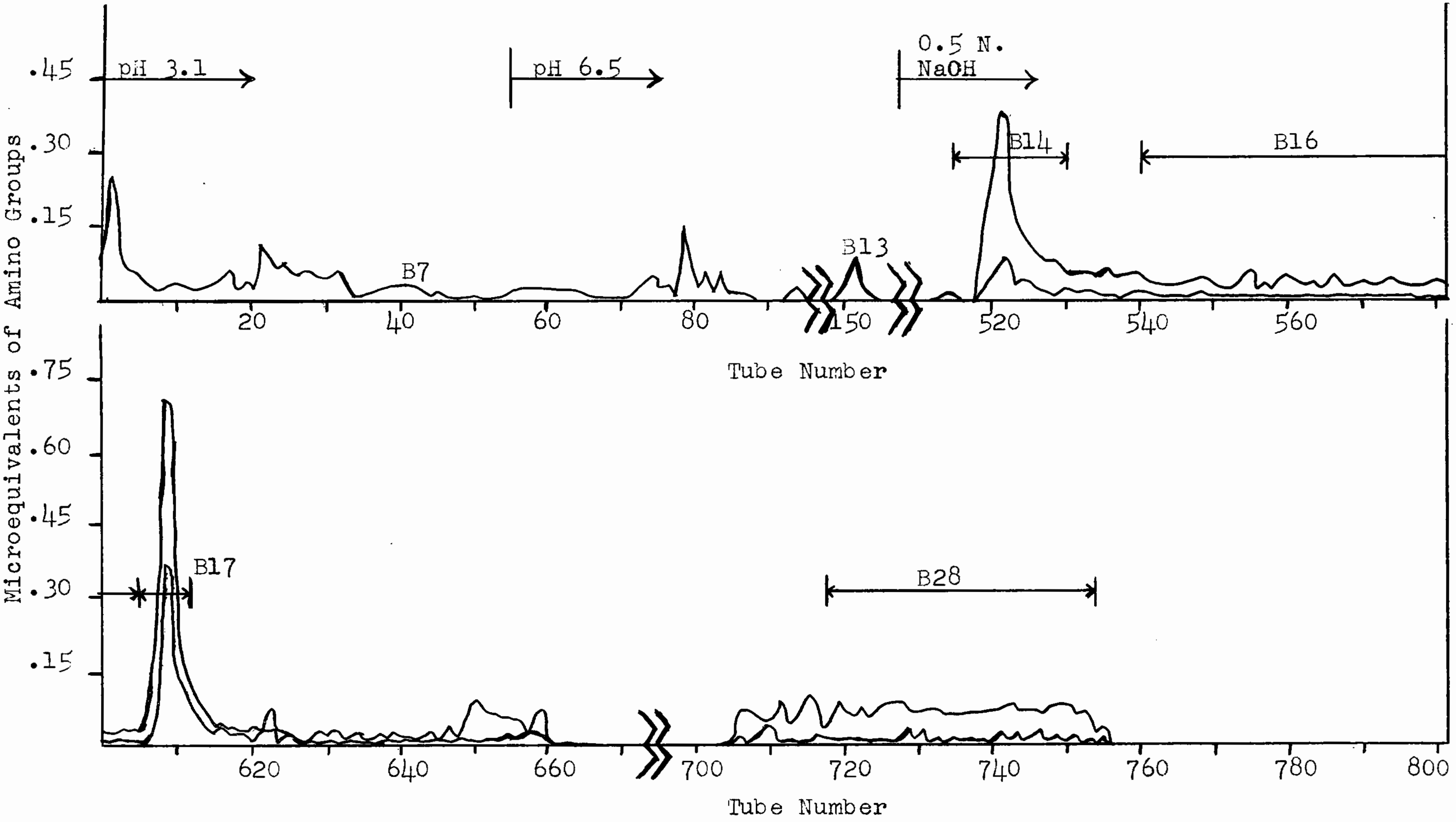


Figure IV. Results of the Ninhydrin Reaction, before and after Hydrolysis, with the Eluate from the Ion Exchange Separation of the 26-hour Hydrolysate of Alpha Casein.

in an effluent at pH 12 or higher.

(e) Experiments on the Separated Fractions:

The estimation of the total amount of the casein hydrolysate, which had been eluted from the column, was difficult. It was not possible to carry out nitrogen determinations on the effluent, due to the large quantities of salts present in association with relatively small amounts of amino acids or peptides. The reaction of the ninhydrin reagent with the amino acids is nearly quantitative, and it is possible to sum the ninhydrin colour values obtained from those portions of the effluent which probably contained amino acids only. This was done and a total quantity of about 20 micromoles of amino acids were estimated to be present. If the average molecular weight of the amino acid residues is 120 grams, then about 2.7 mgm. of amino acids were eluted from the column. This represents 2.3 per cent of the alpha casein hydrolysate which was applied. The colour yield of the peptide materials is dependant on their molecular weight, and there is no quantitative relationship between the optical density of the ninhydrin colour and the amount of peptide present. The colour yield after hydrolysis is also not a quantitative one, but depends upon the degree of hydrolysis of the molecular species involved. There was, therefore, no way that the total recovery could be determined. As will be seen later, however, none of the major peptide bands was finally isolated in amounts greater than 4 mgm. For this reason it is probable that there was still material on the column which was not eluted by the methods employed.

The contents of the tubes containing bands B14, B16, B17, and B28 were pooled and desalted as follows. A glass tube (0.9 x 20 cm.) with a constriction at one end, was plugged with glass wool, and Dowex 50 (2 per cent cross linked, 200-400 mesh, washed as described before) was poured to a depth of 3 cm. The resin was washed with 1 N. ammonium formate buffer adjusted to pH 3.0 with formic acid. The pooled effluent from the column was acidified to pH 3.0 with formic acid and passed through the column. The column was then washed with 1.0 N. ammonium formate buffer (pH 3.0) until no sodium ion could be detected in the effluent by means of the flame test. The column was washed with 5 ml. of water, and then 1.0 N. ammonia solution was passed through and the effluent collected in fractions of 10 ml.

each. The optical absorbance of each fraction was then read at 280 $m\mu$ and at 240 $m\mu$ in the Beckman DU spectrophotometer. The aromatic amino acids absorb in the region of 280 $m\mu$, and at 240 $m\mu$ and below is the region of general absorbance by the peptide bond. Those tubes which gave evidence of containing peptide materials were pooled, frozen, and lyophilized over P_2O_5. A small amount of residual non-peptide solid, which was probably ammonium formate, remained; this was removed by heating at 80° C. *in vacuo*.

It was not possible to recover fraction B28 by the procedure given above. The material was not eluted from the desalting column by 80 ml. of 1.0 N. ammonia, nor was it eluted by the passage through the column of 240 ml. of 9.5 N. ammonia solution. Fraction B28 could not be recovered, and was thus lost.

The dried powders remaining after lyophilization of Fractions B14, B16, and B17 were dissolved in water containing 10 per cent isopropanol at neutral pH. A portion of each solution was removed, and hydrolysed with 6 N. HCl for 12 hours in a sealed tube at 120° C. The HCl was removed under vacuum and the residue dissolved in 50 microliters of 10 per cent isopropanol. The amino acids which were present were identified by two dimensional paper chromatography, using Solvent 1 in the first dimension and Solvent 2 in the second dimension. The patterns which were produced were compared with those produced by a standard mixture of amino acids, and by alpha casein hydrolysed in the same manner. A quantity of hydrolysate equivalent to 200 micrograms of protein was applied to the sheet for each chromatogram of alpha casein.

All the amino acids present in acid hydrolysates of the alpha casein were found to be present in the acid hydrolysate of Fraction B14. As nearly as could be estimated from visual comparison of the chromatograms, the amino acids were present in the same relative proportions as alpha casein.

Fraction B16 contained all the amino acids present in alpha casein, with the exception of tyrosine, methionine, and histidine. Cystine and phenylalanine may or may not have been present. The amino acids which were present varied somewhat in their relative proportions from those found in alpha casein.

Fraction B17 was similar in amino acid composition to B16, but considerably less total material was present.

A visual estimation of the amount of peptide material present in the three fractions by the total colour density of the paper chromatograms indicated that the total quantity of fraction B16 originally separated was about 4 mgm. The quantity of fraction B14 was about 3 mgm., and of fraction B17 about 2 mgm.

The remaining material in each pooled fraction was analysed for end groups by the FDNB procedure. Each fraction, which was dissolved in ½ ml. of 10 per cent isopropanol, was mixed with 1 ml. of 1 M. sodium bicarbonate buffer. To this solution was added 100 microliters of a 6 per cent solution of FDNB, and the mixture was shaken intermittantly for 3 hours. The reaction mixture was extracted with 3 portions of 2 ml. each of ethyl ether, and after removal of the ether *in vacuo*, sufficient HCl was added to make the solution 6 N. in HCl. The solution was placed in a sealed tube and heated for 10 hours at 120° C. The acid hydrolysate was extracted 5 times with an equal volume of ether. The ether extract was evaporated to a small volume and was analysed for the presence of ether soluble DNP amino acids by the paper chromatographic method of Levy. The residue, after the ether extraction, was evaporated to dryness. One drop of water was added to dissolve the residue, and the solution was extracted 8 times, each time with 1 to 2 ml. of butanone. The butanone extract was evaporated to a small volume and was analysed for DNP arginine by the silica gel column chromatography method.

Chromatograms of the ether extract of the hydrolysate of fraction B14 showed the presence of di-DNP-lysine only. Chromatograms of the water soluble DNP amino acids showed the presence of mono-DNP-lysine, but no evidence of DNP arginine.

Chromatograms of the ether extract of the hydrolysate of fraction B16 showed the presence of di-DNP-lysine, and also a small amount of material with R_f values which were identical to DNP glutamic acid. The water soluble DNP amino acid extract contained mono-DNP-lysine, and also DNP arginine. Fraction B17 gave results similar to B16, but the quantities involved were much less, and no DNP glutamic acid was found.

(f) Discussion:

The complete removal of phosphorus from the alpha casein by the unpurified phosphatase enzyme (Enzyme 1) was probably aided by proteolytic breakdown of the alpha casein molecules. The fact that the purified enzyme was much less active than the non-purified enzyme in the hydrolysis of the protein-bound phosphorus, may well be due solely to steric effects. The phosphorus attached to some parts of the protein molecule might have been unapproachable by the enzyme. There is also the possibility that the enzyme preparation employed consisted originally of a mixture of several phosphatase enzymes, one or more of which had specific activity towards protein-bound phosphorus. The process of purification could have resulted in the preferential loss of this phosphoprotein-phosphatase activity.

Perlmann's statement (43a) that alkaline intestinal phosphatase would remove all the phosphorus from alpha casein is not confirmed by the experiments reported here. Also not reconcilable with the theories of the phosphorus distribution in alpha casein published by Perlmann, is the occurrence of just 15 per cent of the phosphorus in a form readily labile with alkaline intestinal phosphatase. These discrepancies may be due to differences in the methods of preparation of the alpha casein and of the phosphatase. Alkaline intestinal phosphatase has never been prepared in a pure crystalline form (34) and the products obtained by different methods may have different specificities.

Of some interest in this connection is a recent paper by Hofman (75). He investigated the effects on alpha and beta caseins of phosphoprotein phosphatase from ox spleen, prostate acid phosphatase, and phosphodiesterase from snake venom. Hofman claimed that the kinetics of the hydrolysis of alpha casein by the phosphoprotein phosphatase did not indicate the presence of more than one type of phosphorus linkage. The results of his experiments on the specificities and reactions of the other enzymes are also incompatible with the theories advanced by Perlmann. The nature of the phosphorus linkages in the casein fractions may be considered to be still an open question.

The appearance of about 2 mgm. of amino acids in the effluent during the ion exchange chromatography of the 26-hour hydrolysate, indicated that quite a large degree of proteolysis had occurred. This degree of proteolysis would certainly not have been expected from the results of the assay for the presence of proteolytic activity. It is possible that the conditions employed for measuring the proteolytic activity (e.g. a high concentration of urea) may have denatured some proteases which were present. It is difficult, however, to account for the appearance of only amino acids as a product of the proteolysis. If, for example, the proteolytic activity was due to trypsin, it would be expected that a large number of small molecular weight peptides would be liberated. This is especially the case when the large amount of lysine and arginine in alpha casein is considered, as trypsin specifically attacks bonds formed by these amino acids. At least some of the smaller molecular weight peptides would be expected to behave on the Dowex 50 resin column in a similar manner to the peptides obtained by Moore and Stein after enzymatic hydrolysis of ribonuclease. However, no bands which were due to peptides were observed during the course of elution with the buffers (pH 3.1 to pH 6.5) employed by Moore and Stein. The bands which appeared when higher pH buffers were used were all due to high molecular weight materials. A possible explanation of these results could be that the phosphatase preparation contained an active carboxypeptidase. This could have removed serially a large number of amino acids from the carboxy-terminal end of the peptide chains without affecting the observed amino terminal end groups of the peptides finally isolated. These end groups, with one exception, were identical to those obtained from alpha casein. The hemoglobin assay procedure for the proteolytic activity may also be rather insensitive to the action of a carboxypeptidase, as it depends on the estimation of the tyrosine present in the filtrate after precipitation of the unhydrolysed hemoglobin with trichloroacetic acid. If the tyrosine in the hemoglobin molecule was not located close to the carboxy-terminal end of the peptide chains, it would not have appeared in the filtrate until a considerable breakdown of the protein had occurred.

The low yield of the products which were finally isolated, indicates that still more of the protein or peptide material was adsorbed very strongly on the resin particles, and was not eluted, even at pH 12 or higher. It is clear that the Dowex 50 used is unsuitable for the separation of such large molecular weight materials, and that probably they should be separated by starch block electrophoresis or on cellulose anion exchange columns (76).

The recovery of one band (number B14), containing only lysine as an end group and with no trace of arginine, is of interest. In view of the proteolysis which has indeed occurred, it is not possible to draw conclusions with certainty; but it seems likely that this peptide must have been freed by the action of the phosphatase upon one or more cross-links between peptide chains. If this is the case, then it should be feasible to prepare larger amounts of peptides released by the phosphatase and to separate them by some other means. The occurrence in the acid hydrolysate of fraction B14 of all the amino acids present in acid hydrolysates of alpha casein, and in nearly the same proportions, clearly indicates that this fraction must have consisted of a major portion of the alpha casein molecule. The other bands probably consisted of materials which were degraded to a greater extent.

4. Kappa Casein

(a) Introduction:

It will be recalled that Waugh and von Hippel (34) separated whole casein, which had been prepared centrifugally, into two fractions by the use of high concentrations of calcium ion. One of these fractions consisted of a mixture of alpha and beta casein, and the other was a mixture of beta casein and a component they designated as kappa casein. They studied the properties and interactions of these fractions at various pH values by the use of the ultracentrifuge, and concluded that kappa casein normally exists combined with alpha casein. The kappa casein formed molecular aggregates with the alpha casein, and these aggregates were not precipitated by low concentrations of calcium ion, although the alpha casein itself was precipitated by similar concentrations of calcium ion. They found that acid-precipitated whole casein contained aggregates of alpha and kappa which were more stable than those of centrifugally-prepared whole casein, but that the stable aggregates could be caused to dissociate by treatment with alkali. Waugh and von Hippel suggested that alpha casein prepared by other workers probably contained, in addition, some kappa casein.

The purpose of the investigations reported here was to prepare kappa casein by separating it from previously prepared alpha casein, and to investigate the properties of the purified alpha and kappa caseins.

(b) Separation of Kappa Casein from Alpha Casein:

Several lots of kappa casein were prepared. In a typical experiment 4 gm. of alpha casein, prepared by the procedure of Warner, was placed in a beaker and suspended in 150 ml. of water. Sodium hydroxide solution (1.0 N.) was added slowly until pH 12 was attained. The solution was left to stand at room temperature for 30 to 45 minutes and then 0.1 N. HCl was slowly added until pH 7 was attained. Enough $CaCl_2.2H_2O$ was added quickly with vigorous stirring to bring the solution to 0.25 M. with respect to calcium ion. The solution and precipitate were left to stand overnight at room temperature, and the precipitate was then removed by centrifugation. The supernatant which contained kappa casein was dialysed against 2 changes of 2 liters of distilled water. This was followed by 2 liters of Versene solution (0.2 per cent) adjusted to pH 8.5, and then another 2 changes of distilled water. HCl

(0.1 N.) was slowly added to the dialysed solution to precipitate the protein. The precipitation appeared to be complete by pH 4.5. The precipitate was washed by decantation with ethanol, followed by peroxide-free ethyl ether, and then was air dried. The yield was 390 mgm. of a fine white powder.

The precipitate, obtained by the addition of calcium chloride, probably consisted of calcium alpha caseinate. This precipitate was dissolved by dialysis against a solution of Versene (0.5 per cent) adjusted to pH 8.5. This procedure was time consuming as the precipitate was gummy, and did not dissolve quickly. The solution was dialysed against two changes of 2 liters each of distilled water. The dialysed solution was diluted to 250 ml. and 0.1 N. HCl was added until pH 4.3 was attained, at which point the flocculation appeared to be complete. The precipitate of alpha casein was allowed to settle, was washed several times by decantation, then dried by decantation with alcohol and peroxide-free ether. The yield was 1.5 grams.

The above separation procedures were also carried out using as a starting material alpha casein which had been prepared by the urea solubility method. From 4 gm. of this material, 51 mgm. of protein which was probably kappa casein was obtained. In another experiment 171 mgm. of this material was obtained from 20 gm. of alpha casein prepared in the same way.

As will be seen from the results of some of the following experiments, the protein prepared by this procedure exhibited properties similar to those of the kappa casein identified by Waugh and von Hippel; it is probable that these two materials are identical.

(c) Experiments on Kappa Casein and Alpha Casein:

In the description of the experiments which follow, abbreviations are employed as listed below.

α1-casein - alpha casein prepared by the procedure of Warner

K1-casein - kappa casein prepared from α1-casein

α2-casein - alpha casein prepared by the urea solubility procedure of Hipp *et al*

K2-casein - kappa casein prepared from α2-casein

α1-K - the alpha casein recovered after the removal of K-casein

α2-K - the alpha casein recovered after treatment to remove the K-casein from α2-casein.

i. Amino acid composition: Ten milligram samples of α1-casein, α1-K, K-casein, and beta casein were placed in small test tubes, 1 ml. of 6 N. HCl was added to each, and the tubes were sealed. The sealed tubes were heated for 12 hours at 120° C. It was noticed that hydrolysates prepared from K1-casein contained the greatest quantity of humin, while those prepared from beta casein and from α1-K contained the least humin. The hydrolysate of α1-casein contained slightly more humin than the hydrolysate of α1-K.

The HCl was removed under vacuum and the residues were dissolved in 0.5 ml. of water containing 10 per cent isopropanol. Ten microliters of each solution was applied to the corners of filter papers (18 x 18 inches). The papers were developed with Solvent 1 in the first dimension and Solvent 2 in the second dimension.

The chromatograms were compared, and with one exception, no differences could be detected between the relative quantities of the amino acids present in K-casein, α1-casein, and in α1-K. The sensitivity of the visual comparison of the chromatograms is such that the differences in amino acid composition between beta casein and α1-casein could be quite easily seen. For example, the quantities of about 10 amino acids in beta casein differ by at least 25 per cent (Table 1) from the quantities present in α1-casein. By visual comparison, differences in concentration of 9 amino acids could be observed in 2-dimensional chromatograms of the two proteins. If, then, no difference whatever could be seen between the chromatograms prepared from K-casein, α1-K, and α1-casein, it is unlikely that the concentrations of the amino acids differ by as much as 25 per cent for any one amino acid between the three protein preparations. The one exception to this was cystine. Chromatograms prepared from hydrolysates of kappa casein all showed strong spots due to cystine. Chromatograms prepared from hydrolysates of α1-casein and α-2 casein all showed weak cystine spots. No cystine spots could be seen on chromatograms prepared from hydrolysates of α1-K and of beta casein.

The occurrence of cystine, in K1-casein but not in α1-K, was also confirmed by the use of the Mulder reaction (77, p.187). Ten milligrams of each lot of casein was placed in a small test tube, and 2 ml. of 20 per cent NaOH and 2 drops of 1 M. lead acetate solution were added. Each tube was heated to 100° C. for

10 minutes. A slight brown colour formed in the tubes containing α1-casein and α2-casein. A very slight colour developed in the tubes containing α2-K. No colour at all was observed in the tube containing beta casein. The tubes containing K1- and K2-caseins developed strong brown colours. This was a very clear demonstration that the cystine of casein is located in the kappa fraction only.

The presence of tryptophane was detected by the Hopkins-Cole Reaction (76, p.119). Two milligrams each of α1-K and of K1-casein were placed in test tubes and 0.5 ml. of 0.04 M. copper sulphate, 1.0 ml. of 5 per cent aqueous glyoxylic acid and 1.0 ml. of concentrated H_2SO_4 were added to each tube, and the tubes heated in a boiling water bath for 10 minutes. The α1-K produced a colour somewhat greater than that produced by the kappa casein.

ii. <u>Phosphorus and nitrogen</u>: The nitrogen contents of α1-casein, α2-casein, α1-K, K1-casein, and K2-casein were determined by the micro-Kjeldahl procedure. After digestion with nitric and perchloric acids, the phosphorus contents were determined by the

Martin and Doty procedure (Table 7).

There was not sufficient kappa casein available to determine the moisture content accurately, so the nitrogen figures are expressed on an air-dry basis. The values for phosphorus, however, are corrected to a basis of 15.6 per cent nitrogen, which is the nitrogen content of α1-casein after drying for 5 hours in a vacuum oven at 100° C.

iii. <u>The carbohydrate content of kappa casein and alpha casein</u>: The occurrence of a much greater quantity of humin in acid hydrolysates of K1-casein than in hydrolysates of α1-casein indicated that the carbohydrate components of casein might be concentrated in the kappa casein fraction.

Hydrolyses of α1-casein, α1-K, K1-casein, α2-casein, α2-K, and K2-casein were carried out by the procedure of Anastassiadis and Common (78) using Dowex 50 resin as a catalyst. The sugars were eluted from the resin with water and determined colorimetrically by means of the anthrone reaction.

Table 7

PHOSPHORUS AND CARBOHYDRATE CONTENTS OF ALPHA AND KAPPA CASEINS

Casein	Per Cent Nitrogen	Per Cent Phosphorus Corr. to 15.6 Per Cent N.	Carbohydrate Mgm./Gm.
α1-casein	14.6	1.06	1.33
α1-K	14.6	1.15	0.50
K1-casein	14.2	0.660	3.79
α2-casein	14.0	1.12	0.89
α2-K	14.8	1.12	0.79
K2-casein	14.1	-	7.64

Table 7 shows that the carbohydrate content of K1-casein is greater than that of either α1-K or α1-casein. Approximately twice the concentration of carbohydrate was found in kappa casein prepared from urea-precipitated alpha casein as that prepared from acid-precipitated alpha casein.

The water eluate from the resin was purified by the method of Reynolds (79), and the sugars present were identified by the use of the chromatographic method published by McFarren <u>et al</u> (80). Two sugars were present, which exhibited R_f values identical to standard samples of galactose and mannose. No other sugars were present.

iv. <u>Electrophoresis of kappa casein and alpha casein</u>: The following samples were subjected to paper electrophoresis under identical conditions:

α1-casein - 400 micrograms/strip
α1-K - 400 micrograms/strip
K1-casein - 400 micrograms/strip
α2-casein - 400 micrograms/strip
α2-K - 400 micrograms/strip
mixture of: α1-K - 400 micrograms/strip
K1-casein - 200 micrograms/strip

All samples produced almost identical patterns. There was no discernable difference in mobility between the various fractions. It was found that there was a slightly greater degree of adsorption of the K1-casein on the paper over which it had travelled than was the case with α1-casein or with α1-K. There was no evidence of a double band on the paper strip with the mixture of α1-K and K1-casein.

v. The end groups of kappa casein and alpha casein: Ten milligrams of K1-casein was dissolved in 1 ml. of 1 M. sodium bicarbonate buffer, and 100 microliters of a 6 per cent solution of FDNB in ethanol was added. The mixture was shaken intermittantly for 4 hours, after which the solution was extracted with 4 changes of 2 ml. each of ethyl ether. Dilute HCl was added until the DNP-derivative of the protein precipitated, and the precipitate was washed with water to remove the salts, care being taken not to lose any of the precipitate. One milliliter of 6 N. HCl was added, and the tube sealed. The tube was heated to 120° C. for 10 hours and, after cooling, the acid solution was extracted with 5 changes of 2 ml. each of ethyl ether. The ether extract was evaporated to a small volume and was applied to a paper for chromatography. The chromatogram was run with the 2-dimensional toluene and phosphate system.

The acid solution remaining after the ether extraction was evaporated to dryness. One drop of water was added to dissolve the residue; this solution was extracted 5 times with one ml. portions of butanone. The butanone extract was evaporated to a small volume, and was chromatographed on a silica gel column.

Samples of α1-casein, α1-K, gamma casein and beta casein were also treated in the same manner as kappa casein, and chromatograms of the DNP amino acids in the hydrolysates were prepared from the derivatives which were formed, as well as from pure samples of the DNP amino acids.

The only amino acid derivatives found on the chromatograms from any of the casein fractions were DNP arginine, di-DNP-lysine, and mono-DNP-lysine. The mono-DNP-lysine is formed by reaction of the FDNP with the ϵ-amino groups of lysine in non-terminal positions in a peptide chain. The other two derivatives are formed by amino terminal amino acids only. It thus appears that all known components of the casein complex contain arginine and lysine as amino terminal end groups.

The quantities of di-DNP-lysine on the paper chromatograms, and DNP arginine recovered from the silica gel column were determined by the method of Levy (52). The spots on the paper chromatogram were cut out, eluted with 4 ml. of water, and the absorbance of the water eluate measured at 350 mμ in the Beckman DU Spectrophotometer. The DNP arginine-containing effluents from the silica gel columns were diluted to an appropriate volume and the absorbance measured.

The amounts of the DNP amino acids isolated are given in Table 8, on the basis of moles of end group per 100,000 grams of protein. The figures as given are uncorrected for losses during hydrolysis.

Table 8

THE AMINO TERMINAL END GROUPS OF SOME CASEIN COMPONENTS

	Moles per 100,000 grams of protein	
	Arginine	Lysine
α1-casein	13.6	0.19
α1-K	15.4	0.21
K1-casein	11.5	0.11
α2-casein	18.7	0.21
β casein	6.6	0.076
γ casein	9.0	0.15

vi. Behaviour towards calcium ion: The behaviour of kappa casein in the presence of calcium ion is different from that of alpha casein. All the following experiments were carried out at pH 7.0, at a protein concentration of approximately 1.0 per cent. The proteins were dissolved by suspending them in water and adding NaOH until pH 7 was attained.

Alpha casein without the kappa removed (α1-casein) was mixed with an equal volume of 0.10 M. calcium chloride solution. There was no precipitate. A white opaque solution, which had the appearance of milk, was formed. This was due to the formation of micelles of calcium caseinate.

Alpha casein (α1-K) was mixed with an equal volume of 0.10 M. calcium chloride solution. A heavy white stringy precipitate formed immediately. Precipitation was apparently complete, as the addition of trichloroacetic acid to the supernatant caused no additional precipitation.

Kappa casein (K1-casein) solution was mixed with equal volumes of 0.10 M. and with 0.50 M. calcium chloride solutions. The mixtures turned slightly opalescent, but no precipitation occurred.

A mixture of α1-K and K1-casein solutions (3 parts α1-K to 1 part K1-casein) turned milky when mixed with an equal volume of 0.10 M. calcium chloride solution. There was no precipitate. This mixture did give a precipitate when mixed with an equal volume of 0.50 M. calcium chloride solution. No white micelles remained in solution, but the supernatant was opalescent.

A mixture of α1-K and K1-casein solutions (4 parts α1-K to 1 part K1) formed white micelles and also a small precipitate when mixed with an equal volume of 0.10 M. calcium chloride solution.

vii. Behaviour towards rennin: Several experiments were carried out to investigate the effect of rennin upon the reactions of alpha and kappa caseins with calcium ion. A commercial rennet extract was employed, and this was added in such concentration that it was diluted to 1 part in 5,000 in the final reaction mixture. The proteins were dissolved by suspending them in water and adding dilute NaOH until pH 6.0 was reached. The protein concentration was about 1.0 per cent, and pH 6.0 was maintained.

The kappa casein (K1) solution was clear. It was mixed with rennet and after 10 minutes it turned opalescent or cloudy.

The solution of K1-casein was mixed with an equal volume of 0.1 M. calcium chloride solution and then with rennet. No change was observed for 10 minutes, but after that a small white precipitate slowly appeared.

Alpha casein (α1-K), when dissolved in water, gave a clear solution. Rennet was added to this solution, and no change could be observed.

Alpha casein (α1-K) and K1-casein solutions were mixed in the proportions of 3 to 1 respectively. The solution was mixed with an equal volume of 0.10 M. calcium chloride solution, and the solution turned milky. Rennet was added to this mixture. A precipitate started to appear in 2 to 5 minutes, and gradually became larger. Eventually most of the protein appeared to have coagulated.

A mixture of α1-K and K1-casein solutions (3 parts of α1-K to 1 part of K1) was mixed with rennet. After 10 minutes this solution was mixed with an equal volume of 0.10 M. calcium chloride solution. A precipitate appeared immediately.

Alpha casein (α1-K) solution was mixed with rennet and allowed to stand for 10 minutes. A solution of K1-casein was then added, followed immediately by an equal volume of 0.10 M. calcium chloride solution. The solution turned milky and no precipitate appeared at first. Precipitation started in 3 to 5 minutes.

A solution of K1-casein was mixed with rennet and allowed to stand for 10 minutes. This solution was then mixed with an equal volume of 0.10 M. calcium chloride solution, and the mixture added to a solution of α1-K. A large precipitate formed immediately.

The results of these experiments show that the curdling of milk by rennin is due chiefly, if not entirely, to the action of this enzyme on kappa casein. Rennin does not affect the reactions of alpha casein with calcium ion. It does, however, destroy the ability of kappa casein to protect alpha casein from coagulation in the presence of calcium ion.

(d) Discussion:

The experiments on the interactions of alpha casein, kappa casein, and calcium ion demonstrate that the kappa casein obtained by the method described here is similar in properties to the kappa casein obtained by Waugh and von Hippel (34). The kappa casein which these workers investigated, however, was mixed with beta casein. The experiments reported here prove that the beta casein is not necessary to the peculiar interactions of kappa and alpha caseins.

The treatment with alkali at pH 12 probably was not severe enough to cause degredation of the protein molecule. There was no loss of phosphorus, which is fairly labile to alkali, due to this treatment. Many proteins are subject to alkali denaturation, which probably involves a disruption of at least the tertiary structure of the molecule. It has been claimed by McMeekin (81) that casein is not subject to denaturation by the usual agents employed for this purpose, so it probably does not possess such a rigid tertiary structure. The optical rotation of solutions of casein is in agreement with this hypothesis (81). Waugh and von Hippel (34) found that the patterns obtained in the ultracentrifuge with alpha casein were not altered by treatment at pH 12. This also lends support to the belief that the molecular structure of the casein has not been damaged by the alkali treatment. The only apparent effect of the alkali treatment is that it loosens the binding (caused by the procedure of acid precipitation) of the kappa casein to the alpha casein. The extract nature of this binding is unknown.

The failure to obtain kappa casein from the alpha casein prepared by the urea solubility method cannot be satisfactorily explained. The values for phosphorus and carbohydrate contents of the urea-separated alpha casein were between the values reported for α1-K and K1-casein. This is evidence that the kappa content of α2-casein may have been reduced by the treatment with urea. The α2-casein had also been precipitated from 50 per cent ethanol before the urea separation, and it is possible that this treatment removed some of the kappa casein. After calcium treatment to separate the kappa casein from the α2-casein, the amounts of the phosphorus and carbohydrate were still intermediate between the values found for α1-K and K-1 casein. This is evidence that the kappa casein remaining bound to the α2-casein was not completely separated by the treatment employed to prepare kappa casein.

The investigations described earlier in this report concerning the action of alkaline intestinal phosphatase on alpha casein should be viewed in the light of these results. The alpha casein employed as a substrate for the phosphatase was the α2-casein, which had been precipitated from 50 per cent ethanol and from urea. The failure to remove all the phosphorus may have been due to the changes in the molecular aggregates which seem to have been caused by one or other of these treatments.

The great similarity in amino acid composition between α1-K and K1-casein probably reflects a similarity in molecular structure, and this in turn may partially explain why the two proteins have not been separated by most of the chemical treatments which have been applied to casein. Seno et al (41) claim to have isolated the N-terminal lysyl peptides from alpha casein and to have established a unique sequence of six amino acids. Since their alpha casein probably contained kappa casein, the finding of a unique sequence may mean that kappa casein possesses this sequence as well as alpha casein. The similarity in structure cannot, of course, be very close, as the proteins have differing phosphorus, cystine, carbohydrate, and possibly tryptophane contents.

The similarity in the amino-terminal end groups is also evidence for a similarity in molecular structure. No particular significance should be attached to the quantitative results reported here for the content of arginine and lysine end groups. Mellon <u>et al</u> (40) found that acid-precipitated alpha casein contained 1.5 moles of lysine end group per 100,000 grams of protein. This figure was arrived at after correction for a loss of 82 per cent during hydrolysis for 24 hours. In the experiments reported here the hydrolyses were carried out for only 10 hours, but the losses were apparently greater. If the figure reported by Mellon <u>et al</u> represents the true quantity of lysine present then the loss in these experiments was close to 88 per cent. If losses of this magnitude do indeed occur, then even the relative values reported in Table 8 probably have little significance. If the minimum molecular weight of 15,000 reported by von Hippel and Waugh (7) is accepted as correct, and if there is one lysine end group per molecule, then there should be 6.7 lysine end groups per 100,000 grams. If this were true then the actual loss on hydrolysis of di-DNP-lysine must have been 97 per cent.

There are at least two possible explanations for these discrepancies: 1) the recovery of the di-DNP-lysine after hydrolysis is much less than was estimated by Mellon _et al_, and was even lower in the experiments reported here or, 2) alpha casein and kappa casein both exist as a mixture of two molecular species, the one being present in the smaller concentration bearing lysine as an amino terminal end group.

These experiments, and most of the experiments reported in the literature with which the author is familiar, have been carried out on casein prepared from pooled milk from several cows. It is suggested that investigations on the structure of the caseins should be carried out on casein obtained from a single animal.

That cystine occurs in kappa casein is of interest. Both beta casein and gamma casein have been reported to lack cystine (35, 36), while whole alpha casein prepared by acid precipitation contains 1.77 moles of cystine per 100,000 grams (35). This value can be reconciled with the minimum molecular weight of 15,000 reported by von Hippel and Waugh (7), if it is assumed that approximately one fourth of alpha casein is kappa casein. This proportion is close to that proposed by Waugh and von Hippel (34).

The carbohydrate-containing peptide isolated by Masamune and Maki (11) was obtained from a hydrolysate of whole casein. Since kappa casein has been shown to contain the highest concentration of carbohydrate of any casein component, it is likely that this peptide originated in the kappa component. It will be recalled that Nitschman _et al_ (13) isolated a glycomacropeptide which was liberated during the curdling of milk by rennet. This peptide also probably originated from the kappa component. The molecular weight of this material was 6,000 to 8,000, which is approximately half the probable molecular weight of kappa casein. As it had a high carbohydrate content it may be that the action of rennin is to split the kappa casein molecule into two halves, one of which normally remains bound to the alpha casein, and the other, containing the carbohydrate, being released into the solution. Now that kappa casein is available it should be possible to obtain higher yields of these peptides, and perhaps study the mode of linkage between the carbohydrate and the protein.

The solubility behaviour of the caseins may be influenced by the carbohydrate content. It was noticed, for example, that the K1-casein was much more readily wetted by water and dissolved at pH 7.0 much more quickly than did α1-K. Alpha casein without the kappa removed was wetted and dissolved at an intermediate rate. The rapid wetting of the kappa casein may be due to the hydrophilic properties of the carbohydrate portion.

The separation of acid-precipitated alpha casein into two components with differing phosphorus contents of course vitiates the conclusions drawn by Perlmann concerning the distribution of phosphorus bonds in alpha casein. Alpha casein probably does consist of at least two peptide chains joined by phosphorus linkages. Kappa casein, however, contains enough cystine to form one cross link per mole. The two purified fractions should be investigated with the same enzymes employed by Perlmann and by Hofman.

Kappa casein contains about two thirds as much phosphorus as α1-K. The high electrophoretic mobility of alpha casein is due in part to the negative charges on the phosphoric acid groups; however, the mobility of kappa casein is the same as that of α1-K. More negative charges must, therefore, be located on the dicarboxylic acids of kappa casein. The difference in the content of the dicarboxylic acids between the two casein components is small or non-existant. Therefore it may be expected that the quantities of amide groups present in kappa casein will be less than in alpha casein.

SUMMARY

1. Alpha casein prepared by the urea solubility method was treated with alkaline intestinal phosphatase which had been purified to remove proteolytic activity. Not more than 80 per cent of the phosphorus was removed. The results of this experiment were not in agreement with those reported by Perlmann.

2. The hydrolysate of alpha casein was chromatographed on Dowex 50 ion exchange resin. The large molecular weight products were eluted only at pH 9 or above. Some of the products were irreversibly bound to the resin. Several peptides were isolated; the proportions of the amino acids present in these peptides indicated that they must have possessed a high molecular weight, and thus they must have been major portions of the alpha casein. These peptides were isolated with a very low yield.

3. At least one of the peptides which was isolated possessed only one amino-terminal end group, indicating that the peptide chains of alpha casein can be separated after reaction with alkaline intestinal phosphatase.

4. It may be concluded from these experiments that the alkaline intestinal phosphatase is not a suitable enzyme for removing the phosphorus from the alpha casein preparation employed here. Dowex 50 is not a suitable resin for the separation of such high molecular weight peptides as were formed in these experiments.

5. A method for the preparation of kappa casein from acid-precipitated alpha casein is described. This method is simple and does not require the use of a high speed centrifuge. The kappa casein prepared by this procedure exhibits physical and chemical properties which would be expected from the results of the experiments of Waugh and von Hippel.

6. Kappa casein could not be obtained, except in very low yield, from urea-precipitated alpha casein.

7. The amino-terminal end groups of the kappa and alpha caseins, as well as those of gamma and beta caseins, were found to be arginine and lysine. This confirms the work of Mellon *et al* on beta casein, but the end groups of the separated alpha and kappa caseins, and gamma casein, have not previously been reported.

8. Several of the properties of kappa casein prepared from acid-precipitated alpha casein were investigated.

The cystine present in casein appears to be located exclusively in the kappa component.

With the exception of cystine, the amino acid composition of kappa casein is very similar to that of alpha casein.

The carbohydrate portion of alpha casein is found chiefly in the kappa component. Galactose and mannose were identified as constituents of this carbohydrate.

The phosphorus content of kappa casein is two thirds as great as that of alpha casein.

The electrophoretic mobility of kappa casein on paper is the same as that of alpha casein.

The chief action of rennin seems to be confined to the kappa casein. Rennin destroys the capacity of the kappa casein to protect alpha casein from precipitation by calcium ion.

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