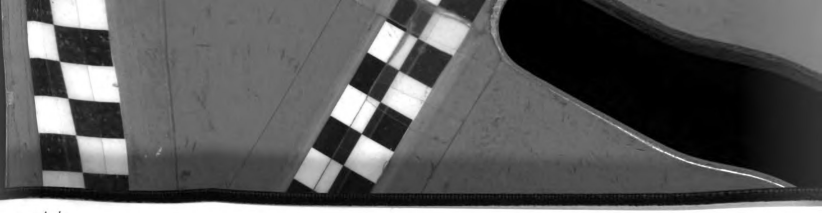

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PHYSICAL AND CHEMICAL CHANGES PRODUCED BY CHYMOTRYPTIC PROTEOLYSIS OF CASEINS

By

Rashid Ahmad Anwar

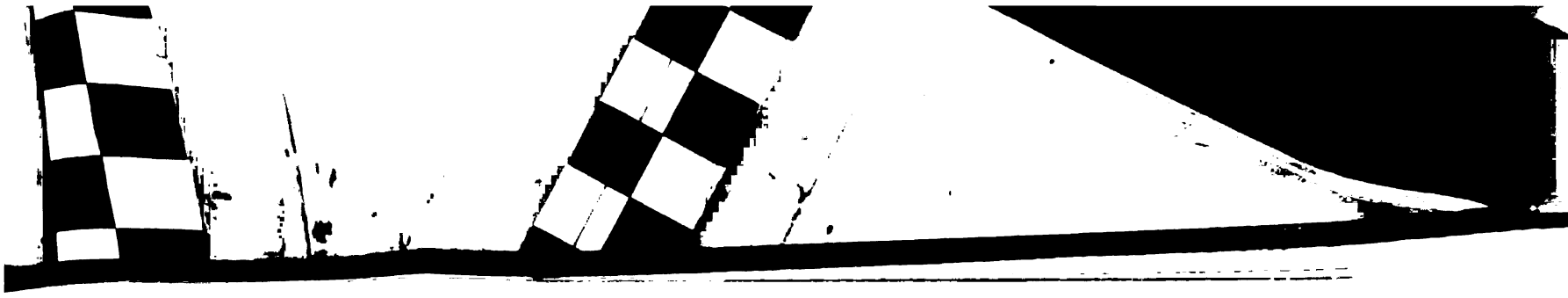
A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1957



ACKNOWLEDGMENTS

The author wishes to express his most sincere appreciation to Dr. Hans A. Lillevik for his patience, deep interest, and active guidance without which it would have been practically impossible to complete this work.

Acknowledgment is also due to other members of the chemistry department for help and advice from time to time, and the American Dairy Association for providing the funds in support of this work.

In addition the author wishes to thank Miss Jane Rhae Smith for technical assistance in the ultracentrifuge analysis.

Finally, he is grateful to all those who helped in the completion of this manuscript, especially the one who helped tracing the figures.

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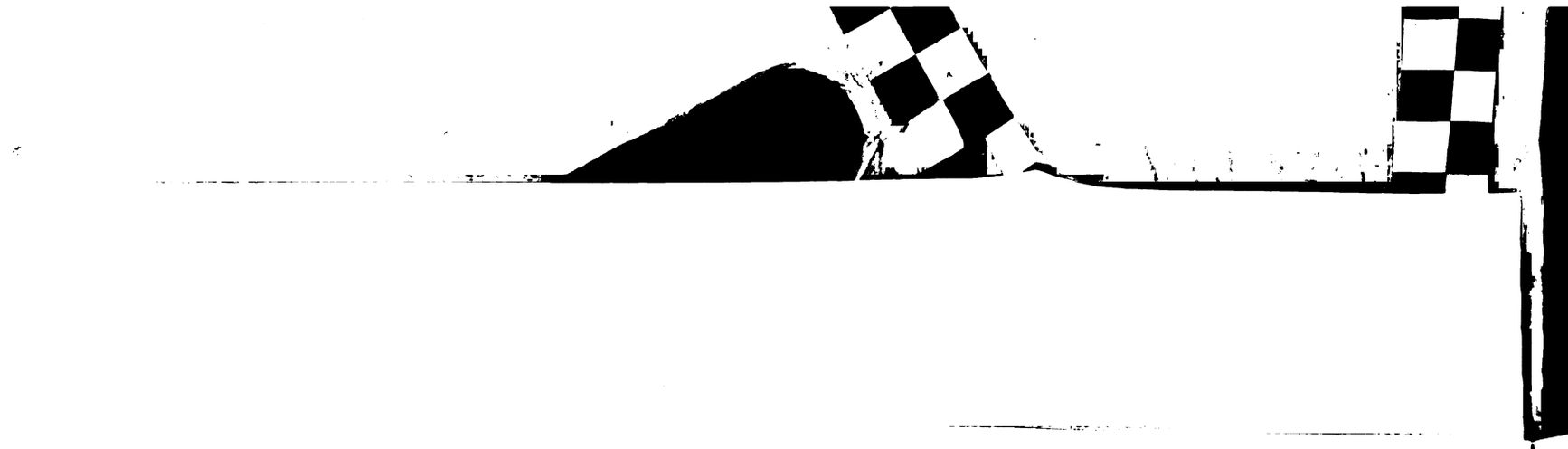

PHYSICAL AND CHEMICAL CHANGES PRODUCED BY CHYMOTRYPTIC PROTEOLYSIS OF CASEINS .

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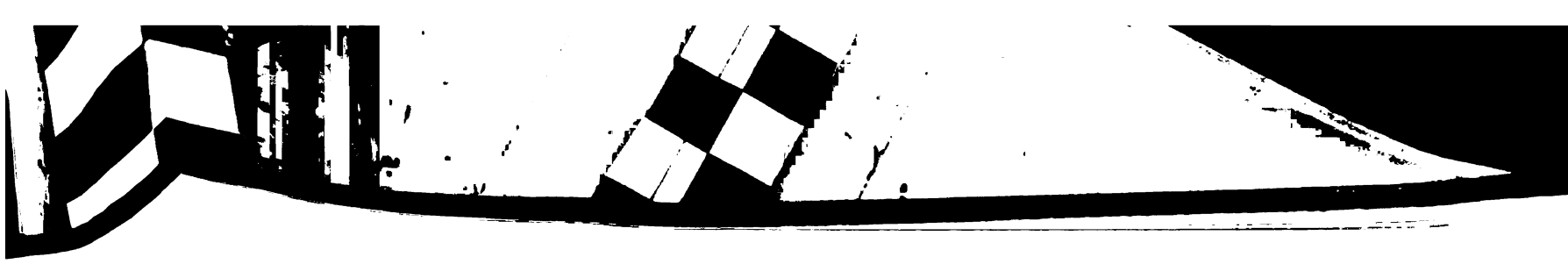
ABSTRACT

A study was made of the action of chymotrypsin upon whole casein and its purified alpha and beta fractions in more or less systematic manner, since very little such work appears to have been reported with this enzyme.

Proteolysis of 3 per cent caseins (whole, alpha or beta) with 0.010 or 0.0165 mg. crystalline chymotrypsin per ml. of digest at pH 7.5 was studied by: electrophoresis; titration in aqueous, alcohol or acetone media; conductivity change; and analysis for nitrogen and phosphorus products made soluble in 10 per cent trichloroacetic acid (TCA).

By moving boundary electrophoretic analysis of isoelectric precipitable products in 0.1M veronal, pH 8.6, it was noticed that both major components of whole casein gradually disappeared. Initially a split in the alpha peak was observed but this was followed by increasing development of both faster and slower peaks.

The same digestion mixture run at pH 5.6 by dilution with an equal volume of 1 M acetate buffer produced a precipitate (at 30°C.) which, after washing and reprecipitations, showed electrophoretically a single component with a mobility of 5.3 Tiselius units. Repeating the experiment upon pure preparations of alpha or beta casein produced the same result. If a sample of the precipitate from alpha or beta casein was mixed with a sample of the precipitate from whole casein the electrophoretic pattern of the mixture again showed a single peak of the same

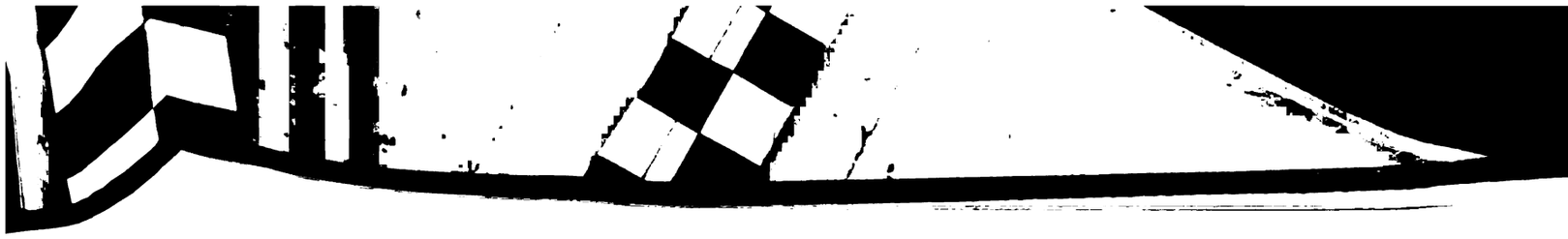


mobility. This casein derivative from whole casein showed two peaks in the ultracentrifuge with sedimentation coefficients of 7.45 (Svedbergs) in peak 1, and 36.4 (Svedbergs) in peak 2, when extrapolated to zero concentration. Its isoelectric point was found to be pH 6.1 and its phosphorus and nitrogen were 0.3 and 15.1 per cent respectively.

The greater titration increaments of potassium hydroxide in alcohol, shown by all casein chymotryptic digests (at 30°C) at pH 7.5, compared with those in either aqueous medium or with hydrochloric acid in acetone, indicate the liberation of acid groups additional to those derived from peptide bond hydrolysis. This suggestion is further substantiated by the finding in these casein digests of phosphorus products (mostly inorganic P) soluble in 10 per cent TCA.

The rate and extent of liberation of TCA soluble phosphorus was greatest from digests of alpha casein and least from those of beta. The inorganic portion of the total acid soluble phosphorus was greater from all preparations. The organic phosphorus portion which was the least of the total TCA soluble, was released more from whole casein than that from alpha casein.

One dimensional paper chromatography of TCA soluble products from whole and alpha caseins showed 2 ninhydrin spots (peptides) with high R_f values. The same two spots could be detected from early stages and upwards to 4 hours of digestion. Beta casein TCA soluble products showed mainly one spot with an R_f corresponding to the faster derived from whole or alpha casein.



The hydrolysates of TCA soluble peptides from whole alpha and beta caseins showed in each case identically 12 amino acid spots by two dimensional paper chromatography which were positively identified. In addition to these residues TCA soluble peptides from whole and alpha casein were found to contain tryptophan, showing that the fast moving peptide(s) common to all 3 proteins did not contain tryptophan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - Continued

I. INTRODUCTION

Since the last quarter of the nineteenth century the biochemically catalyzed reactions of casein with various enzyme preparations have been the subject of many investigations. The principal aims of such work have been of seeking information with regards to protein structure, mechanism of reaction and biological significance of the protein and its derivatives.

Very little of such information has been reported with catalytic effect of crystalline chymotrypsin (an important proteolytic enzyme of pancreatic juice) and no systematic examination appears to have been done with this biocatalyst either on whole casein or its purified fractions.

The importance of casein in nutrition, the important role which chymotrypsin plays in intestinal digestion and lack of information concerning the action of chymotrypsin on casein and the nature of the products resulting therefrom are strong enough basis to justify the investigations carried out in this research.

In the experiments to be described that follows, attempts have been made to study the action of chymotrypsin on whole casein and on its purified alpha and beta fractions in more or less systematic manner with regards to proteolysis, liberation of phosphorus and products formed at different stages of digestion.

II. HISTORICAL

A. Casein and its Fractions

Casein, a phosphorus containing protein of milk, was one of the first proteins to be isolated in relatively pure form. It is present in the milk of all animals so far investigated and can be readily precipitated by the addition of acid. Cow's milk, due to its availability, has been most completely investigated and same is true with the principal protein product thereof; namely, bovine casein. Therefore, the word casein generally refers to bovine casein unless otherwise specified.

Mulder (66) in 1838 published a method for the separation of casein from milk by acidification. Hammarsten (32) prepared casein from diluted skim milk by the addition of acetic acid. In general practice, dilute hydrochloric acid is used for the preparation of acid precipitated casein.

In 1956 Waugh (92) obtained a patent for the preparation of water soluble casein. According to this procedure, casein was not exposed to a higher hydrogen concentration than that of milk. He precipitated casein by the addition of calcium chloride to skim milk and showed his preparation to be highly water soluble (30%) as compared to the acid precipitated preparation (9-10%). Waugh's water soluble casein was recently shown to have close resemblence to acid precipitated casein by Nielsen (67) from his studies on its osmotic pressure, molecular weight and electrophoretic behavior.

Casein was considered to be a pure protein for a long time largely due to the work of Hammarsten. The first evidence of its heterogeneity can be attributed to the work of Osborn and Wakeman (72), who in 1918 isolated a small amount of alcohol soluble protein from isoelectric casein. At that time this protein was merely considered to be the contaminant. In 1925 Linderstrøm-Lang and Kodama (51) from their solubility studies of casein in acid solutions, showed that it is a mixture. Later in 1929 Linderstrøm-Lang (52) was able to obtain fractions, differing greatly in phosphorus content and several other properties, by treatment of casein with ethyl alcohol and hydrochloric acid and precipitating the protein from the extracts with sodium hydroxide. The fractionation studies of Cherbuliez and Meyer in 1933 (9) and Cherbuleiz and Schneider in 1932 (10) also give a strong support to the heterogeneity of casein.

Groh et al. (28) in 1934 reported on the separation of casein by three different methods, namely fractionation by 1) urea, 2) phenol, and 3) alcoholic ammonium hydroxide.

Mellander in 1939 (64) was the first person to demonstrate electrophoretically and beyond doubt the presence of at least three distinct and more or less homogeneous components of casein, and designated them as alpha, beta, and gamma in the decreasing order of their mobilities. At about the same time Cherbuliez and Jeanerat in 1939 (11), had successes in isolating a fourth component of casein and named it delta casein, which appeared to be identical with the whey protein of Hammarsten.

Warner in 1944 (91) reported the isolation of each of the two distinct fractions alpha and beta from whole casein, based on the higher solubility of beta casein at pH 4.5 and 2° C. He, however, pointed out that his fractions although distinct were not electrophoretically homogeneous at all pH's, particularly below their isoelectric points.

In 1950 Hipp et al. (38) separated the gamma fraction from whole casein by taking advantage of its solubility in fifty per cent ethyl alcohol. It was shown to be identical with the alcohol soluble protein earlier described by Osborn and Wakeman (72). Based on the solubility in 5 per cent ammonium sulfate at pH 6 and 40°C Cherbuliez and Baudet (12) the same year isolated two subfractions from alpha casein, with almost identical phosphorus, tyrosine and tryptophan content. The soluble portion was designated as alpha-1 and insoluble as alpha-2 casein.

Hipp et al. in 1951 (17) and 1952 (18) published several successful methods for the fractionation of casein. Their urea method was patented in 1955 and appears to be the most practical one at the present time. Von Tavel and Signer (89) separated alpha and beta casein by counter current distribution, using phenol-water-ethanol or phenol-water-acetic acid as solvent system.

Waugh et al. (93) in 1956 reported the presence of another component in casein which they designated as kappa casein. On treatment of once calcium precipitated casein described earlier (92) with 0.25M calcium chloride at 37°C and pH 7 the alpha component was observed to

dissociate and show a new component (named kappa) in the ultracentrifuge. Alpha and beta caseins rapidly precipitated by this treatment but kappa casein tended to remain in the supernatant. They have reported its phosphorus content to be less than 0.5%.

McMeekin and co-workers (63) at the American Chemical Society Meeting of April, 1957, in Miami, Florida, reported the isolation of fourth component from acid precipitated casein, which was other than alpha, beta or gamma, and designated it as alpha-2 casein. The original alpha casein minus the alpha-2 casein has been designated by them as alpha-1 casein. Alpha-2 casein of McMeekin has phosphorus content of 0.1 to 0.15% and electrophoretic mobility of 5.0 Tiselius Units at pH 8.4 in 0.1M veronal buffer.

The properties of several casein fractions isolated thus far by various workers are summarized by Nielsen (67) in the form of a table.

In conclusion, nothing can be said as to the total number of components present in casein. However, it may be pointed out that for the purpose of investigations to be reported in the following pages, the alpha and beta components which comprise the major share of casein were isolated by the urea method of Hipp et al. (37) and used.

B. Enzyme Catalyzed Hydrolytic Reactions of Casein

(1) Proteolytic Enzymes.

The progress or research in the field of protein hydrolysis by proteolytic enzymes has been slow in spite of the fact that the proteins are the natural substrates for many proteolytic enzymes.

This is mainly due to the complex nature of proteins.

Enzyme catalyzed hydrolysis reactions upon casein have been under investigation for a long time and the clotting of milk is perhaps the oldest such enzymatic reaction known. Rimington and Kay (79) have written a comprehensive historical summary of the work prior to 1926 concerning the action of pepsin and trypsin on casein.

Lubavin (56) in 1871 reported that a greyish deposit was gradually formed when gastric juice was allowed to act upon casein. It contained phosphorus varying with the conditions of experiment. In 1891 this greyish precipitate was given the name of paranuclein by Kossel (45) and pseudonuclein by Hammarsten in 1893 (31) due to its physical similarity with the insoluble nuclein produced from nucleoproteins by the action of pepsin.

Salkowski and Hahn (1895) showed that in the presence of sufficient pepsin the whole of the precipitate (paranuclein) goes into solution (80). This observation was confirmed by Krehl and Matthes (46) in the same year and three years later by Alexander (3), but questioned by Moraczewski (65).

Sebelien (83) also in 1895, working with crude pancreatic enzymes (at that time called trypsin) showed that, unlike the action of pepsin, no paranuclein was formed but that the whole of the casein, except for a negligible residue, went into solution. During tryptic proteolysis of casein Biffi in 1898 found that about 27% of the soluble phosphorus could be precipitated by magnesia mixture (7). This observation was later confirmed by Plimmer and Bayliss (1906) who reported the presence

of, on the average, 35% of the phosphorus as phosphoric acid after tryptic digestion (76).

According to Salkowski, 1899, pepsin first transforms casein in such a way that no precipitate is obtained by the addition of acetic acid and after that the separation of paranuclein begins gradually (80).

Plimmer and Bayliss (76) during 1906 also studied the rates of separation of phosphorus from casein by trypsin, pepsin, papin and alkali. From their studies they concluded that total trichloroacetic acid (TCA) soluble phosphate was released in a way similar to that of the acid soluble nitrogen. Papain when allowed to react in neutral media upon casein produced results similar to trypsin, whereas pepsin was much slower and did not completely solubilize the protein phosphorus.

Rimington and Kay (1926) while studying the action of pepsin, trypsin, bone and kidney phosphatases on casein made the following observations: 1) No inorganic phosphorus was liberated by the action of pepsin even after nine days. 2) Paranuclein containing a large proportion of the original casein phosphorus was obtained. 3) Trypsin brought about the complete hydrolysis of the organic to inorganic phosphorus in a slow process through an intermediate phosphopeptone stage. 4) No hydrolysis was observed with bone phosphatase but there was slight action with kidney phosphatase.

In 1927 Posternak (77) digested casein with trypsin and from the digest isolated a phosphopeptone, containing 5.9% phosphorus, 11.9% nitrogen, and it was composed of glutamic and aspartic acids, serine and isoleucine. He indicated that phosphoric acid was bound to the hydroxyl group of serine.

For the degradation of casein Levene and Hill (49) in 1933 used trypsin and isolated a phosphodipeptide with the aim of finding out where phosphate is attached and to what amino acid. They proposed the structure of this dipeptide to be either phospho-seryl-glutamic acid or glutamyl-serine-phosphate. At about the same time Lipmann (55) isolated phosphoserine from casein and thus proved that phosphoric acid is attached to serine.

In 1935 the effect of various substances e.g. carbohydrates, heavy metal salts, bile salts, etc., on the hydrolysis of casein by pancreatic proteases was studied by Farber and Wynne (22). They found that carbohydrates and bile salts inhibited the enzymes whereas the heavy metals had no effect. Damodaran and Ramachandran (16) in 1941 digested casein initially with pepsin to a paranuclein containing 50 to 60% of the total phosphorus and 20% nitrogen and then with trypsin until constant amino nitrogen was obtained. From such a digest they were able to isolate the barium salt of a phosphopeptone, containing 4.34% phosphorus and 6.46% nitrogen, which was composed of glutamic, isoleucine and serine residues.

Horwitt in 1944 (40), studied the first stage of casein hydrolysis by chymotrypsin and crystalline trypsin. He observed that after the addition of 1 mg. of chymotrypsin in 1 ml. of water to 10 ml. of 6 per cent casein, pH 7.5 at 50°C, the solution became opaque in 1 minute and suggested that this could be used to determine the amount of chymotrypsin. Similar reaction was observed with trypsin when used in greater amount. Winnik (96) in 1944 studied the action of pepsin, trypsin, chymotrypsin,

papain and ficin on casein. After prolonged treatment the average molecules were approximately pentapeptides in digests with chymotrypsin, ficin or papin and heptapeptides in the pepsin and trypsin digests. They also reported the liberation of 1 to 3 per cent of the total nitrogen as free amino acids, determined as carboxyl nitrogen. This study was made on prolonged hydrolysis and with large quantities of the enzymes as compared to the work to be described in this investigation.

The effect of proteolytic enzymes on raw and heated casein was investigated by Eldred and Rodney (21) in 1946. They first treated casein with pepsin at pH 1.8 and then with trypsin and chymotrypsin at pH 7.8 for three to four days. The digestibility of raw and heated casein did not differ; whereas available lysine was found to be less in the case of heated casein as determined by the specific enzyme lysine decarboxylase. Reisen et al. (78), using pepsin, whole "pancrease" and erepsin, observed that longer heating of casein decreased the rate of enzymatic liberation of amino acids.

A comparative study in 1947 of the liberation by pancreatin of four amino acids from casein; namely, tyrosine, tryotophan, histidine, and arginine was made by Beck (5), and he observed that tyrosine was liberated most rapidly.

Hoover and Kokes (39) the same year observed that the digestion of casein by papain was characterized by a rapid production of peptides, averaging four to six units, followed by the release of amino acids without much change in the average length of the peptides present.

They also pointed out that Winnik's experiments were concluded at a point when the production of amino acids was just becoming appreciable.

After six days of digestion with trypsin Sullivan et al. (86) showed that 46 per cent of the total amino acids were liberated from raw casein, whereas 35 per cent from Vitamin free and 26 per cent from commercial dried casein became released. Denton and Elvehjem (18) digested bovine casein and zein with pepsin at pH 2.0 followed by pancrease and duodenal powder at pH 8.0. They noticed wide variations in the liberation of amino acids from casein and zein when measured by biological assay methods, whereas by chemical methods the rate and extent of liberation were approximately the same.

Christensen (13) in 1954 reported his observations about the action of the proteolytic enzymes plasmin, trypsin and chymotrypsin on casein proteins. He studied the action of these enzymes on whole, alpha, and beta caseins, with respect to viscosity changes and liberation of acid soluble material as determined by E280 absorbancy measurements. From the data he concluded that proteolytic hydrolysis of casein does not follow a simple course but that several apparently independent reactions occur and that the complexity of the reaction is due to factors in addition to the presence of several proteins hydrolyzing at different rates.

Peterson et al. (75) in 1954 separated the primary products formed from beta casein by the action of trypsin. They were able to obtain a fraction free of phosphorus and another fraction containing 3 per cent phosphorus. They also reported the further fractionation of the

digest into components, which were essentially electrophoretically homogeneous.

The isolation of a pressor material, pepsitensin, produced by the action of pepsin on casein was reported in 1955 by McGlory et al. (62). It is apparently a polypeptide or a mixture of similar polypeptides and a true product of enzymatic action rather than of autolysis.

Phosphopeptones, obtained from alpha and beta casein by partial hydrolysis with pepsin were isolated by Grove et al. (29) in 1956. They showed that a phosphopeptone gel from alpha casein was insoluble at pH 4.7 and contained essentially one component by electrophoresis, whereas the phosphopeptone from beta casein was largely soluble at pH 4.7, insoluble at pH 3.5 and contained two components in equal amounts.

(2) Effect of Phosphatases on Casein.

An extensive investigation on the effect of phosphatase enzymes on casein and its separated fractions was summarized in 1956 by Perlman (73). From her results, she has been able to throw light on the nature of phosphate bonds in alpha and beta caseins and has also explained why in certain cases phosphatase has not released inorganic phosphorus from whole casein.

Sundrarajan and Sarma (87) in 1956 reported the formation of dephosphorylated casein by the action of ox spleen phosphoproteinphosphatase upon whole casein. They also studied the nature of acid soluble nitrogenous products formed during enzymic dephosphorylation of casein by one dimentional paper chromatography. They concluded that during

the enzymic dephosphorylation of casein, the protein remained relatively intact.

(3) Clotting of Casein.

The enzyme studied most for the clotting of casein is rennin. Chymotrypsin also has milk cotting activity. The nature of the change produced in casein when enzymically clotted is not yet fully understood. Nitschmann and co-workers (1, 61, 61a, 69, 69a) have published a series of papers in recent years, concerning the action of rennin on casein. According to the knowledge at hand, clotting of casein with rennin is considered to be a three step reaction; 1) Casein is changed to modified casein with the simultaneous liberation of non protein nitrogen, 2) Modified casein undergoes moderate thermal denaturation which takes place above 15° C., and 3) Denatured casein crosslinks with calcium ions and gives a clot.

C. Chymotrypsin

Chymotrypsin is one of the three major proteolytic enzymes of pancreatic juice, the others being trypsin and carboxypeptidase. Prior to the individual separation of these enzymes, the combined activity was believed to be due to a single enzyme called trypsin. The term trypsin is now applied to only one enzyme of this group.

Although the isolation and characterization of chymotrypsin was first reported by Kunitz and Northrop (48) in 1935, it was shown as early as 1902 by Vernon (90) that the activity of pancreatic extract as determined by the clotting of milk could be separated from proteolytic

activity as determined by direct methods detecting hydrolysis. He concluded that there were two enzymes. He also showed that one of these was more stable than the other and that the activation of the extract was caused by the less stable one.

Trypsin and chymotrypsin do not exist as such in the pancreas but as proenzymes, called trypsinogen and chymotrypsinogen. The activation of chymotrypsinogen was first studied in 1935 by Kunitz and Northrop (48). Jacobson (42) studied rather in some detail in 1947 the activation of chymotrypsinogen. Since then a number of investigators have worked in this field and have isolated several different chymotrypsins, with practically the same activity and specificity. Their findings indicate the complexity involved in the activation of this proenzyme. According to Janddorf and Michel (43) in 1956 "Many of the postulated intermediates may well be the result of proteolytic processes or changes in the physical state of the proteins, and their importance in the main pathway of activation is largely unknown at present." Some of the different chymotrypsins are alpha, beta, gamma, delta and pi.

Sometimes, in enzymic studies, it becomes desirable to inhibit the enzyme in such a way that the inhibiting agent does not effect the substrate. Inhibition of chymotrypsin has been studied by a number of workers among which Ball and co-workers (44) are the leading investigators. Ball and Jansen (4) wrote a comprehensive review on the stoichiometric inhibition of chymotrypsin. Their own work was mainly concerned with the inhibition of chymotrypsin in the absence of substrate, with a view towards finding the active site.

In 1945 Sizer (84) concluded from his work that sulfhydryl or disulfide groups are not essential for chymotrypsin activity. Wood and Ball (97) in 1955 using partially purified horseradish enzyme showed that oxidation of tryptophan residues reduces the enzymatic activity of chymotrypsin.

Cohen et al. (14) on the basis of their work in 1955 suggested that the final position of the dialkyl phosphoryl group introduced into the chymotrypsin molecule by di-isopropyl fluorophosphate (DFP) was at the hydroxyl group of a serine residue. On the other hand photoxidation studies in 1953 by Weil et al. (94) showed that chymotrypsin was completely inactivated and no longer reacted with DFP when one histidine and three tryptophan residues were destroyed.

Gutfreund and Sturtevant (30) in 1956 presented the evidence that both, serine hydroxyl and imidazole groups are important for proteolytic enzyme activity. Also in the same year, Massey and Hartley (60) supported the view that histidine is the active center of chymotrypsins.

In spite of all this work, no suitable procedure for the inhibition of chymotrypsin in the presence of substrate appears to have been worked out. Schwert et al. (81) while studying the amidase activity of trypsin and chymotrypsin in 1948 used saturated potassium carbonate to liberate ammonia and assumed that this kind of enzyme activity stopped when the reaction mixture came in contact with the reagent.

Gergely et al. (25) in 1955 used di-isopropylfluorophosphate to stop the chymotryptic digestion of myosin. Li et al. (50) a year later stated but without any evidence that one drop of glacial acetic acid

served to stop the reaction of this enzyme upon hypophyseal growth harmone, a polypeptide.

In 1956, Harris (33) demonstrated that chymotrypsin is irreversibly denatured with 8 M urea. However, he expressed the view that in the presence of substrate, the enzyme is stabilized to a considerable extent against this urea inactivation.

Most of the detailed physicochemical measurements have been carried out with alpha chymotrypsin and some of them are as reported below.

Nitrogen	15.5% (71)
Isoelectric point	8.1-8.3 (47)
Sedimentation constant S_{20w}	2.5 S (79)
Molecular weight	27,000 (94)
Crystalline form	Rhombohedrons (71)
pH optimum for casein digestion	7-9 (71)
pH optimum for coagulation	6.5-7.0 (57)

III. EXPERIMENTAL

A. Apparatus

Temperature Control -- A constant temperature bath with a 1/4 inch plate glass front window and constructed in the Kedzie Chemical Laboratory was used for controlling the temperature. It was provided with a reservoir bottle to maintain automatically a constant level of water. The R. B. Instrument Company thermoregulator with Fisher Serfass electronic relay controlled the temperature at 30 ± 0.03°C.

pH Meter -- A Beckman Model H2, glass electrode, line operated pH meter was used for hydrogen ion activity measurements.

Timer -- A Meylan stop watch was used to time the reaction periods.

Glassware -- All pipetts and volumetric glassware used were of Kimble glass brand.

Spectrophotometers -- Absorbance measurements at 280 mμ were made using the Beckman Model DU spectrophotometer. The Beckman Model B spectrophotometer was used in the determination of total and inorganic phosphorus.

Centrifuges -- The International Model 2 centrifuge was used in preparation of acid precipitated casein. This was equipped with a basket attachment. For the determination of inorganic phosphorus an International clinical centrifuge with a size 213 rotor for 15 ml.

centrifuge tubes was used. The Servall refrigerated centrifuge with size SS-1 rotor for 50 ml. stainless steel tubes was used in the preparation and purification of protein precipitated at pH 5.6 after action of chymotrypsin on whole, alpha and beta caseins.

Dialysis -- All the dialyses were made in Visking cellophane tubing, on an external rotating liquid dialyzer constructed by Djang, Lillevik and Ball (19).

Electrophoretic Analyses -- Were made with the Tiselius electrophoresis apparatus Model 138 (Perkin Elmer Corp.). For conductivity measurements, the Model RC-IB conductivity bridge (Industrial Instruments Inc.) equipped with a cell (Perkin Elmer) of 0.4893 constant was used.

Freeze Drying -- Was carried out with the Virtis Freeze Dryer (Virtus Co.).

Digestion Rack -- Was one manufactured by the American Instrument Co., and was used in the digestion of samples for nitrogen and total phosphorus analysis.

Semi-micro Kjeldahl Apparatus -- Fifty ml. digestion flasks were used for the digestion of total phosphorus and nitrogen samples. The distillation apparatus employed was one modified and used in the Kedzie Chemical Laboratory.

Chromatography -- The Chromatocab Model B, (Research Equipment Corp.) was used for descending runs and ascending chromatograms were

developed in the chromatography cabinet manufactured by University Apparatus Co. The chromatograms were dried in the (Research Equipment Corp.) oven constructed for this purpose.

Electromagnetic Stirrer -- An electromagnetic stirrer (Labline Inc.) was used in the alcohol, acetone and water media titration work.

Analytical Ultracentrifuge -- The Spinco Model E (Specialized Instruments Corp.) was utilized for studying the sedimentation behavior of proteins.

B. Materials and Reagents

Chemicals -- All inorganic and organic chemicals used were either C. P. or reagent grade unless otherwise specified.

Enzyme Source -- Crystalline chymotrypsin (salt free from ethanol) supplied by the Nutritional Biochemicals Corp., Cleveland, Ohio, in one gram quantities was used.

Substrates -- Acid precipitated casein was prepared from cow's fresh raw skim milk by the procedure described by Dunn (20). It contained 15.9 per cent moisture and 15.29 per cent nitrogen on moisture free basis, and was stored at -20°C until used. Electrophoretic behavior of the preparation in 0.1M veronal buffer of pH 8.6 is seen from (Figure 17) and appears similar to the one shown by Hipp et al. (37).

Alpha and beta caseins were very kindly supplied by H. C. Nielson who prepared these according to a modification described in his doctoral dissertation (67) of the urea procedure of Hipp et al. (37).

Alpha casein contained 2.6 per cent moisture whereas beta had 3.8 per cent moisture. Their electrophoretic patterns were also similar to those obtained by Hipp et al. (37) under the same conditions.

Casein Stock Solutions -- Six grams of air dried whole, alpha or beta casein was weighed into a 125 ml. erlenmeyer flask. Sixty to seventy ml. of glass distilled water was added in small portions until a smooth paste. (Distilled water mentioned hereafter refers to glass distilled water.) To this was then gradually added 16 ml. of 0.2N sodium hydroxide in the case of whole and beta caseins and 20 ml. of 0.2N sodium hydroxide was used in the case of alpha casein. A mechanical shaker was used to disperse the proteins. After dispersion the solution was heated in a boiling water bath for 15 minutes, cooled to room temperature and its pH was adjusted to 7.5 by the dropwise addition of 0.2N sodium hydroxide. The pH 7.5 solution was then quantitatively transferred to a 100 ml. volumetric flask, made to volume, filtered and supplied with a crystal of thymol as preservative. It was always stored in cold room at 5°C and used within two weeks of its preparation. Thus was produced a 6 per cent (w/v), pH 7.5 stock solution for use as substrate in the enzymatic studies.

Chymotrypsin Stock Solution -- Five mg. of crystalline chymotrypsin was weighed into a 50 ml. volumetric flask and dissolved to volume with distilled water.

Twenty Per Cent (w/v) Trichloroacetic Acid -- Twenty grams of trichloroacetic acid was dissolved in water and volume made to 100 ml.

For ten per cent trichloroacetic acid, the above solution was diluted with equal volume of distilled water.

Fisk-Subbarow Phosphorus Analysis Reagents -- These reagents were prepared as described by Hawk, Oser and Summerson in the thirteenth edition of their text Practical Physiological Chemistry (34).

Reagents for Separation and Analysis of Inorganic Phosphorus --

Five N sodium hydroxide--one hundred grams of sodium hydroxide was weighed out on an analytical balance, transferred quantitatively to a 500 ml. volumetric flask, and diluted to the mark.

0.5N sodium hydroxide--twenty grams of sodium hydroxide was weighed on an analytical balance and transferred quantitatively to a 1.0 liter volumetric flask, and diluted to the mark.

10 Per cent (w/v) calcium chloride reagent--ten grams of calcium chloride was dissolved in an ammonium chloride buffer pH 9.0 (prepared as below), diluted to 100 ml., and saturated with ammonium hydroxide. The reagent was good for one week, when stored in Pyrex bottle and filtered just before use.

Wash reagent was a one to five dilution of the above ten per cent calcium chloride with distilled water.

Ammonium Chloride buffer pH 9.0--was prepared by dissolving 26.7 gms. of ammonium chloride in water and adding concentrated ammonium hydroxide gradually until it reached pH 9.0.

Bromothymol Blue Indicator for use in inorganic phosphorus analysis was prepared by dissolving 0.04 gm. bromothymol blue in 100 ml. of 95 per cent ethanol.

10 N Sulfuric Acid--Accurately measured 280 ml. of concentrated sulfuric acid was diluted with water transferred quantitatively to one liter volumetric flask and made to volume by the addition of distilled water.

Isobutanol Benzene mixture was prepared by mixing equal volumes of isobutanol and thiophene free benzene.

10 Per cent Ammonium Molybdate--Accurately weighed 10 gms. of ammonium molybdate was dissolved in distilled water and volume made to 100 ml.

3.2 Per cent (v/v) Sulfuric acid in Absolute Ethanol--Thirty-two ml. of concentrated sulfuric acid was dissolved in 968 ml. absolute ethanol.

Stannous Chloride stock solution--Ten grams of stannous chloride (Dihydrated) was dissolved in 25 ml. concentrated hydrochloric acid and kept in a refrigerator.

Stannous Chloride working solution--Stock solution of stannous chloride was diluted 200 times with 1N sulfuric acid. This was always freshly prepared immediately before use.

1 N Sulfuric Acid was prepared by diluting 10 N sulfuric acid ten times with distilled water.

Reagents for Nitrogen Determinations

Digestion Mixture--Five hundred ml. of concentrated sulfuric acid was added to 500 ml. of distilled water containing 100 gms. of sodium sulfate and two gms. of copper sulfate.

2 Per cent (w/v) Boric acid was prepared by dissolving 20 gms. of boric acid in distilled water and making the volume to one liter.

Boric Acid--Indicator solution--Two ml. of freshly prepared 0.02 per cent methyl red and one ml. of 0.02 per cent methylene blue was added to 100 ml. of two per cent boric acid solution. It was always prepared just before use.

6 N Sodium Hydroxide--Accurately weighed 240 gms. of sodium hydroxide was quantitatively transferred to one liter volumetric flask, dissolved in distilled water and volume made to the mark.

Veronal buffer pH 8.6 ionic strength--0.1 was prepared by dissolving 21.197 gms. of veronal (5.5 diethyl barbituri-c acid U.S.P.) and 0.1 mole of sodium hydroxide in distilled water and making the volume to one liter.

0.05 N Alcoholic Potassium Hydroxide--3.75 gms. potassium hydroxide was dissolved in 62.5 ml. distilled water and diluted to one liter with 95 per cent ethanol. The reagent was standardized against 0.1067 N hydrochloric acid with phenolphthalein as indicator.

Thymolphthalein Indicator--The indicator solution for the

Willstatter and Waldschmidt-Leitz (1921) titration was prepared by diluting six ml. of 0.5 per cent thymolphthalein in 95 per cent ethanol to 100 ml. with absolute alcohol.

0.05 N Alcoholic Hydrochloric Acid--0.2 ml. of concentrated hydrochloric acid was diluted to one liter with 90 per cent ethanol and finally standardized against 0.05 N alcoholic potassium hydroxide using phenolphthalein as indicator.

Naphthyl Red Indicator--0.1 gm. of Naphthyl red (4-benzene-azo-naphthylamine-1) was dissolved in 96 per cent alcohol and volume made to 100 ml.

1 M Acetate Buffer--Was prepared by the gradual addition of one normal sodium hydroxide to one mole of acetic acid (57.4 ml. of glacial acetic acid) until the required pH 5.6 was attained. A buffer of pH 4.6 was also similarly prepared. Approximate amounts of sodium hydroxide required in each case were precalculated using Henderson-Hasselbach equation as described by Gortner (26).

Other Buffers--All other buffers used in electrophoretic determinations were of ionic strength 0.1 and necessary amount of monobasic acid required for 0.1M sodium hydroxide was calculated using Henderson-Hasselbach equation. For phosphate buffers both the Lewis ionic strength equation (58) and the Henderson-Hasselbach equation (26) were solved simultaneously to get the necessary amounts of acid and alkali. In every case the pH was checked and adjusted on the pH meter.

Solvent Systems for Paper Chromatography

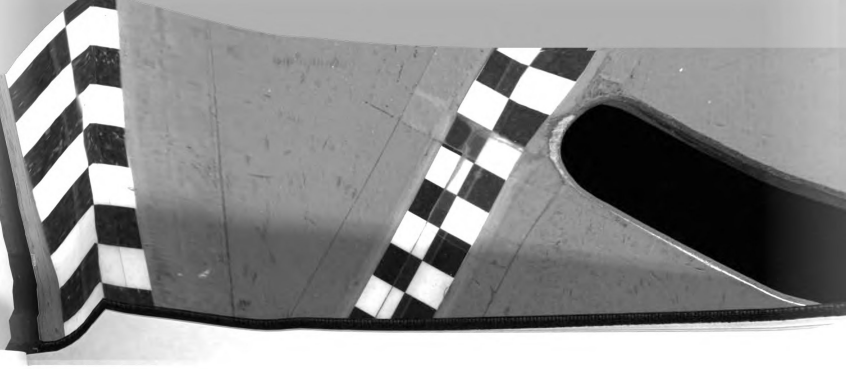
Butanol : acetic acid : water (4:1:5) was prepared according to Slotta (1951) (85). For the preparation of water saturated phenol, 39 ml. of distilled water was added to 100 gm. of phenol and made 0.1 per cent with respect to alpha benzoin oxime as recommended by Consden et al. (15).

2,6-Lutidine : collidine : water (1:1:1) was made according to Dent (17), and was added 1-2 per cent diethylamine.

Ninhydrin solution for the detection of spot was prepared by dissolving 0.1 gm. of ninhydrin in 100 ml. of ethanol containing 5 per cent v/v collidine.

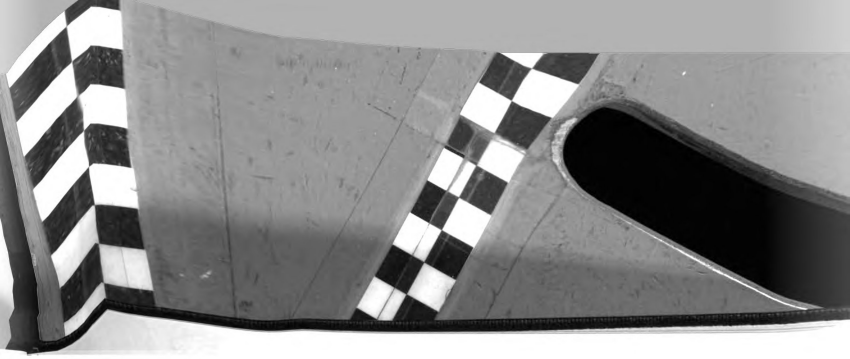
C. Experimental Procedures

Enzymatic Digestion -- A suitable volume of 6 per cent casein solution (alpha, beta or whole) was pipetted into one arm (50 ml. capacity) of a bifurcated test tube. Into the other arm was added an equal volume of suitable diluted chymotrypsin solution (0.01 per cent w/v stock solution diluted one to three or one to five was used). When the total volume of the digestion mixture was expected to be more than 40 ml., two 125 ml. Erlenmeyer flasks, one for the substrate and the other for the enzyme solution, were used. The digestion vessel(s) was/were placed into a constant temperature water bath held at 30°C. Before mixing, the solutions were allowed to stand for 20 minutes in the bath to bring them to temperature. The digestion was started by mixing the two solutions thoroughly. The time of initial contact of



the two solutions was taken as zero digestion time and was noted by starting the stop watch. Appropriate aliquots of digestion mixture were removed at specified times and proteolysis arrested for the type of analysis to be described.

Alcoholic potassium hydroxide titration for total acidity change— The enzyme concentration used was 0.0165 mg./ml. of digest (stock solution diluted one to three). One ml. aliquots were removed from the digestion mixture at intervals and were immediately titrated in alcohol according to the method of Willstatter and Waldschmidt-Leitz (95). This method is a modification of Foreman's (24a) original alcoholic sodium hydroxide titration. The aliquots removed were directly pipetted into three ml. of absolute alcohol-indicator mixture contained in 25 x 100 mm. test tubes. Each sample was then titrated against 0.05 N alcoholic potassium hydroxide solution to a distinct blue color; six ml. of absolute alcohol was added and the sample again titrated to the appearance of permanent blue color. A five ml. burette calibrated to 0.02 ml. was used. The sample was kept well stirred during the process of titration with the aid of electromagnetic stirrer which also aids in keeping minimum time for minimum carbon dioxide interference. The initial titer obtained from the aliquot taken immediately after mixing (zero time) was subtracted from subsequent titers to get the increment in ml. (Δ ml.) of standard alcoholic potassium hydroxide required for titration of the acid groups produced during digestion, per ml. of the digest. The results obtained are reported as μM of potassium hydroxide required to neutralize the acid groups produced



during digestion, per ml. of the digest in Table I and shown in Figure 1.

Aqueous potassium hydroxide titration -- For aqueous titrations, one ml. aliquots were removed from the same digestion mixture at specified intervals of time and added directly to 9 ml. of distilled water containing thymolphthalein indicator, already placed in 25 x 100 mm. test tubes. Each aliquot thus removed was immediately titrated against the same standard alcoholic potassium hydroxide, using the same burette as mentioned above, to the appearance of blue color. The solution was kept well stirred during titration with the help of an electromagnetic stirrer. The initial titer obtained from the aliquot taken immediately after mixing was subtracted from the subsequent titers as mentioned in alcoholic potassium hydroxide titration. The results were also treated and expressed in the same manner as in alcoholic potassium hydroxide titration. See Table I and Figure 1.

Linderstrøm-Lang's Acetone titration with hydrochloric acid (53)-- In principle the procedure followed was the same with minor modifications as discussed in detail by Jacobson (42). The concentration of chymotrypsin was 0.0165 mg./ml. of digestion mixture. Two ml. aliquots of digest were removed at intervals and quantitatively transferred to 25 x 100 mm. test tubes, containing a pre-determined quantity of aqueous 0.1067 N hydrochloric acid. This amount (0.3-0.6 ml.) was such that the mixture of acid, protein solution and acetone together with indicator, in the beginning of the experiment showed a suitable

red-yellow color. Each aliquot was then immediately titrated with small increments of 0.05 N hydrochloric acid to the appearance of a permanent red color, during which time eight ml. of acetone was gradually supplied. Not all of the acetone was added to the sample tube before starting the titration but was added in portions to avoid protein precipitation. A burette of one ml. capacity and calibrated to read to 0.01 ml. was used. During titration the solution was well stirred with the aid of an electromagnetic stirrer. The initial titer obtained from the aliquot taken immediately after mixing (zero time) was subtracted from the subsequent titers. Thus the increment ml. (Δ ml.) of standard alcoholic hydrochloric acid required to titrate the basic groups produced during digestion was obtained from 2 ml. of the digest. The results obtained are reported in terms of μM of hydrochloric acid required to neutralize the basic groups produced during digestion per ml. of the digest in Table I and shown in Figure 1.

Conductivity changes -- The resistance changes during proteolysis of digests containing 0.0165 mg. of chymotrypsin per ml. of the digest were recorded at desired time intervals. The specific conductance was calculated from the ohms measured and cell constant value of 0.4893 and is reported in Table V.

Inhibition Studies of Chymotrypsin Activity on Caseins -- To study the electrophoretic changes produced during digestion, it was desirable to inhibit the chymotrypsin activity at desired time intervals and yet produce practically no effect on the protein or products under

investigation. A number of possibilities were tested. The exact procedure was as follows. To three ml. of six per cent casein solution was added an equal volume of diluted 0.1 per cent (w/v) chymotrypsin solution (stock solution diluted one to five). Immediately after mixing, a three ml. aliquot of the solution (pH 7.5) was removed and accorded the inhibition treatment under consideration. (These treatments are listed in the next paragraph.) The solution was then adjusted to a protein concentration of about 1.5 per cent by the addition of the appropriate buffer (3 ml.), dialyzed against this buffer to equilibrium, and electrophoretically analyzed (electrophoresis procedure to be described). When the electrophoretic patterns of the inhibitor treated casein-enzyme mixture and the normal casein (no enzyme) solution were similar, it was regarded as having produced inhibition of chymotrypsin activity. When digests inhibited by hydrogen ion concentrations of pH 6 and less formed a precipitate during dialysis against buffers of same pH, no electrophoretic analysis was made. Since the appearance of such a precipitate was not evident for enzyme free casein solutions under similar conditions, it was taken to be a sufficient proof that chymotrypsin was not inhibited.

Inhibition Treatments of an aliquot of the digestion mixture were made by:

1. Addition of three drops of glacial acetic acid to one ml. of digest, dialysis against 0.1M veronal buffer pH 8.6.
2. Digestion mixture plus three drops of glacial acetic acid and

heated to boiling for 30 seconds. It was dialyzed against veronal buffer pH 8.6.

3. Digestion mixture was heated to boiling for 30 seconds on direct flame and then supplied with three drops of glacial acetic acid. Dialysis was done against veronal buffer pH 8.6.
4. Digestion mixture was precipitated with trichloroacetic acid, filtered, precipitate redissolved in veronal buffer pH 8.6 and dialyzed against veronal buffer.
5. Digestion mixture was brought to pH 5.6 by the addition of 1 M acetate buffer pH 5.6 and dialyzed against the same buffer.
6. Digestion mixture was brought to pH 12 by the addition of phosphate buffer and dialyzed against the same buffer.
7. Three ml. of digestion mixture was added directly to 3 ml. of distilled water containing enough urea so that the total concentration of urea after the addition of digestion mixture was 8 M. The mixture was heated to bring all the urea in solution and allowed to stand for a few hours. It was then dialyzed against veronal buffer pH 8.6. (Harris 1956).
8. The digestion mixture was added to 1 M acetate buffer pH 4.6 to produce isoelectric precipitation. The precipitate formed was washed, reprecipitated, and finally dissolved and dialyzed in veronal buffer pH 8.6.

Isoelectric precipitation, washing five times with two reprecipitations was found to be the only satisfactory way of getting rid of and producing inhibition of chymotrypsin. Thus a study of proteolytic

changes by electrophorectic analyses of the digests was accomplished by taking 3 ml. of aliquots (which were removed from the digestion mixture) at desired intervals of time and then the enzyme was removed by iso-electric precipitation procedure with washing, etc., as described above. The precipitate from each aliquot rendered practically free from enzyme, was dissolved, dialyzed and electrophoretically analyzed in six ml. of 0.1 M veronal buffer pH 8.6, ionic strength 0.1.

Electrophoresis -- The procedure described in the instruction manual for the Perkin-Elmer electrophoresis instrument was used. Usually, one to one and a half per cent protein solution was equilibrated by dialysis, against 300 ml. of the selected buffer (mostly 0.1M veronal of pH 8.6), at 5°C.

Digestion Products Soluble in 10 per cent (w/v) Trichloroacetic Acid -- Ten to 15 ml. aliquots were removed from the digestion mixture at selected time intervals and pipetted directly into an equal volume of 20 per cent (w/v) of trichloroacetic acid. These samples were shaken intermittantly during a 30 minute period and then filtered through Whatmann No. 2 filter paper. The filtrates were analyzed for total acid soluble phosphorus, inorganic phosphorus, products absorbing at 280 mμ, non-protein nitrogen, peptides and amino acids hydrolysable therefrom by paper chromatography, which is described as follows:

1. Absorbancy at 280 mμ -- The filtrate from the zero digestion time sample was set at 100 per cent transmission or (absorbancy) in the DU Beckman spectrophotometer at 280 mμ as a blank. The subsequent time

samples were compared against the blank and the changes found in absorbancy units are reported in Tables VI, VII and VIII and shown in Figures 5 and 6.

2. Total Phosphorus (T.C.A. Soluble) -- The procedure followed was essentially the same as described in the text by Hawk and co-authors (34). Five ml. of the above protein free filtrate was pipetted into a 50 ml. micro Kjeldahl digestion flask and 2.5 ml. of 5N sulfuric acid (along with two glass beads to prevent bumping) was added. The flask was heated on the micro-Kjeldahl digestion rack until the evaporation was complete and the mixture turned brown or black, with no further change. The sample was cooled slightly, treated with one drop of 30 per cent hydrogen peroxide (Baker's) and heated again. The addition of hydrogen peroxide and heating was repeated until the contents of the flask were colorless. About 3 to 4 ml. of distilled water was then added to the cooled flask and heated momentarily to boiling. The flask was cooled again and its contents were rinsed into a 25 ml. volumetric flask. The total phosphorus present was determined by the Fisk and Subborow (23) method. A blank and phosphorus standard solutions were run in the same manner. The results reported in ug./ml. (or per cent) are given in Tables II, III and IV and shown in Figures 2 and 3.

3. Inorganic phosphorus.-- The method devised and found applicable to proteolysates for the analysis of inorganic phosphorus is the result of parts of procedures described by Norberg (70) and the Berenblum and Chain method (8) as modified by Martin and Doty (59). None of these methods alone could give recoverable results upon analysis of known

amounts of phosphate added to proteolytic digests. One ml. of protein-free trichloroacetic acid filtrate was pipetted into an 11 ml. glass stoppered conical centrifuge tube. The filtrate was neutralized by initial dropwise addition of 5 N sodium hydroxide and finally 0.5N sodium hydroxide to the green color of bromo thymol blue indicator (pH ca 7.0).

To the neutralized aliquot was added one ml. of precipitating agent, which consisted of 1 ml. of 10 per cent (w/v) calcium chloride in 0.5M ammonium chloride buffer of pH 9.0, saturated with calcium hydroxide. After the mixture stood for 30 minutes, the precipitate which formed was centrifuged and washed with 5 ml. of a one to five dilution of the precipitating reagent. The washed precipitate was redissolved in 3 ml. of 10 per cent trichloroacetic acid, and treated with 5 ml. of 1:1 isobutanol-benzene mixture, 0.5 ml. of 10N sulfuric acid and 0.5 ml. of 10 per cent ammonium molybdate. The mixture was well shaken in the glass stoppered centrifuge tube for 15 seconds.

After separation of the two layers which occurred after mild centrifugation, 3 ml. of the upper phase was transferred with the aid of pipette into a new 15 ml. glass centrifuge tube. To this was then added 2 ml. of 3.2 per cent sulfuric acid in absolute ethanol and 0.5 ml. of diluted stannous chloride solution. Immediate mixing produced a blue color whose intensity was measured at 625 mμ in the Beckman Model B spectrophotometer. A blank and a phosphorus standard solutions were run in the same manner. The results obtained in terms of ug./ml. digest and per cent of total protein phosphorus are given in Tables II, III and IV and shown in Figures 2 and 3.

4. Non-Protein Nitrogen -- Five ml. of trichloroacetic acid filtrate was pipetted into 50 ml. digestion flask and two ml. of digestion mixture added. The sample was digested for several hours on micro-Kjeldahl digestion rack to a pale blue-green color. The flask was placed on distillation apparatus, and 10 to 12 ml. of 6N sodium hydroxide was used to liberate the ammonia. The ammonia was distilled into 10 ml. of a boric acid-indicator mixture and titrated against 0.01N hydrochloric acid. A reagent blank was also run along with the unknown samples. The results are given in Tables VI, VII and VIII and shown in Figures 5 and 6.

5. Paper Chromatography -- An attempt was made to characterize the trichloroacetic acid soluble split products produced during digestion by paper chromatography. Eight to 10 ml. of each of the filtrates, taken at different time intervals, was extracted six times with ether to remove T.C.A. The aqueous layer, after final extraction, was separated and evaporated to dryness under vacuum in a desiccator. The residue was dissolved in about 2 ml. of distilled water. About 10 µl. of this concentrated solution was applied as a spot onto a sheet of Whatman No. 1 paper ($18\frac{1}{4}$" x $22\frac{1}{2}$") and the chromatograms were developed by the ascending technique. Three different solvent systems were tried. Butanol : acetic acid : water (4:1:5) and phenol saturated with water gave almost identical results, whereas no movement of the material could be detected using the lutidine-collidine solvent system. Two spots very close to each other and with high R_f values were detected in case of whole and alpha caseins, whereas beta casein gave a single spot

corresponding to the faster of the two derived from either whole or alpha casein. From the chromatographic analysis it appeared that the split products were the same throughout the digestion (up to four hours), only increasing in amount. Traced patterns of the chromatograms are shown in Figures 7 to 16.

The remainder of the concentrated filtrate solution was again evaporated to dryness under reduced pressure. Five ml. of 6N hydrochloric acid was added to the residue and it was hydrolyzed in sealed glass tubes at 110°C for 40 to 48 hours. During hydrolysis black humin was formed in the samples only from whole and alpha caseins, but no humin was observed in the samples from beta casein.

After hydrolysis, hydrochloric acid was repeatedly evaporated in vacuo and the residue was taken up in about 2 to 3 ml. of water. One directional ascending chromatograms were run using water-saturated-phenol as the solvent system. At least eight spots were detected with ninhydrin reagent and were similar from all the samples and from all three caseins (whole, alpha and beta). To completely identify the amino acids, two dimensional chromatograms were run. Butanol : acetic acid : water solvent system was used on a descending run and water saturated phenol for ascending (other right angular direction) run. For the detection of the spots, the chromatograms were sprayed with 0.1 per cent ninhydrin in a mixture of 5 per cent collidine and 95 per cent ethanol and heated in a chromatography oven at 100°C for a few minutes.

At least 12 spots could be detected with the aid of two dimensional chromatograms and these spots were identified as specific known amino acids by running standard amino acids along with the unknown solution. The results are shown in Figures 11 to 16.

In addition to the 12 amino acids found, the presence of tryptophan was indicated by the formation of humin in the case of whole and alpha caseins (see Lillevik and Sandstrom (54)).

Preparation of A Casein Derivative Precipitable at pH 5.6 with Chrymotrypsin -- This precipitate was first observed while trying to inhibit chymotrypsin below pH 6.0 as suggested from the data of Northrop (71). Appropriate amount of 6 per cent casein solution was mixed with equal volume of diluted chymotrypsin solution (stock solution diluted one to five). One molar acetate buffer pH 5.6 equal to the combined volume of casein and chymotrypsin solutions was then added and the mixture allowed to stand at 30°C. A white precipitate separated out after three hours. The appearance of the precipitate was much earlier in the case of pure beta casein and quite slower in the case of pure alpha casein. In every case it was observed that the digestion mixture when allowed to stand overnight gave cleaner precipitate.

The precipitate thus formed was separated in the refrigerated centrifuge operated at 8 to 10 thousand R.P.M. and 0°C. This precipitate was washed 5 times with water containing a small amount of pH 5.6 acetate buffer, then redissolved with aid of 0.2N sodium hydroxide and reprecipitated with the same buffer twice more. Finally the precipitate

was lyophyllized and stored in the deep freezer for further studies. The same precipitate was also obtained, as indicated by electrophoretic analysis, by digesting at pH 7.5 for 35 minutes and then adding an equal volume of pH 5.6 acetate buffer.

Studies on the Casein Derivative Precipitatable at pH 5.6 --

1. Electrophoretic studies.--Between a 1.0 to 1.5 per cent (w/v) solution of the casein derivative (or pH 5.6 precipitate) was electrophoretically analyzed in 0.1M veronal pH 8.6 as previously described and this precipitate from whole casein showed essentially a single peak. Preparations from pure alpha and beta caseins under similar conditions, gave similar results. For further proof of similarity in the precipitates from all three protein preparations, the precipitate derived from whole casein was mixed with that from alpha. The mixture was electrophoretically analyzed and found to show again a single peak. Similar treatment accorded to the precipitate mixture from whole and beta caseins gave same results.

2. Isoelectric pH of the casein derivative. This was determined by running the electrophoretic analysis at different hydrogen ion concentrations, both below and above the isoelectric point. The results are shown in Figure 23 and given in Table IX.

3. Nitrogen and Phosphorus Content. An accurately weighed 30 mg. quantity of pH 5.6 precipitate from whole casein was dissolved in three ml. of concentrated hydrochloric acid. One ml. of this solution was digested and analyzed for nitrogen content, as described under non-protein nitrogen analysis, and 1 ml. was digested for phosphorus

determination as described under total phosphorus procedure. The results are given in Table XI.

4. Analysis in the Ultracentrifuge. The sedimentation behavior of the casein derivative when dissolved in 0.1M veronal buffer of pH 8.6 was studied using the Spinco analytical ultracentrifuge run at 24°C and 59780 R.P.M. For comparison, pure alpha and beta preparations were similarly studied for their sedimentation behaviors under identical conditions. The results are given in Table X and shown in Figures 25 to 27.

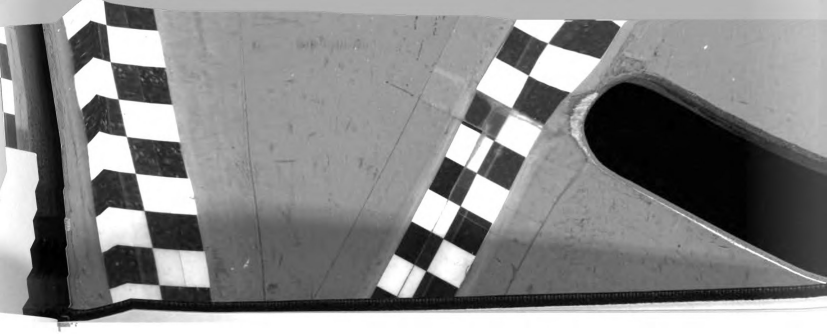


TABLE I

LIBERATION OF ACIDIC AND BASIC GROUPS DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS, USING 0.0165 MG. OF CHYMOTRYPSIN PER ML. OF DIGEST, AS DETERMINED BY TITRATION IN ALCOHOL, WATER AND ACETONE MEDIA

Digestion Time (minutes)	Alcohol Medium: Δ ml. of 0.045N KOH/ml.	Alcohol Medium: δμM KOH	Water Medium: Δ ml. of 0.045N KOH/ml.	Water Medium: δμM KOH	Acetone Medium: Δ ml. of 0.06N HCl/ml.	Acetone Medium: δμM HCl
			Whole Casein			
0	0	0	0	0	0	0
15	0.03	1.35	0.03	1.35	0.02	1.20
30	0.07	3.15	0.065	2.92	0.03	1.80
60	0.13	5.85	0.11	4.95	0.05	3.00
120	0.16	7.20	0.13	5.85	0.06	3.60
240	0.20	9.00	0.14	6.30	0.09	5.40
			Alpha Casein			
0	0	0	0	0	0	0
15	0.06	2.7	0.03	1.35	0.07	4.20
30	0.14	6.3	0.065	2.93	0.105	6.30
60	0.21	9.45	0.115	5.20	0.14	8.40
120	0.28	12.62	0.16	7.22	0.17	10.20
240	0.32	14.43	0.20	9.02	0.191	11.46
			Beta Casein			
0	0	0	0	0	0	0
15	0.02	0.90	0.00	0.00	0.015	0.90
30	0.03	1.35	0.00	0.00	0.03	1.95
60	0.07	3.15	0.03	1.35	0.045	2.7
120	0.09	4.05	0.06	2.70	0.055	3.30
240	0.11	4.95	0.08	3.6	0.075	4.5

TABLE II

LIBERATION OF ACID SOLUBLE PHOSPHORUS DURING PROTEOLYSIS OF 3% WHOLE CASEIN WITH CHYMOTRYPSIN

Digestion Time (minutes)	Total Acid Soluble Phosphorus		Inorganic Phosphorus		Organic Phosphorus	
	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.
	0.0165 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	5.95	5.54	5.94	5.53	0.01	0.01
30	9.86	9.19	7.39	6.87	2.47	2.32
60	10.36	9.65	7.58	7.05	2.78	2.60
120	11.41	10.61	7.48	6.96	3.93	3.65
240	11.82	11.00	7.91	7.36	3.91	3.64
	0.010 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	4.1	3.82	2.1	1.96	2.00	1.86
30	7.5	7.0	4.13	3.85	3.37	3.15
60	10.58	9.85	6.2	5.78	4.38	4.07
120	11.8	11.0	6.9	6.44	4.9	4.56
240	12.00	11.2	7.1	6.62	4.9	4.56

TABLE III

LIBERATION OF ACID SOLUBLE PHOSPHORUS DURING PROTEOLYSIS OF 3% ALPHA CASEIN WITH CHYMOTRYPSIN

Digestion Time (minutes)	Total Acid Soluble Phosphorus		Inorganic Phosphorus		Organic Phosphorus	
	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.
	0.0165 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	5.5	3.8	3.0	2.07	2.5	1.73
30	10.0	6.92	7.6	5.26	2.4	1.66
60	15.16	10.48	10.840	7.5	4.32	2.98
120	15.4	10.65	12.40	8.58	3.0	2.07
240	15.4	10.65	12.6	8.6	2.8	2.05
	0.010 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	2.8	1.935	2.4	1.66	0.4	0.275
30	6.5	4.49	4.56	3.16	1.94	1.33
60	11.0	7.60	8.2	5.67	2.8	1.93
120	14.0	9.67	10.3	7.11	3.7	2.56
240	14.0	9.67	10.6	7.33	3.4	2.34

TABLE IV

LIBERATION OF ACID SOLUBLE PHOSPHORUS DURING PROTEOLYSIS OF 3% BETA CASEIN WITH CHYMOTRYPSIN

Digestion Time (minutes)	Total Acid Soluble Phosphorus		Inorganic Phosphorus		Organic Phosphorus	
	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.	µg/ml.	Percent of total Protein P.
	0.0165 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	0.73	0.83	0.48	0.54	0.25	0.29
30	1.1	1.25	0.94	1.07	0.16	0.18
60	1.3	1.475	1.0	1.14	0.3	0.34
120	1.5	1.7	1.1	1.25	0.4	0.45
240	1.88	2.14	1.3	1.48	0.58	0.66
	0.010 mg. Chymotrypsin per ml. of Digest					
0	0	0	0	0	0	0
15	0.60	0.68	0.30	0.34	0.30	0.34
30	1.0	1.135	0.50	0.57	0.5	0.565
60	1.4	1.59	0.80	0.91	0.6	0.68
120	1.56	1.77	1.0	1.14	0.56	0.63
240	2.6	2.945	1.26	1.43	1.34	1.515

TABLE V

CHANGE IN CONDUCTIVITY DURING PROTEOLYSIS OF 3% WHOLE, ALPHA, AND BETA CASEINS, WITH 0.0165 MG. OF CHYMOTRYPSIN PER ML. OF DIGEST

Digestion Time (minutes)	Whole Casein		Alpha Casein		Beta Casein	
	Resistance (ohms)	Specific Conductance (mhos)	Resistance (ohms)	Specific Conductance (mhos)	Resistance (ohms)	Specific Conductance (mhos)
0	450	1.085	326	1.5	480	1.02
15	440	1.11	321	1.52	461	1.06
30	430	1.137	316	1.545	450	1.085
60	420	1.16	311	1.57	448	1.09
120	420	1.16	309	1.58	447	1.092
240	419	1.163	309	1.58	447	1.092

TABLE VI

NON-PROTEIN NITROGEN DURING PROTEOLYSIS OF 3% WHOLE CASEIN WITH CHYMOTRYPSIN

Digestion Time (mimutes)	Kjeldahl N		E_{280}
	µg./ml.	Percent of Total Protein N	Absorbancy Units
	0.0165 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	6.16	3.19	0.402
30	10.78	5.57	0.74
60	14.56	7.58	1.24
120	21.08	10.93	1.93
240	28.21	14.7	∞
	0.010 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	5.65	2.92	0.257
30	9.10	4.70	0.568
60	13.77	7.12	0.89
120	20.30	10.49	1.5
240	26.09	13.48	> 2.0

TABLE VII

NON-PROTEIN NITROGEN DURING PROTEOLYSIS OF
3% ALPHA CASEIN WITH CHYMOTRYPSIN

Digestion Time (minutes)	Kjeldahl N		E_{280}
	µg./ml.	Percent of Total Protein N	Absorbancy Units
	0.0165 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	6.41	2.82	0.503
30	11.16	4.92	1.02
60	16.34	7.20	1.92
120	25.07	11.05	∞
240	35.24	15.46	∞
	0.010 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	4.54	1.99	0.308
30	8.60	3.78	0.635
60	12.74	5.60	1.20
120	18.80	8.27	2.00
240	27.60	12.15	∞

TABLE VIII

NON-PROTEIN NITROGEN DURING PROTEOLYSIS OF
3% BETA CASEIN WITH CHYMOTRYPSIN

Digestion Time (minutes)	Kjeldahl N		E_{280}
	Δμg./ml.	Percent of Total Protein N	Absorbancy Units
	0.0165 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	2.58	1.15	0.139
30	4.82	2.15	0.238
60	7.76	3.46	0.358
120	11.96	5.34	0.522
240	17.98	8.02	0.790
	0.010 mg. Chymotrypsin per ml. of Digest		
0	0	0	0
15	1.93	0.86	0.048
30	3.50	1.56	0.107
60	5.60	2.49	0.195
120	8.45	3.76	0.31
240	13.30	5.92	0.473

TABLE IX

ELECTROPHORETIC MOBILITIES OF A CASEIN DERIVATIVE PRODUCED BY CHYMOTRYPSIN AND PRECIPITABLE AT pH 5.6 AT DIFFERENT HYDROGEN ION CONCENTRATIONS

pH	Buffer 0.1 M	Mobility (Tiselius Units)
3.50	Acetate	5.44
4.00	Acetate	4.55
8.60	Veronal	-5.30
9.00	Veronal	-6.05

TABLE X

APPROXIMATE SEDIMENTATION COEFFICIENTS OF THE CASEIN DERIVATIVE, ALPHA CASEIN AND BETA CASEIN AT 24° IN 0.1 M VERONAL BUFFER pH 8.6

Protein	Concentration	Sedimentation Coefficient (Svedbergs)
Casein derivative	3%	
Peak 1		5.96
Peak 2		18.04
Casein derivative	1.5%	
Peak 1		6.73
Peak 2		27.3
Alpha casein	3%	4.44
Alpha casein	1.0%	4.58
Beta casein	3%	4.9
Beta casein	1.5%	7.8

TABLE XI

SOME PHYSICOCHEMICAL PROPERTIES OF THE CASEIN DERIVATIVE PRODUCED BY CHYMOTRYPSIN AND PRECIPITABLE AT pH 5.6

Nitrogen	15.10%
Phosphorus	0.30%
Electrophoretic mobility in 0.1M veronal buffer pH 8.6	5.3 Tiselius units
Isoelectric point	pH 6.10 (Figure 23)
Sedimentation coefficient in 0.1M veronal buffer pH 8.6, at 24°	Peak 1. 7.45 (Svedbergs) (Figure 24) Peak 2. 36.4 (Svedbergs)

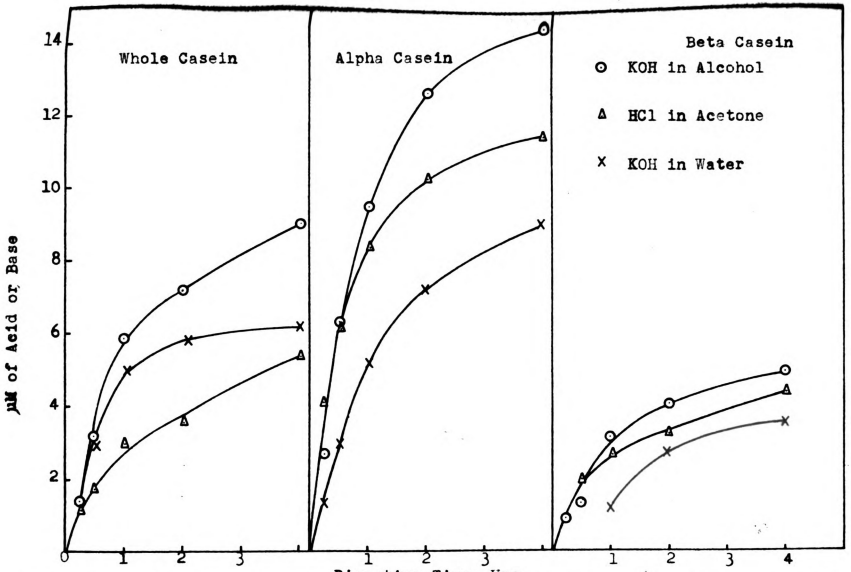


Figure 1. CONSUMPTION OF ACID OR BASE IN ALCOHOL, ACETONE AND WATER MEDIA, DURING DIGESTION OF 3% CASEIN SOLUTIONS. With 0.0165 mg. of chymotrypsin per ml. of digest.

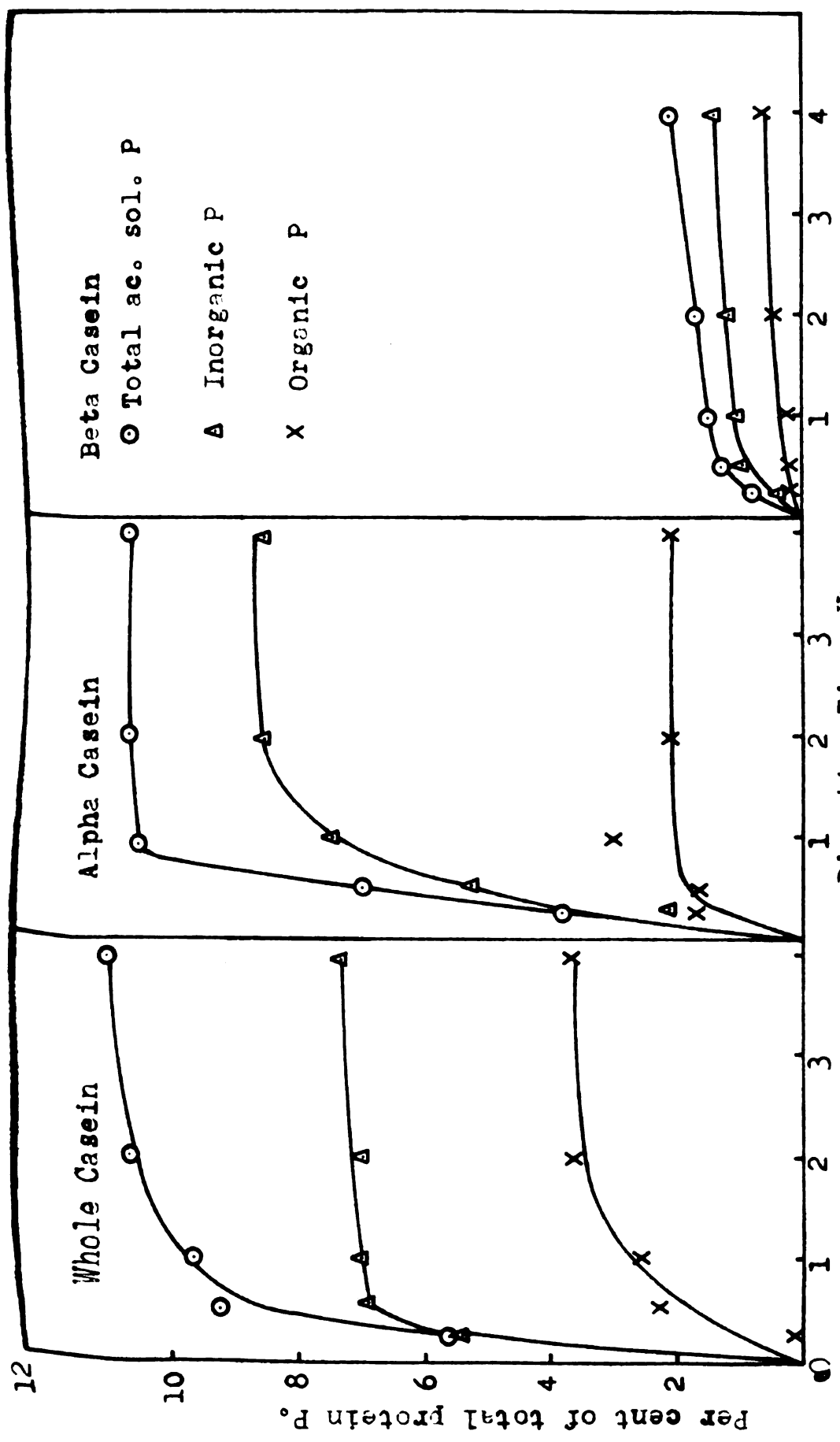


Figure 2. LIBERATION OF PHOSPHORUS DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS. Chymotrypsin concentration of 0.0165 mg. per ml. of digest.

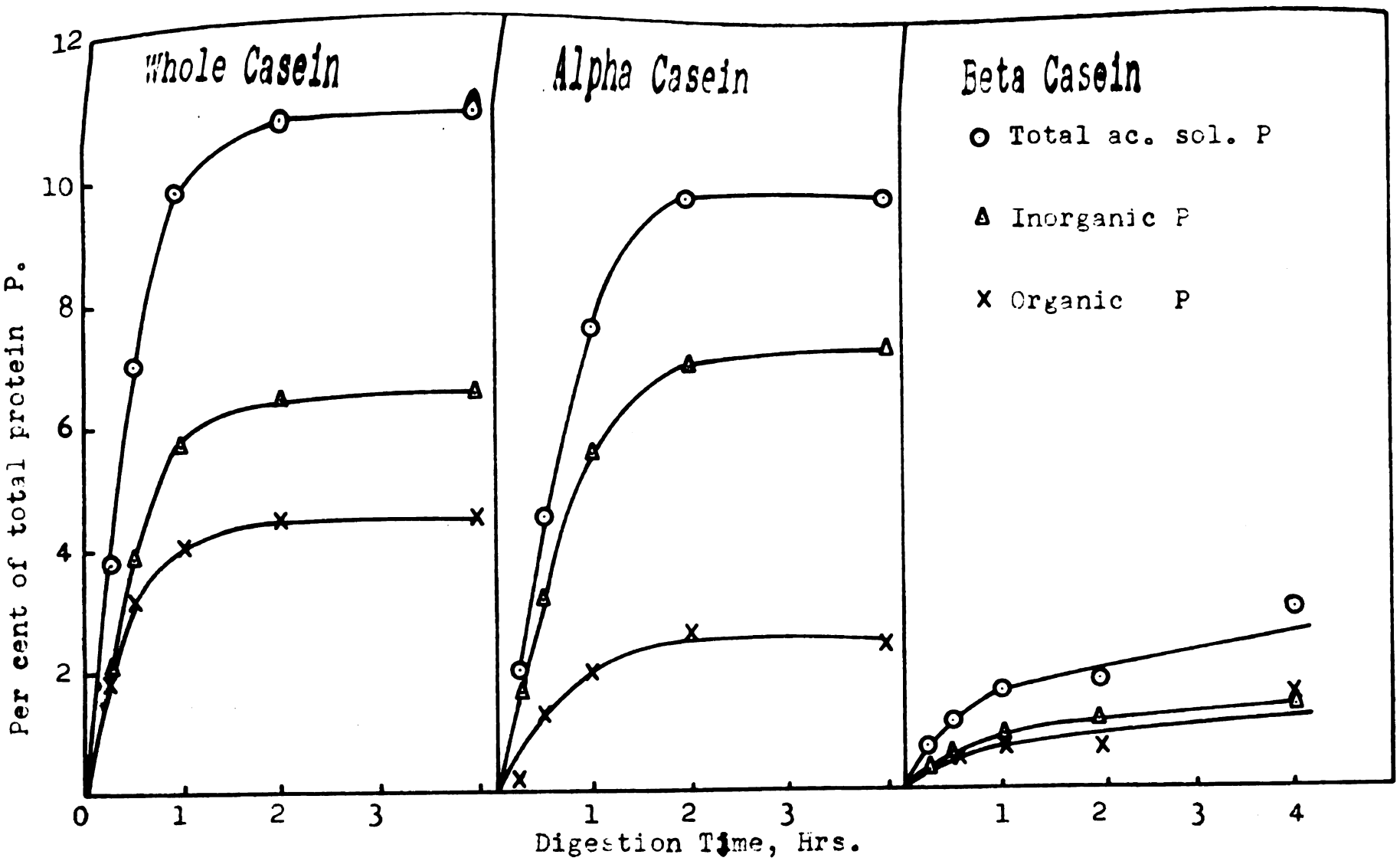


Figure 3. LIBERATION OF PHOSPHORUS DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS. Chymotrypsin concentration of 0.010 mg. per ml. of digest.

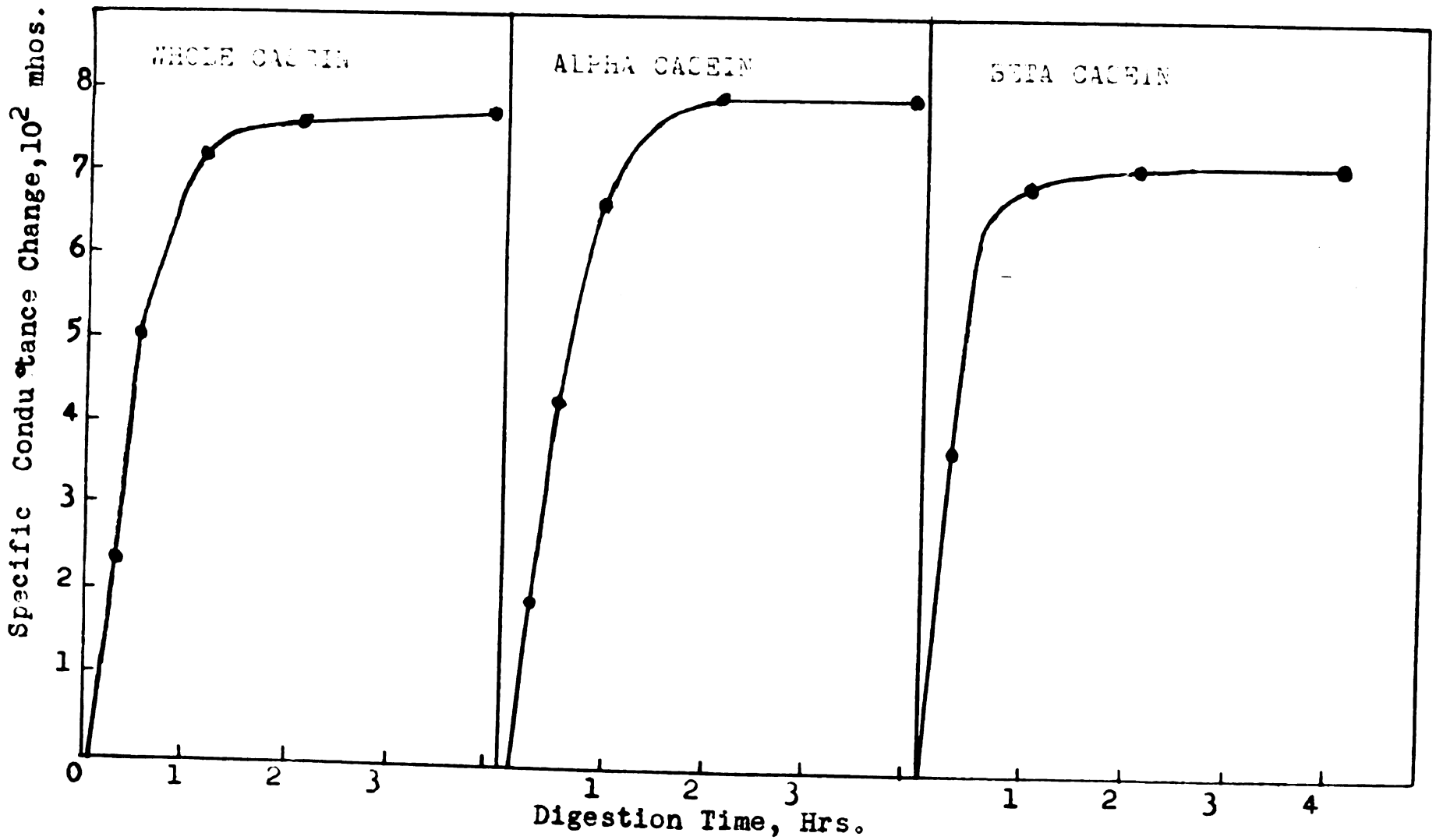


Figure 4. SPECIFIC CONDUCTANCE CHANGE DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS. Chymotrypsin concentration of 0.0165 mg. per ml. of digest.

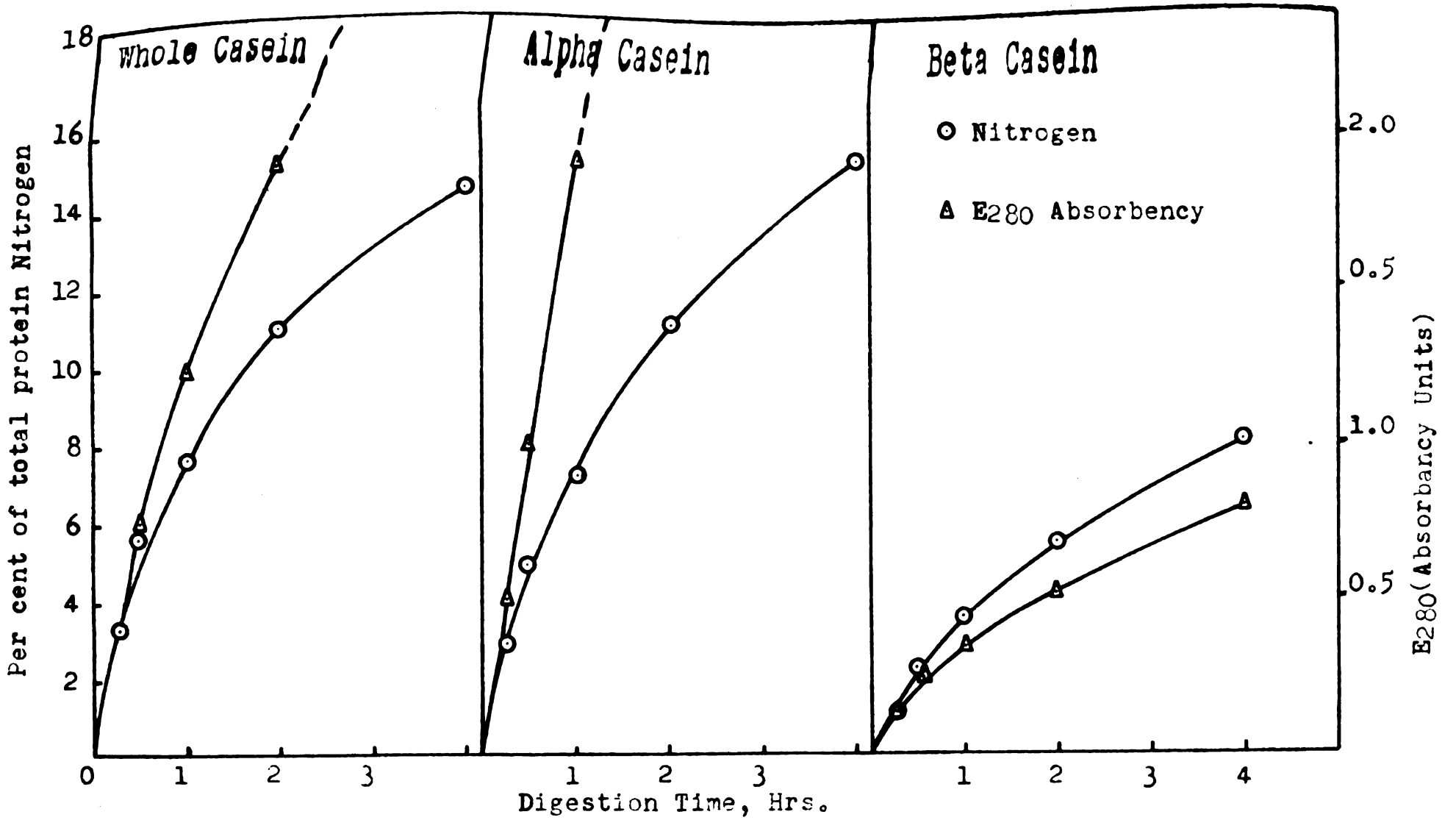


Figure 5. LIBERATION OF TCA SOLUBLE NITROGEN AND E_{280} ABSORBING SUBSTANCES DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS. Chymotrypsin concentration of 0.0165 mg. per ml. of digest.

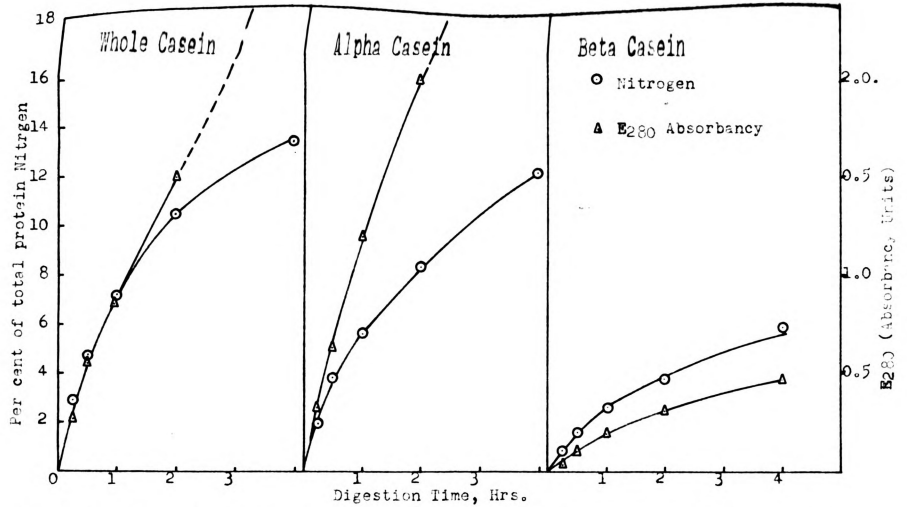


Figure 6. LIBERATION OF TCA SOLUBLE NITROGEN AND E_{280} ABSORBING SUBSTANCES, DURING PROTEOLYSIS OF 3% CASEIN SOLUTIONS. Chymotrypsin concentration of 0.010 mg. per ml. of digest.

Figure 7. ONE DIMENSIONAL PAPER CHROMATOGRAM OF TCA SOLUBLE PEPTIDES. Released during digestion of 3% whole casein with 0.010 mg. of chymotrypsin per ml. of digest. 0, 1, 2, 3, 4, and 5 represent 0, 15, 30, 60, 120, and 240 min. digest samples.

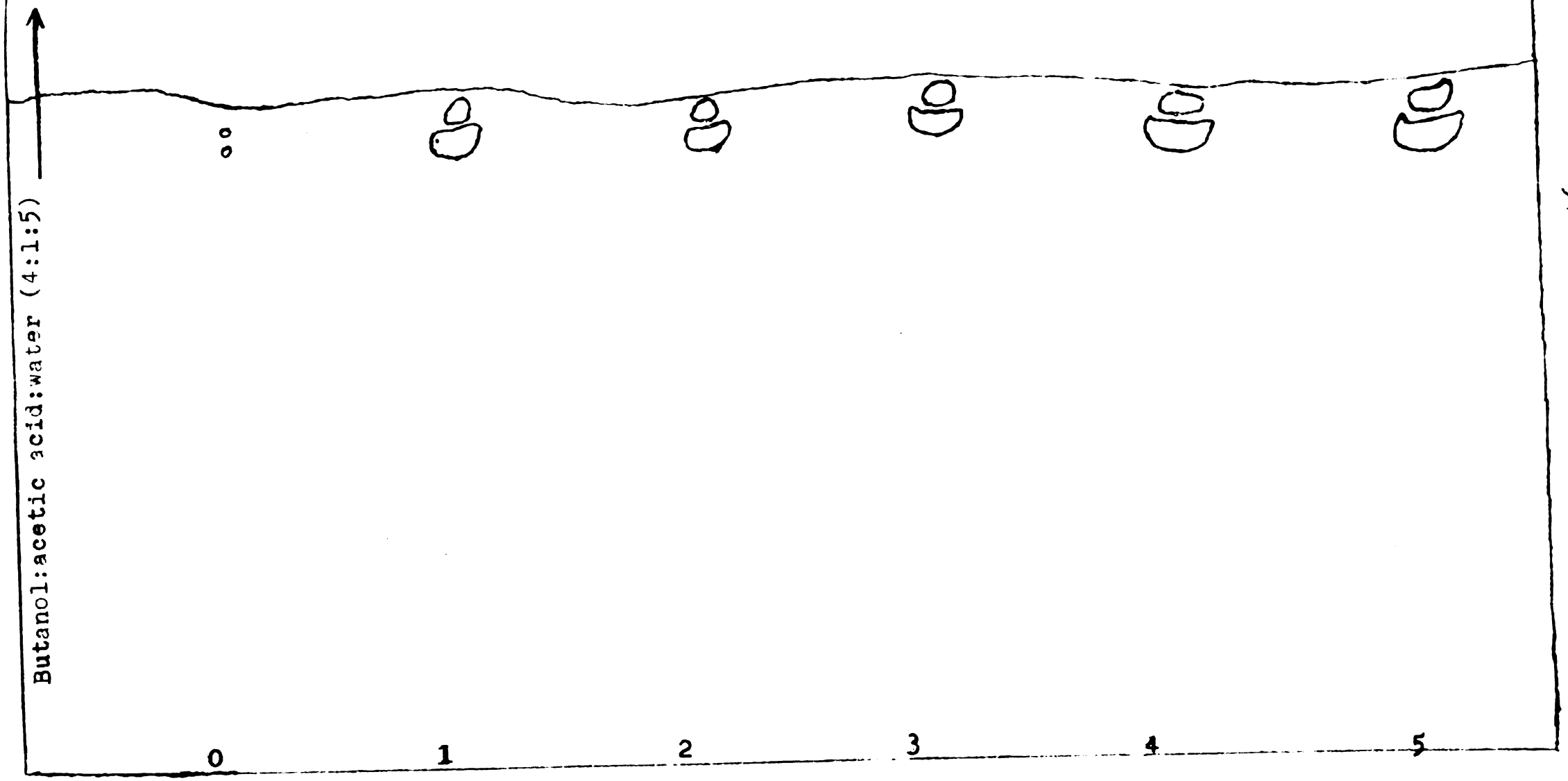


Figure 8. ONE DIMENSIONAL PAPER CHROMATOGRAM OF TCA SOLUBLE PEPTIDES.Released during proteolysis of 3% casein solutions. 1,3,and 5 represent 4 hrs. digests of whole,alpha and beta caseins with 0.010 mg. chymotrypsin per ml.of digest. 6,2,and 4 represent 4 hrs. digests of whole,alpha and beta caseins with 0.0165 mg. of chymotrypsin per ml. of digest.

Figure 9. ONE DIMENSIONAL PAPER CHROMATOGRAM OF HYDROLYSATES OF TCA SOLUBLE PEPTIDES. Released during proteolysis of casein solutions. 1,2,and 3 represent 15 min.digests of whole, alpha and beta caseins. 4,5,and 6 represent 4 hrs.digests of whole,alpha and beta caseins with 0.010 mg. chymotrypsin per ml.of digest.

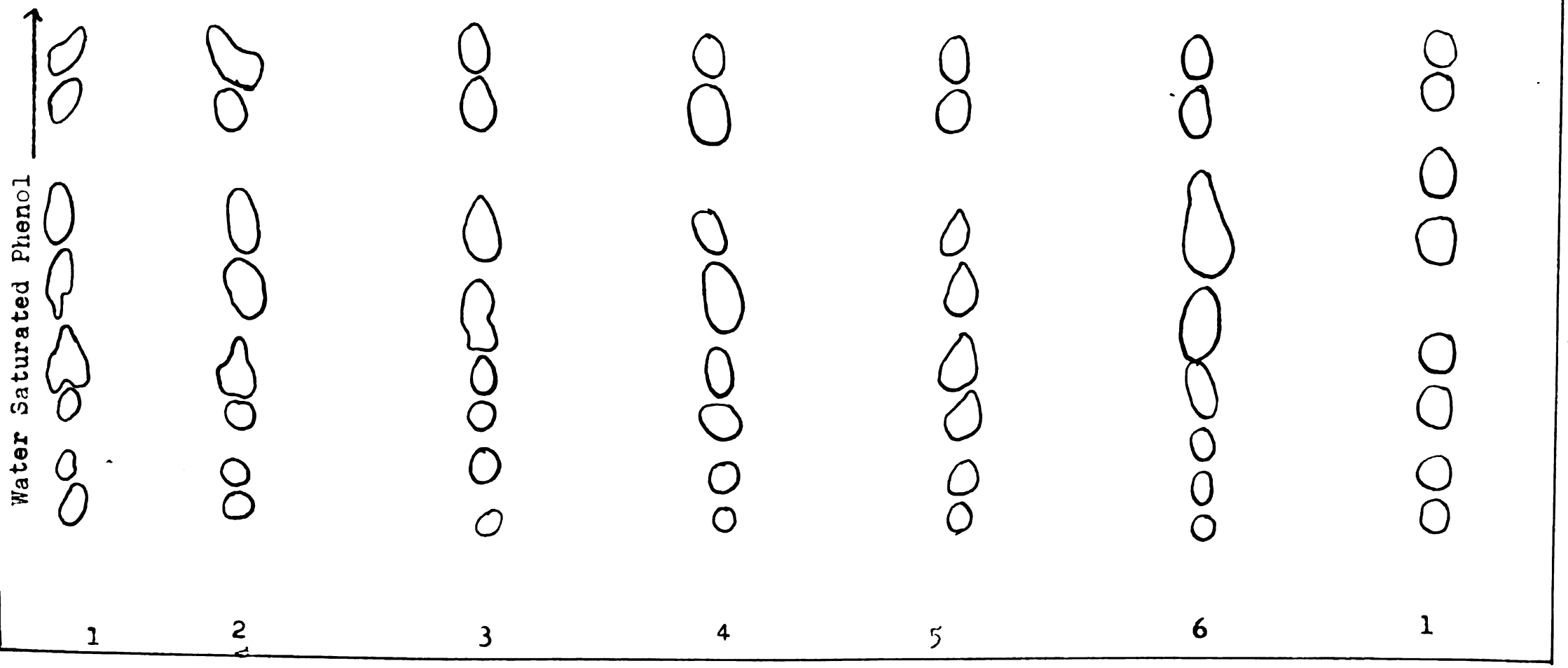


Figure 1). [illegible] PAPER CHROMATOGRAM OF HYDROLYSATES OF TCA SOLUBLE PEPTIDES. Released during proteolysis of casein solutions. 1,2,and 5 represent 10 min. digests of whole, alpha and beta caseins. 3,4,and 6 represent 4 hrs. digests of whole, alpha and beta caseins with 0.01(5) mg. chymotrypsin per ml. of digest.

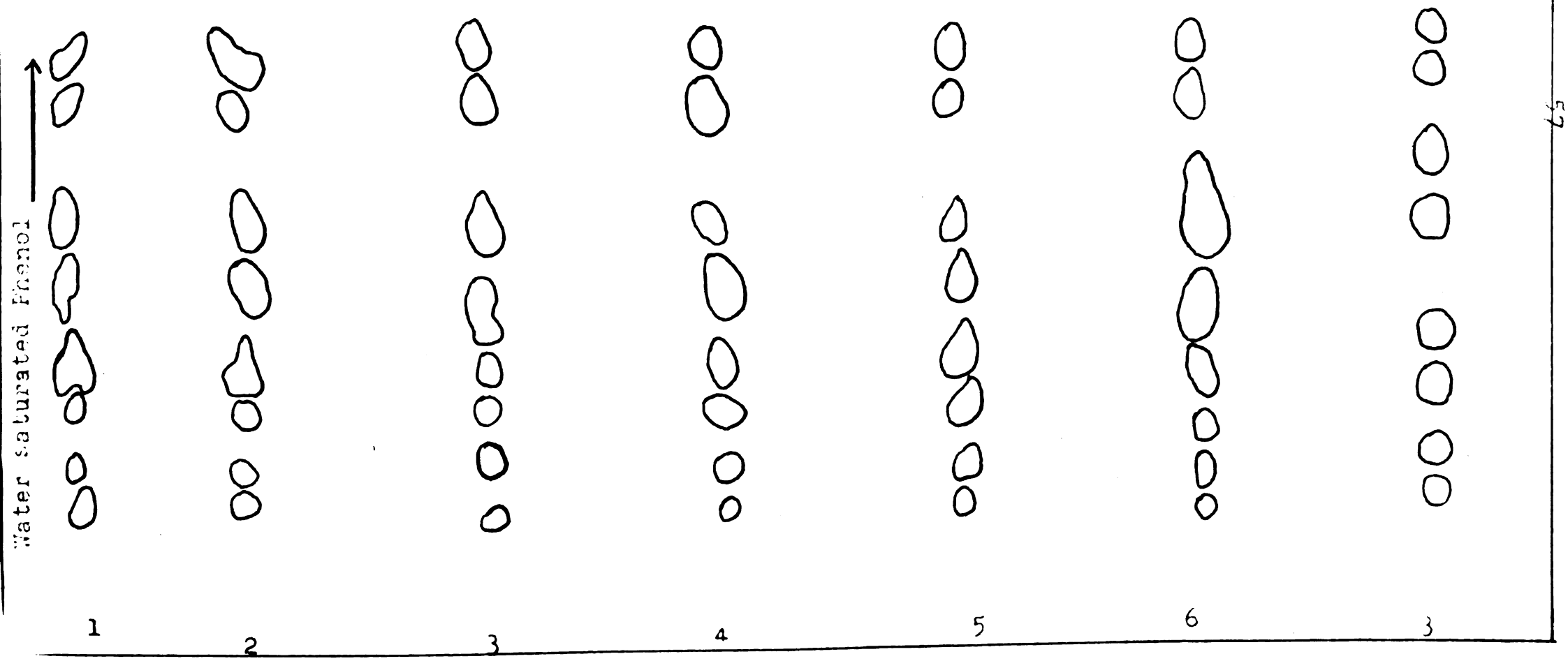


Figure 11. TWO DIMENSIONAL CHROMATOGRAM OF HYDROLYSATE OF TCA SOLUBLE PEPTIDES.Released from whole casein after 15 min. digestion with 0.010 mg.chymotrypsin per ml. of digest

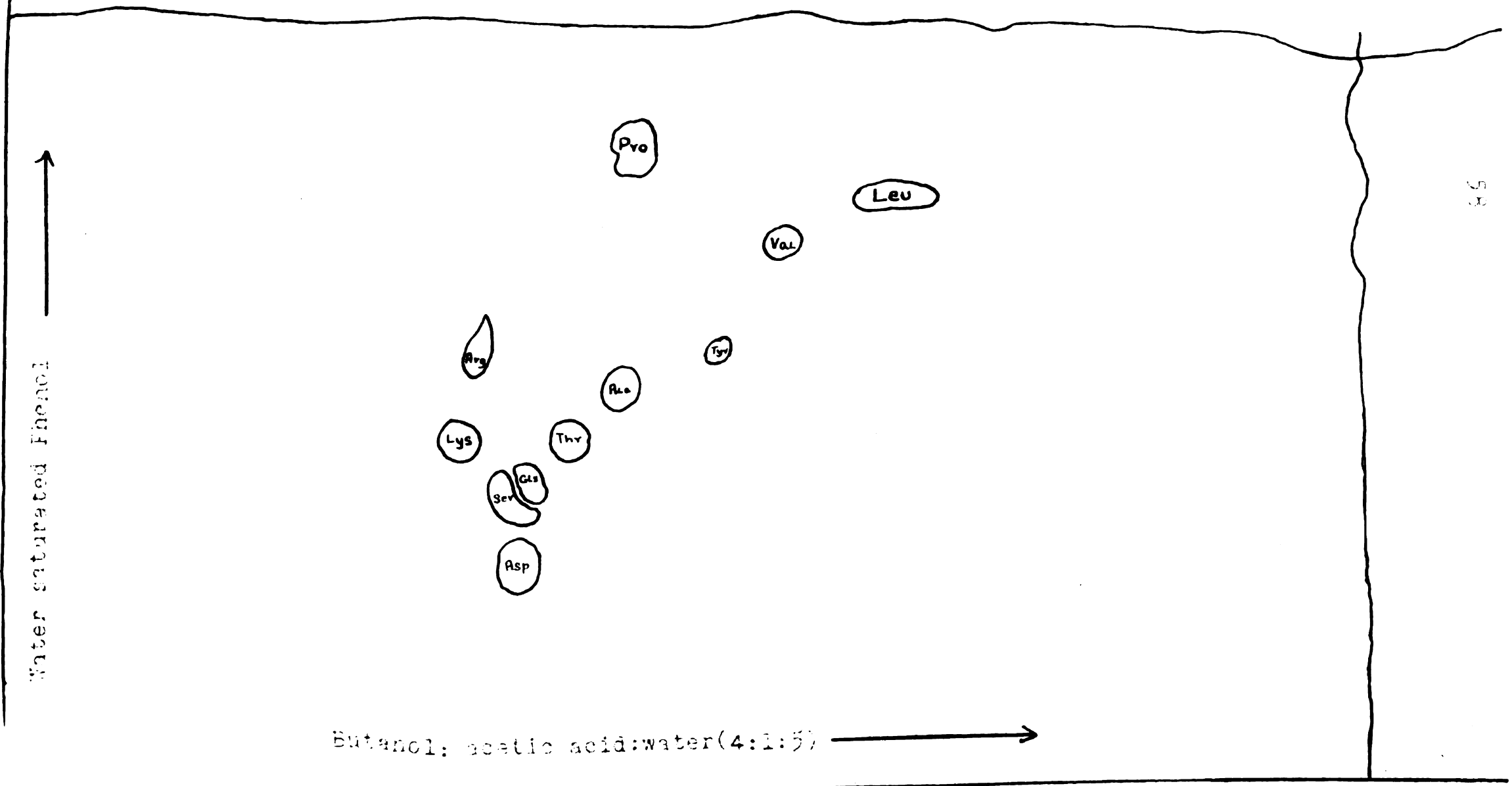


Figure 12. TWO DIMENSIONAL CHROMATOGRAM OF HYDROLYSATE OF TCA SOLUBLE PEPTIDES.Released from whole casein after 4 hours digestion with 0.0165 mg.chymotrypsin per ml. of digest.

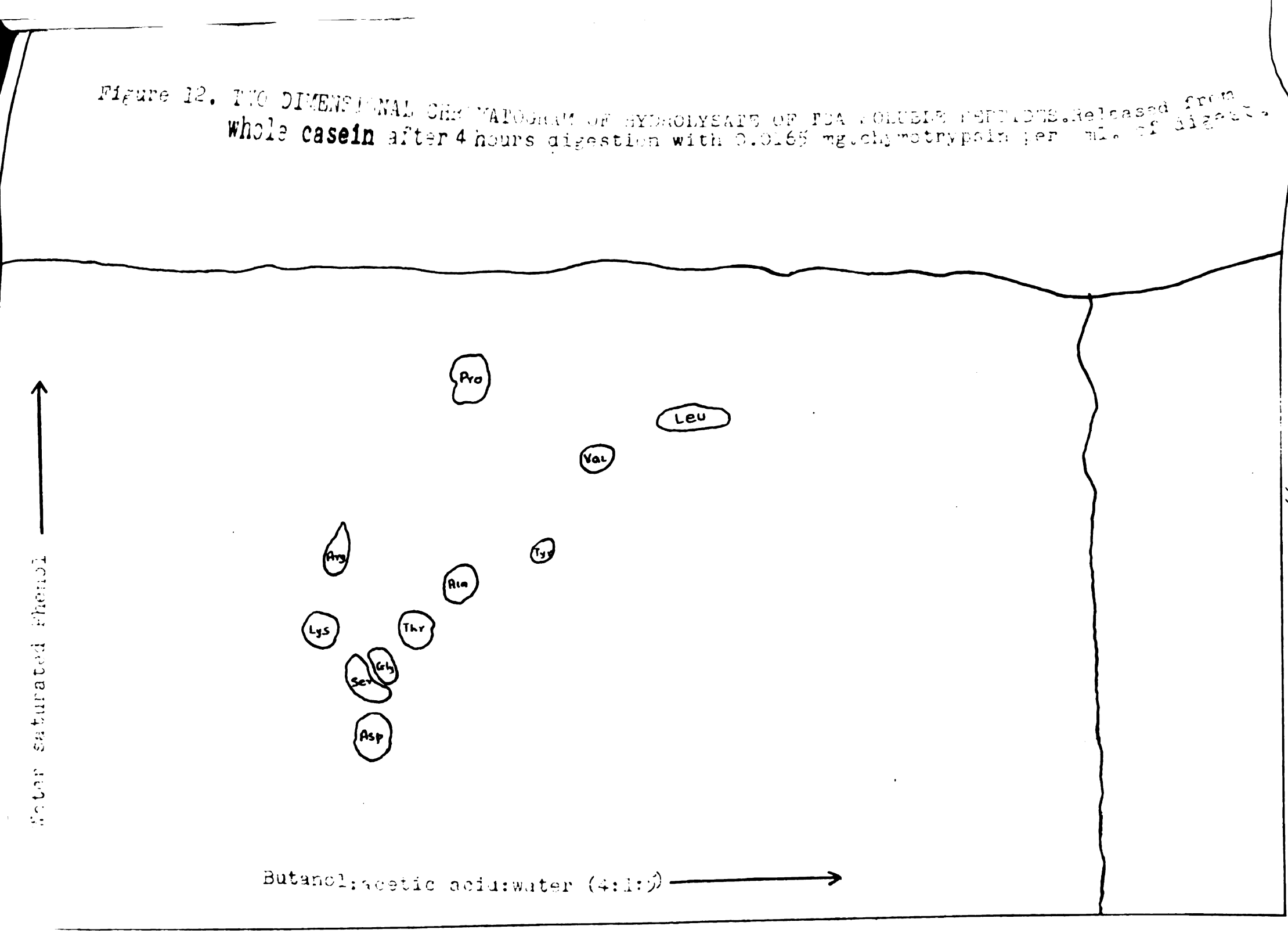


Figure 13. TWO DIMENSIONAL CHROMATOGRAM OF HYDROLYSATE OF TCA SOLUBLE PEPTIDES. Released from alpha casein after 4 hrs. digestion with 0.0165 mg. chymotrypsin per ml. of digest.

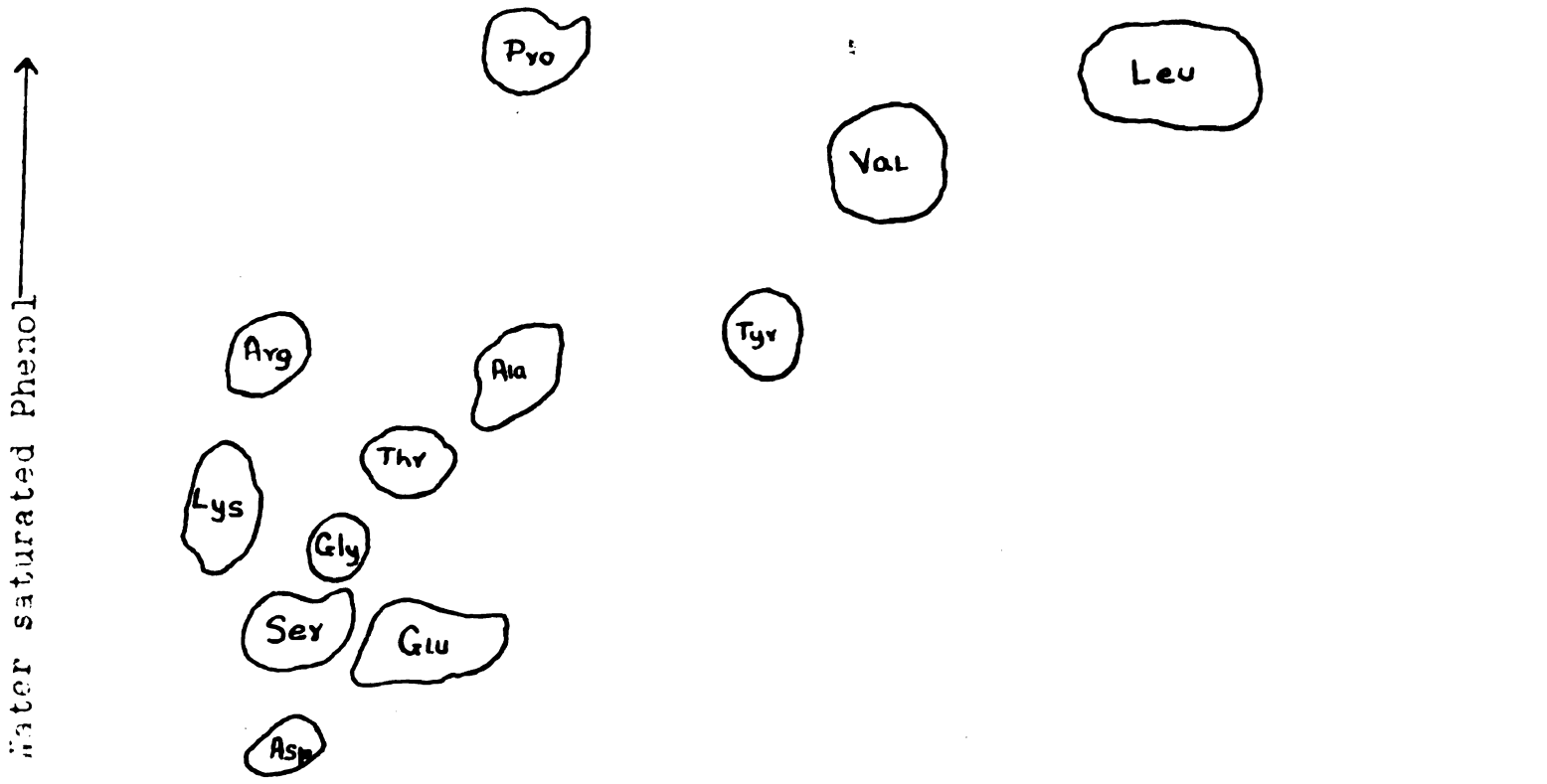


Figure 14. TWO DIMENSIONAL CHROMATOGRAM OF HYDROLYSATE OF TCA SOLUBLE PEPTIDES. Released from beta casein after 4 hrs. digestion with 0.0165 mg.chymotrypsin per ml. of digest.

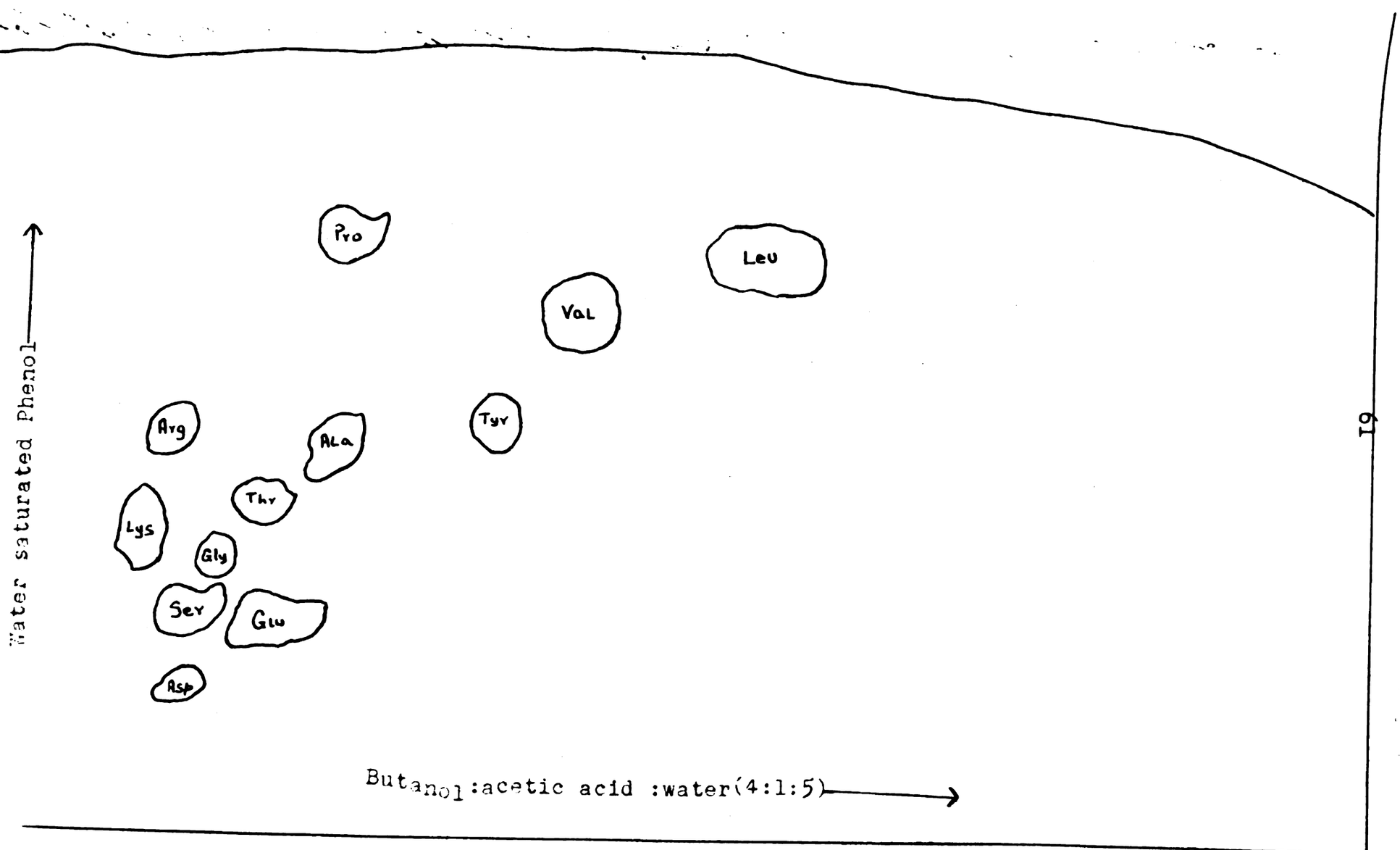


Figure 15. TWO DIMENSIONAL CHROMATOGRAM OF KNOWN AMINO ACIDS.

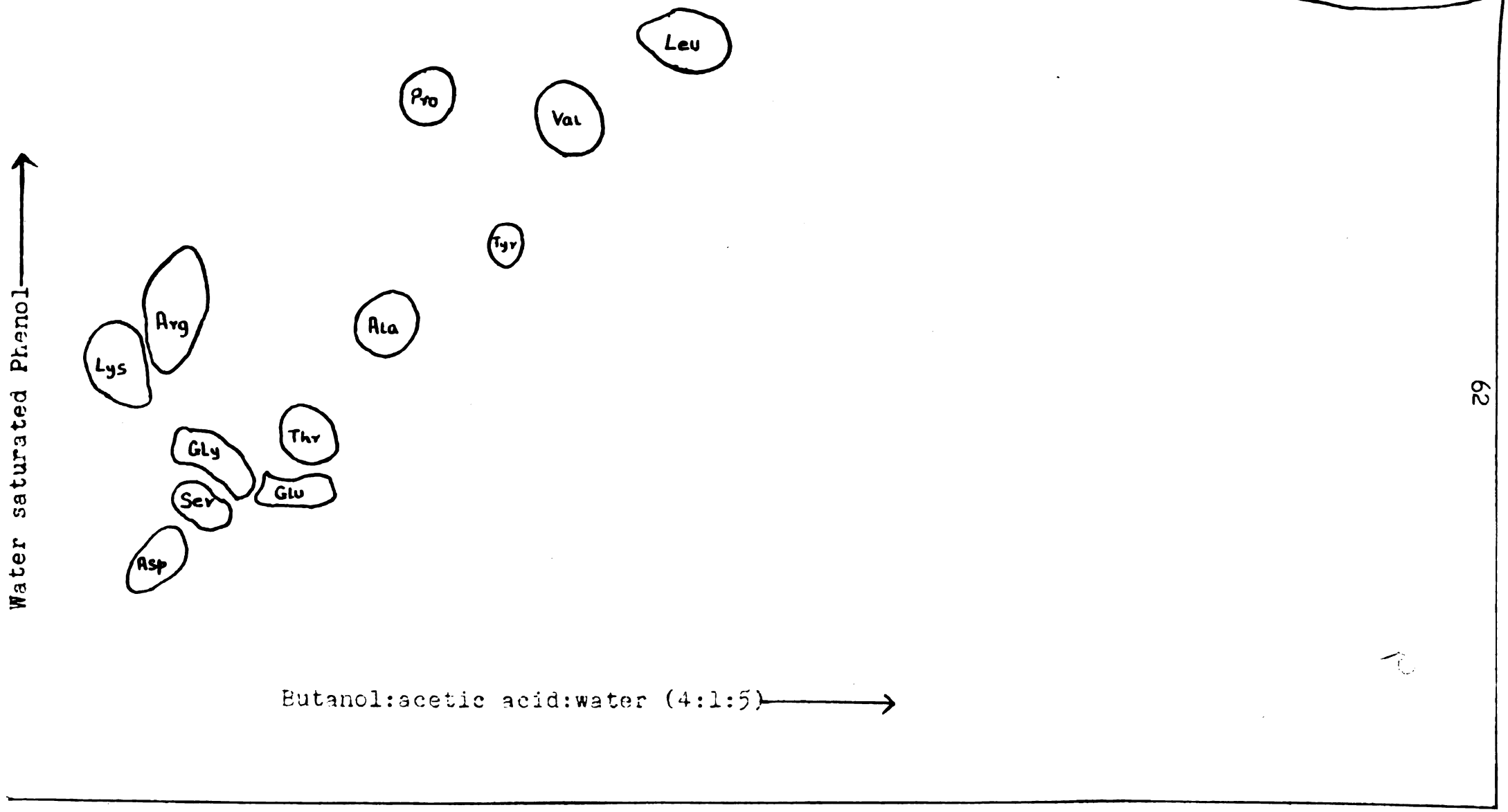


Figure 16. TWO DIMENSIONAL CHROMATOGRAM OF MIXTURE OF KNOWN AMINO ACIDS AND HYDROLYSATE OF TCA SOLUBLE PEPTIDES. Released after 4 hrs. 3% whole casein digestion with 0.0165 mg. chymotrypsin per ml. of digest.

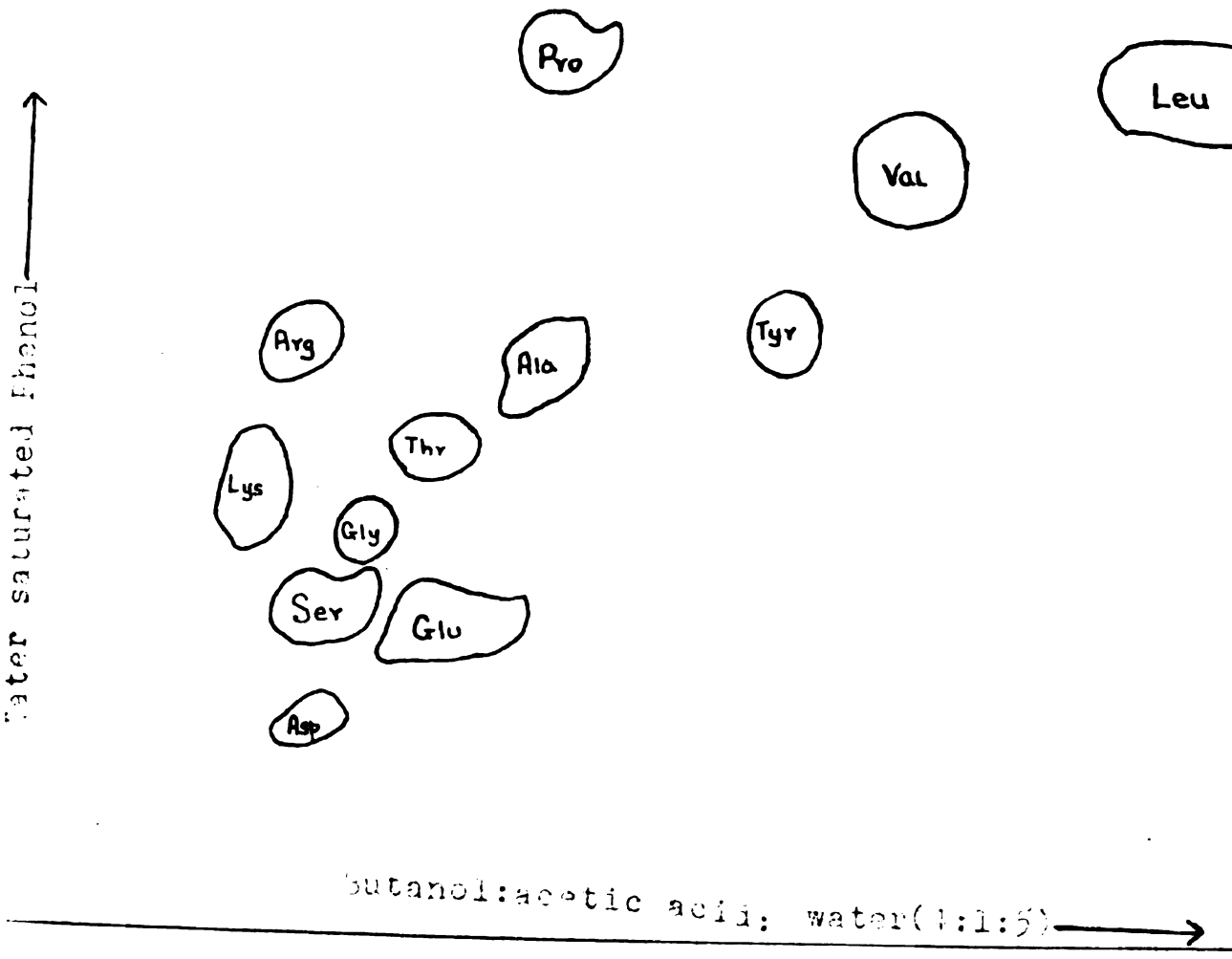
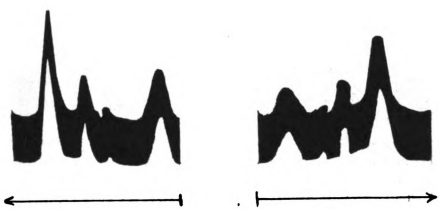
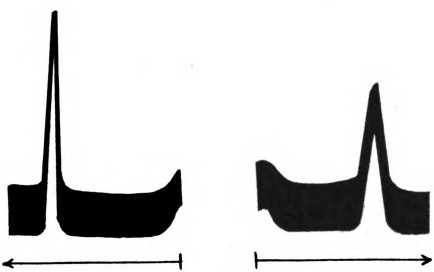


Figure 17 ELECTROPHORETIC PATTERNS.(Top)WHOLE CASEIN (Bottom)ALPHA CASEIN



4500 Sec.; 11.4 Volts per cm.; 1.5% Protein in 0.1 M veronal buffer pH 8.6.



7200 Sec.; 7.76 Volts per cm.; 1% Protein in veronal buffer pH 8.4,ionic strength 0.15 (0.1 veronal and 0.05 sodium chloride).

Figure 18. ELECTROPHORETIC PATTERNS OF WHOLE CASEIN DURING DIGESTION. In veronal buffer pH 8.6, ionic strength 0.1.

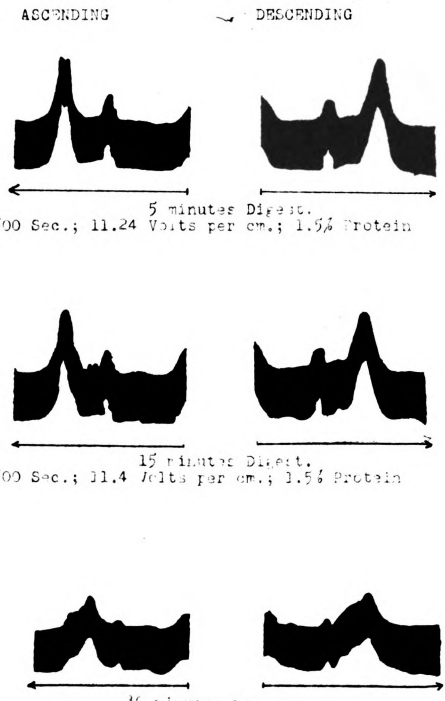


Figure 19. ELECTROPHORETIC PEAK OF ALPHA CASEIN
[illegible] DIGESTED DISMILK TIMES. In veronal
buffer of 8.6 and ionic strength 0.1.

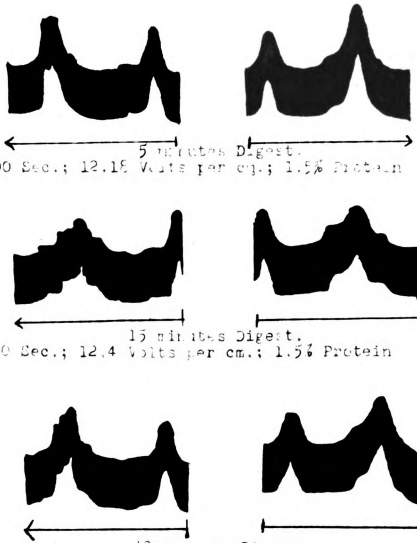
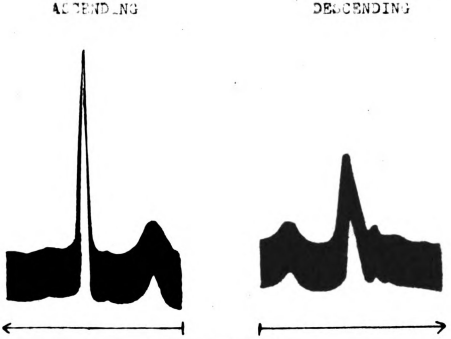
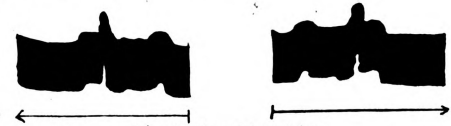


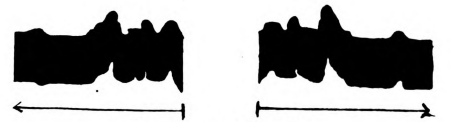
Figure 20. ELECTROPHORETIC PATTERNS OF BETA CASEIN DURING DIGESTION.



No Digestion.
7200 Sec.; 8.26 Volts per cm.; 1% Protein in veronal buffer pH 8.4 ionic strength 0.15(0.1 Veronal and 0.05 sodium chloride)



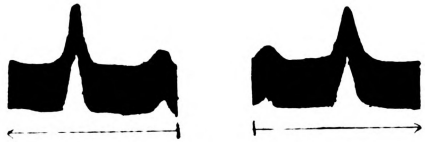
15 minutes Digestion.
3600 Sec.; 12.4 Volts per cm.;1.5% Protein in veronal buffer pH 8.6 and ionic strength 0.1.



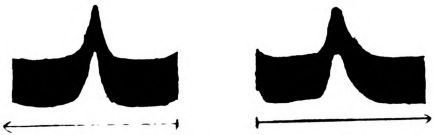
30 minutes Digestion.
3600 Sec.; 12.48 Volts per cm.;1.5% Protein in veronal buffer pH 8.6,ionic strength 0.1

Figure 21. ELECTROPHORETIC PATTERNS OF THE CASEIN DERIVATIVES OF WHEAT (.OL), ALPHA (.07LB), AND BETA (.03LB) GLIADIN in veronal buffer pH 8.6, ionic strength 0.1 and protein concentration 1%

ASCENDING DESCENDING



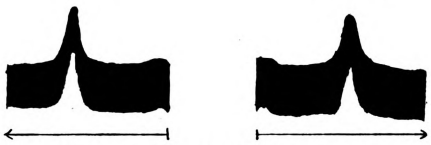
3600 Sec.; 12.5 Volts per cm.



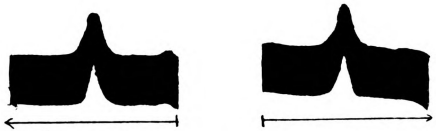
3600 Sec.; 12.5 Volts per cm.

3600 Sec.; 21.55 Volts per cm.

Figure 22. ELECTROPHORETIC PATTERNS OF THE MIXTURE OF T[illegible] CASEIN DERIVATIVE FROM WHOLE AND ALPHA (Iq) AND FROM WHOLE AND BETA CASEINS(bottom) In veronal buffer pH 8.6.ionic strength 0.1 and protein Conc. ca 1%.



3800 Sec.; 12.10 Volts per cm.



3600 Sec.; 11.86 Volts per cm.

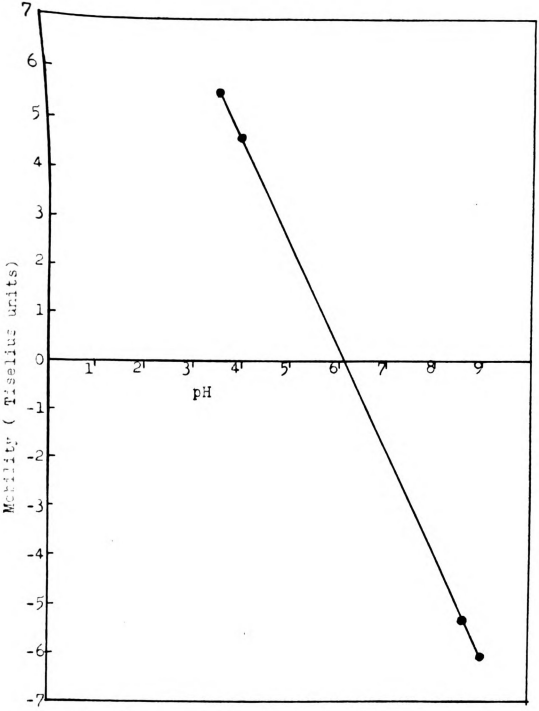


Figure 23. pH- MOBILITY CURVE OF THE CASEIN DERIVATIVE FROM WHOLE CASEIN.

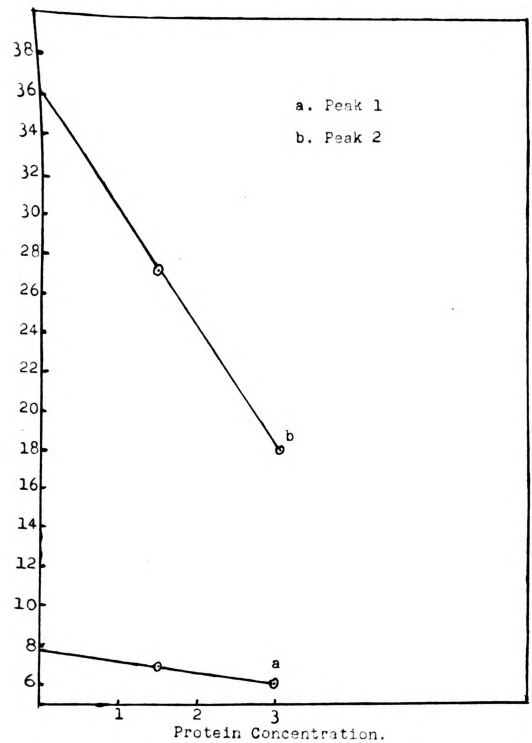
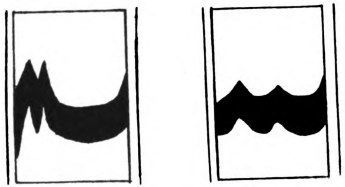
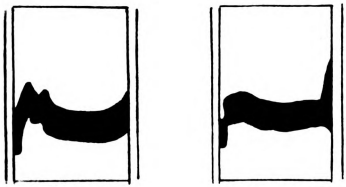


Figure 24. CONCENTRATION- SEDIMENTATION COEFFICIENT (Svedbergs) CURVE OF THE CASEIN DERIVATIVE FROM WHOLE CASEIN. In veronal buffer pH 8.6, ionic strength 0.1, at 24°C and 59,780 r.p.m.

Figure 25. ULTRACENTRIFUGAL PATTERNS OF THE CASEIN DERIVATIVE FROM WHOLE CASEIN, in veronal buffer pH 8.6, ionic strength 0.1, at 24°C and 59,780 r.p.m.

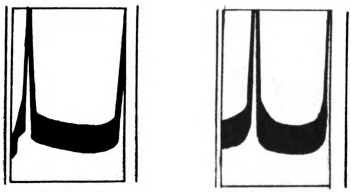


16 minutes 48 minutes
Protein Conc. 3%.

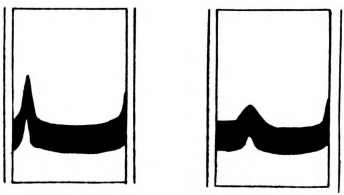


16 minutes 48 minutes
Protein Conc. 1.5%.

Figure 26. ULTRACENTRIFUGE PATTERNS OF ALPHA CASEIN. In veronal buffer pH 8.6, ionic strength 0.1, at 24°C and 59,780 r.p.m.

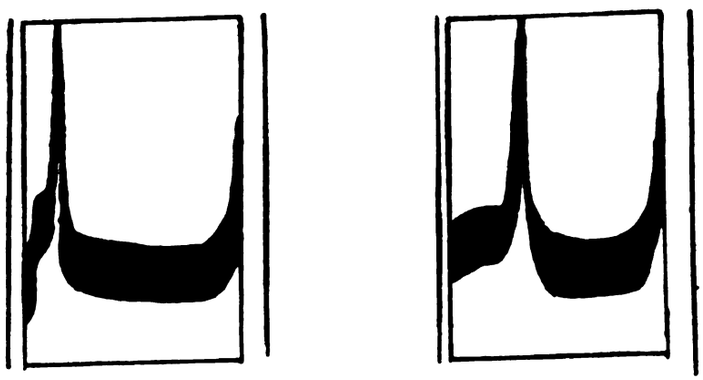


32 minutes 96 minutes
Protein Conc. 3%.

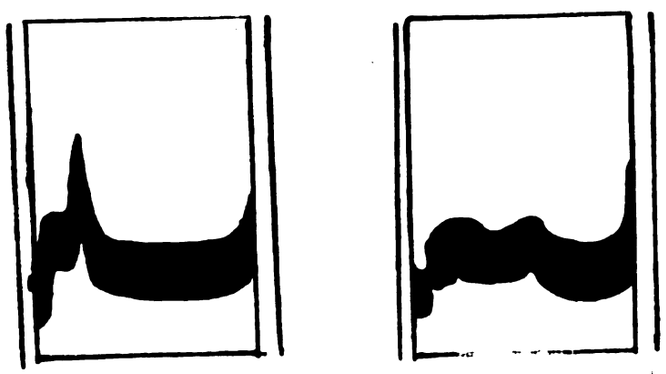


32 minutes 96 minutes
Protein Conc. 1.0%.

Figure 27. [illegible] of β-LACTOGLOBULIN. In veronal buffer pH 8.6, ionic strength 0.1, at 24°C and 59,780 r.p.m.



32 minutes 96 minutes
Protein Conc. 3%.



32 minutes 96 minutes
Protein Conc. 1.5%.

IV. DISCUSSION

1. Age Stability of Enzyme Stock Solution.

Jacobson (42) for chymotrypsinogen conversion studies stored the alpha chymotrypsin stock solution at pH 3.2 and 2° C, and noticed no decrease in clotting activity after 25 days. Christensen (13) prepared stock solutions in 0.025 N hydrochloric acid and stored them in the refrigerator to avoid any loss in activity. To keep the system as simple as possible, it was thought desirable to make stock solutions of the enzyme in distilled water and to store these in the cold room at around 5° C. Thus, if such were possible and if no appreciable loss in activity occurred, no change in pH would be necessary.

In orienting experiments, the activity of a one month old enzyme solution as measured by E_{280} absorbancy of trichloroacetic acid (TCA) soluble products was found to be the same (within experimental error) as that of a new solution. This observation is supported by the work of Kunitz and Northrop (71), who in the preparation of their beta and gamma chymotrypsins stored alpha chymotrypsin at pH 8.0 and 5° C. for over two weeks. They also showed that beta and gamma chymotrypsins have the same activity and specificity as alpha. Thus storing of stock solution for about three weeks at 5° C. was regarded as not effecting the specificity and activity, although alpha chymotrypsin might have changed to beta or gamma forms during this period.

2. Method for Inorganic Phosphate Analysis.

Even today the most frequently employed procedure for inorganic phosphate analysis is that of Fiske and Subbarow (23). Nicholson (68) while trying this method upon the TCA filtrate from casein proteolysate with taka-diastase, observed that addition of the molybdate reagent produced a white precipitate. This precipitate contained most of the phosphorus. She tried numerous modifications (68) in treatment of the filtrate but without much success. Finally she adopted a modification by Norberg (70) of the Fiske and Subborow method (24). According to this procedure, the inorganic phosphate is separated from interfering substances as a precipitate (hydroxy apatite) with a reagent, consisting of calcium chloride dissolved in ammonium chloride buffer of pH 9.0 and saturated with calcium hydroxide. This precipitate is dissolved in 0.05 N sulfuric acid and using Fiske and Subborow reagents, analyzed for ortho-phosphate. As is evident, any other substance which forms an insoluble compound with calcium will also precipitate along with inorganic phosphate. Organo-phosphorus compounds like diphenyl phosphate, were found, during this investigation, to precipitate on treatment by the Norberg modification. High results were then found for inorganic phosphorus due to hydrolysis of the organic phosphorus compound in the presence of sulfuric acid. As further difficulty, the calcium precipitate from TCA filtrates of casein digests with chymotrypsin either did not dissolve completely or else dissolved with great difficulty in 0.05 N sulfuric acid.

Berenblum and Chain (8) investigated in 1938 the interfering substances of non-phosphate character such as those which form molybdenum complexes, e.g., fluorides, citrates, oxalates, etc. To avoid such interference, they offered a new procedure for inorganic phosphate analysis by taking advantage of the extreme solubility of phosphomolybdic acid in organic solvents. Their method was simplified in 1949 by Martin and Doty (59) who improved the choice of solvent and decreased the time of analysis. This simplified procedure when tried on TCA filtrates obtained from casein digests with chymotrypsin proved utterly unsuccessful. Even known quantities of phosphate added to the filtrates could not be recovered (see appendix Table I). Hence it demonstrated that some interfering material was released from casein during digestion. However a combination of the precipitation feature of Norberg's method with the simplified organic solvent extraction technique of Martin and Doty was then tried and found to be quite successful. The procedure in detail, as adopted in its final form is given on pages 31 and 32 of this dissertation.

Quantitative analytical recoveries of the known amounts of phosphate solutions added to the filtrates were obtained (see appendix Table II) by this method. Another advantage of the method adopted in this work was the small amount of TCA filtrates required for each determination.

3. Alcohol, Acetone and Water Media Titrations.

According to Green and Neurath (27), titration methods give more significant information about the course of the proteolytic reaction

than do the other methods, but they are more tedious to use and less suited to routine work. The Willstatter and Waldschmidt-Leitz (95) modification of Foreman's (24a) alcoholic sodium hydroxide titration, which determines the total acid group's liberated during proteolysis, is very sensitive to carbon dioxide contamination (42). To minimize this error all the aliquots were titrated as quickly as possible and under identical conditions. The time interval between the removal of aliquots and completion of titration was most carefully controlled in the case of titration in water medium. The period was the same for each sample, in view of the fact that chymotrypsin was expected to continue its action even in very dilute solutions.

Linderstrøm-Lang's acetone titration (53) determines the basic groups released during digestion. Incidently and as also reported by Jacobsen (42) it was observed that acetone titrations were less tedious to perform as compared to the alcohol titrations.

From theoretical considerations, if during proteolysis there is only peptide bond cleavage, the moles of hydrochloric acid consumed in an acetone titration should equal the moles of potassium hydroxide consumed in an alcoholic titration and there should be practically no consumption of either base or acid in water medium when using the same indicator.

Titration data for whole, alpha, and beta caseins (see Table I and Figure 1) clearly show that consumption of base in alcohol was higher as compared to the consumption of hydrochloric acid in acetone. Also, there was definite and noticeable consumption of base in

aqueous medium and it was more pronounced in the digests of whole and alpha caseins.

From these results it is quite logical to submit that the action of chymotrypsin was not limited to the hydrolysis of peptide bonds only, but in addition some other acid groups were also liberated. More such acid groups which must be considered as other than those from peptide bond cleavage were liberated during the early stages of digestion because then the aqueous medium titration corresponded more closely to the alcohol titration values. It may also be deduced from the same observation that during later stages of proteolysis most of the additional acid groups now came from principally peptide bond cleavage.

From all methods of determination employed the rate and extent of proteolysis with respect to beta casein appeared considerably less than that for either of the other two preparations. Beta casein titration values, although low, are significant enough to also support the contention that acid groups other than those from peptide bond cleavage are released during proteolysis.

The aqueous titration values in the digests from whole casein are higher than the corresponding acetone titration values. This might be attributed to the effect of presence of one casein fraction upon the other in a mixture as shown by Perlmann (74) in connection with phosphorus liberation by certain enzymes. Thus it could be the result of more phosphodiester cleavage from the beta fraction or/and less phosphoamide bond hydrolysis from within alpha casein. According to

Perlmann (73) alpha casein is practically devoid of phosphodiester groups whereas ca 80 per cent of the beta casein phosphorus is regarded to be phosphodiester linked.

It may be remarked that the water medium titration results obtained from beta casein digests were not as reliable as others, on account of the solutions being quite milky.

4. Liberation of Phosphorus.

The liberation of 10 per cent TCA soluble total phosphorus from whole and alpha casein during proteolysis by chymotrypsin did not correspond to the release of non-protein nitrogen. Where there was progressive increase in nitrogen release, the phosphorus reached a maximum and then practically levelled off (cf. Tables II, III, and IV and Figures 2 and 3), especially the organic phosphorus. The maximum point in the case of whole casein represented solubilization of about 11 per cent of the total protein phosphorus and in the case of alpha casein it amounted to about 10 per cent.

The rate and extent of total acid soluble phosphorus liberation from beta casein was very low and the same was true for its nitrogen liberation. Only about 2 per cent of its total protein phosphorus was released during the 4 hours of digestion by chymotrypsin.

Most of the phosphorus so released was found to be in the form of inorganic phosphorus. More than three-fourths of the total phosphorus liberated from alpha casein and about two-thirds the phosphorus from whole casein was found as inorganic phosphorus (See Tables II and III and Figures 2 and 3).

More organic phosphorus as compared to inorganic phosphorus was released from whole casein rather than from alpha casein. This may be attributed to the presence of the beta fraction contained in whole casein and absent in pure alpha. Furthermore, as shown by Perlman (73) the presence of one fraction in a mixture may possibly effect the behavior of enzymes upon the other. In all three cases organic phosphorus liberated as compared to inorganic was greater with lower concentration of the enzyme than that with higher concentration (Tables II, III and IV).

Simultaneous conductivity change curves (Figure 4) did not support the suggestion made by Nicholson (68) that inorganic phosphorus might be determined by the much simpler resistance measurements. It may be noted that the liberation of inorganic phosphorus during digestion was a maximum (Figures 2 and 3) from alpha casein, whereas conductivity change was the least when compared with whole and beta caseins (Table V, Figure 4).

5. Nitrogen Products in the Trichloroacetic Acid Soluble Portion.

a) Acid soluble nitrogen products.

As determined by measurement of absorption at 280 mu or Kjeldahl nitrogen of their TCA filtrates, these products showed with all three caseins and both chymotrypsin concentrations, a progressive increase during 4 hours of digestion. (Tables VI, VII and VIII, Figures 5 and 6.) The rate and extent was highest with alpha casein and lowest with beta casein.

b) Paper chromatography.

For further specific characterization of the acid soluble nitrogen products resulting from proteolysis, TCA filtrates from various casein digests were examined after prescribed treatment by paper chromatography (cf. page 33). The solvent systems successfully employed were: 1) butano : acetic acid : water (4:1:5) and 2) water saturated phenol. Another solvent system tried was: 2,6 lutidine : collidine : water (1:1:1), but was found to be unsuitable as no movement of the peptides could be observed.

With respect to proteolysates of whole casein, only two ninhydrin positive spots of high R_f values (presumably from peptides) and of close proximity could be detected (Figures 7 and 8). It may be noted that the same two spots could be obtained from early stages and upwards to 4 hours of digestion. Such was true for both concentrations of the enzyme. This observation may be taken to support the work of Tiselius and Eriksson-Quensel (88) and others such as Haugaard and Roberts (41), Bellof and Anfinsen (6), etc., who have used various enzyme-substrate systems. On the basis of their work these investigators have stated that protein molecules are broken down one by one to the ultimate peptide stage without the accumulation of intermediate products.

Similar filtrates from alpha casein by the same treatment also showed 2 spots (Figure 8) of R_f values identical to those from whole casein spots whereas with beta casein (Figure 8) only one spot corresponding to the faster of the two in the other cases could be detected.

When filtrates from whole casein digests were first hydrolyzed with 6 N hydrochloric acid and then examined in the same manner by one dimensional paper chromatography, they showed 8 spots which were identical for all the samples resulting from early stages and up to 4 hours of digestion. This again proved that the same peptides were liberated throughout the digestion period under investigation.

Products from filtrates of alpha and beta casein digests when similarly hydrolyzed and treated, also showed identical 8 spots. The only difference was that the TCA soluble products from whole and alpha caseins during acid hydrolysis formed humin showing the presence of tryptophan whereas no humin was observed when beta casein products were involved, showing the absence of typtophan (54).

This demonstrated that the faster moving peptide which was a common product of all three proteins did not contain tryptophan; whereas tryptophan was present in the slower moving peptide(s) which came only from alpha and whole casein. In whole casein this slower tryptophan containing spot might originate from alpha casein since beta casein did not give the said spot. It may also be remarked that apart from tryptophan, the two acid soluble peptides had some amino acids in common.

When two dimensional chromatograms were run with the hydrolyzed peptides of the filtrates, 12 spots could be detected (Figures 11 to 14). These spots were positively identified by comparing and running them in mixture with known amino acids (Figures 15 and 16).

Thus it may be said in summary that the faster moving peptide (or peptides) was common to all three protein digests (in reality common to both alpha and beta caseins) and contained 12 amino acids of which 5 or 6 are nutritionally essential. These are all named upon Figures 11 to 16. The slow moving peptide (or peptides) absent from beta casein contained tryptophan as one of the amino acid residues and other amino acid residues contained herein were also present in the fast moving peptide (or peptides).

6. The Acid Soluble Nitrogen and Titration Data Compared to Phosphorus Analysis.

Comparing the titration data with phosphorus liberation results (previously mentioned separately), it may be proposed that the acid groups found released during digestion, other than those from peptide bond cleavage, were the result of phosphate bond cleavage.

Examining the phosphorus data along with the chromatography findings the following observations can be made. Two identical spots were obtained from the filtrates of whole and alpha casein and only one spot corresponding to the faster was given by beta casein filtrates. Thus one spot (the faster one) was common to all three proteins. All three proteins released organic phosphorus and the proportion of inorganic to organic phosphorus was the highest in alpha casein (compared to the total phosphorus liberated). With all three proteins there was progressive increase of acid soluble nitrogen throughout the 4 hour digestion period. Organic phosphorus did not follow the same pattern. It leveled off substantially during the same period in the case of alpha

and whole casein but did not go as far with beta casein. (See Figures 2 and 3, Tables II, III and IV.)

Now, there are two possibilities offered as to course of reaction, either the organic phosphorus was associated with both the spots or perhaps the faster moving one. If the organic phosphorus was associated with both the spots, there should have been no leveling off of phosphorus in any protein digest and its liberation should always have corresponded to the release of nitrogen, which was not the case. Thus this possibility of both spots containing phosphorus cannot be justified on the basis of the data obtained.

The second alternative possibility; namely, that the organic phosphorus was associated with one spot (evidently with the faster moving one which was common to all) is the only choice left and needs further direct confirmation. To justify this choice, one assumption will have to be made--that the liberation of the fast moving peptide(s) will level off simultaneously with the organic phosphorus in the case of alpha casein. Then if the continued release of nitrogen is to be accounted for it may be due to the continuous liberation of slow moving non-phosphorus peptide(s).

7. Inhibition of Chymotrypsin Activity with Casein.

Stopping the chymotrypsin action was found necessary for studying the electrophoretic changes produced during different stages of proteolysis of casein. Jansen et al. (44) studied the inhibition of this enzyme in its pure form and in the absence of any substrate.

They found that chymotrypsin was irreversibly inhibited by di-isopropyl fluorophosphate (DFP). The reaction was fast but not instantaneous. This reagent is highly toxic and very reactive. Additionally, the groups with which DFP is supposed to react, i.e., hydroxyl of serine and/or imidazole (60) are also present in casein. Thus the use of this inhibitor reagent was undesirable.

Gergely and co-workers (25) while studying the digestion of myosin with chymotrypsin used DFP to stop the reaction but made no mention about the effect of DFP on the substrate, if any.

Li et al. (50) in their paper pertaining to the action of chymotrypsin on hypophyseal growth hormone stated without any further details that one drop of glacial acetic acid stopped the reaction. A number of possible inhibition treatments with glacial acetic acid as reported on page were tried but without any such success.

According to the data of Kunitz and Northrop (71), chymotrypsin should be practically inactive at pH below 5.9. When casein-chymotrypsin mixture after bringing to pH 5.6 by the addition of acetate buffer were allowed to dialyze over night at 5° C. against the same buffer, a white precipitate appeared, showing that chymotrypsin was still active. Although it did not lead to successful inhibition, this observation opened a new avenue of investigation as to proteolytic changes on casein. The precipitate thus obtained was further examined and findings are discussed in later paragraphs.

High alkalinity produced by saturated potassium carbonate as mentioned by Schwert et al. (81) was not tried due to the fact that alkali dephosphorylates casein (76).

Inhibition of chymotrypsin by strong urea solutions was tried in 1956 by Harris (33). He found that the enzyme was irreversibly inhibited even in the presence of synthetic substrate, when treated with 8 M urea. However, he made the following statement. "Nevertheless for practical purposes it is possible to take advantage of the fact that both enzymes (chymotrypsin and trypsin) are stabilized to a considerable extent against urea inactivation in the presence of a substrate and can thus be used to degrade proteins which are themselves susceptible to denaturation in urea but which resist digestion in their native state." This statement was found to be valid as chymotrypsin was not inhibited by 8 M urea in the presence of casein.

The only successful method for stopping the chymotrypsin activity was found to be by isoelectric precipitation (adjusted to pH 4.6, with acetate buffer) of the casein digests. The derived proteins thus precipitated were washed practically free of enzyme (page 29) which remained soluble under these conditions.

One disadvantage of this procedure was the loss of other digestion products which were soluble at pH 4.6. Thus electrophoretic patterns represent only those digestion products which were precipitable at pH 4.6. It may be stated that this procedure provided considerable assurance that the changes produced were due to chymotrypsin action only, as no drastic treatment or reagent was involved.

8. Electrophoretic Changes.

Electrophoretic analysis of proteins precipitated from digest aliquots, removed at different time intervals showed (Figures 18 to 20)

that initially there was a split in the alpha peak. This was true for whole as well as pure alpha caseins. Similar split in the alpha peak by the action of rennin and pepsin was observed by Nitschmann and Lehmann (69). With advancing digestion, both faster and slower moving components started appearing from all three casein preparations. It suggests that the first step involved a change in the alpha fraction complex to produce the observed split in the alpha peak.

After this first step, the rate and extent of appearance of new components was greater from the beta fraction substrate than those with the alpha one. It may be noted that the concentrations mentioned with the figures were based on the original protein concentrations in the digest and the changes produced due to washing away of digestion products soluble at pH 4.6 which would be greater with advanced digestion were not taken into account.

On comparing the electrophoretic patterns of the digest precipitates from the various caseins it may be remarked that a greater amount of soluble products was produced from beta casein than from alpha casein.

9. A Casein Derivative Produced by Chymotrypsin and Precipitable at pH 5.6.

As mentioned above, a white precipitate was observed when trying to inhibit chymotrypsin around pH 5.6. This precipitate after washing and reprecipitating showed, electrophoretically, at pH 8.6, a single peak. Alpha and beta caseins similarly treated also gave a precipitate,

which on electrophoretic analyses again showed a single peak of about the same mobility (Figures 5 and 6).

It may be remarked that from beta casein a white precipitate appeared after 30 minutes of reaction but was not removed since the aforementioned derivative could not be obtained in pure form. It was only after the reaction proceeded for more than three hours, preferably over night that the precipitate showing single peak could be obtained.

When a precipitate from alpha or beta casein was mixed with that from whole casein and electrophoretically analyzed, again a single peak was revealed.

This evidence along with chromatography results discussed earlier, suggests that both alpha and beta fractions may have some common units or segments in their molecular chains. Complete proof of this postulate requires further investigations.

For the determination of its isoelectric point, the precipitate from whole casein was electrophoretically analyzed at different hydrogen ion concentrations (Table IX, Figure 23). All the buffers used, contained simple monovalent ions, as the polyvalent ions affect the mobility of the compounds under investigation (2). Thus the mobility of the derivative under discussion in phosphate buffer at pH 7.0 was found to be almost equal to its mobility in veronal buffer pH 8.6. The casein derivative showed essentially a single peak at all the hydrogen ion concentrations tried.

The same precipitate, when analyzed by the ultracentrifuge, showed two peaks (Figure 25). Both the peaks had sedimentation rates

which were different from the sedimentation rates of pure alpha and beta caseins, when run under identical conditions (Table X). This indicated that the components present were different from the original components of casein. Perhaps the faster moving component was a polymer of the slower component but that their net charge was the same thus accounting the single peak observed by electrophoresis.

Its phosphorus content (Table XI), mobility at pH 8.4 (Figure 22) and minimum solubility show some resemblance to the properties of alpha-2 casein reported by McMeekin (63). This similarity association also needs further investigation. It may turn out that the kappa casein of Waugh (93) and alpha-2 casein of McMeekin (63) are the enzymatic cleavage products of alpha and beta caseins, instead of being regular casein components.

V. SUMMARY

1. A method for analysis of inorganic phosphate in trichloroacetic acid filtrates of chymotryptic casein hydrolysates with chymotrypsin was developed.

2. Alcohol, acetone and water media titration results showed that acid groups, other than those coming from peptide bond cleavage, were liberated during chymotryptic digestion of whole, alpha and beta caseins.

3. These acid groups appeared to be coming from phosphate bond cleavage.

4. The liberation of total acid soluble phosphorus from whole and alpha casein did not parallel the liberation of non-protein nitrogen.

5. The rate and extent of liberation of acid soluble phosphorus and non-protein nitrogen was the lowest with beta and highest with alpha casein fractions.

6. It was noted that more organic phosphorus (of the total acid soluble) was released from all three proteins with the lower concentration of the enzyme.

7. Paper chromatography of nitrogen products, soluble in 10% trichloroacetic acid (TCA), from alpha and whole casein digests produced with chymotrypsin showed mainly two ninhydrin spots with high R_f values.

8. Beta casein TCA soluble products showed mainly one spot with an R_f value corresponding to the faster of the two derived from whole

or alpha caseins.

9. Acid soluble nitrogen products from alpha and whole casein were found to contain 13 amino acid residues, namely: 1) leucine, 2) valine, 3) tyrosine, 4) proline, 5) alanine, 6) threonine, 7) serine, 8) glycine, 9) glutamic acid, 10) aspartic acid, 11) arginine, 12) lysine and 13) tryptophan.

10. Acid soluble digestion products from beta casein showed the presence of all the amino acid residues, as mentioned above, with the exception of tryptophan.

11. It is postulated that the faster moving peptide(s) which was common to all three proteins, contained the organic phosphorus.

12. Based on isoelectric precipitation procedures a method of stopping chymotryptic action, for electrophoretic analysis, upon casein digestion products was worked out.

13. At early stages of digestion a split in the alpha peak was observed which was followed by increasing development of both faster and slower peaks, from both the original components.

14. On treatment of whole casein with chymotrypsin at pH 5.6, a white precipitate was obtained which on washing and reprecipitation showed electrophoretically a single peak at various pH.

15. Alpha and beta caseins when similarly treated also gave precipitate which again showed a single peak of about the same mobility as that from whole casein.

16. Some physicoehemical properties of the casein derivative obtained from whole casein by the action of chymotrypsin at pH 5.6 are

reported (Table XI).

17. It is postulated that alpha and beta caseins have some common units or segments in their molecular chains.

BIBLIOGRAPHY

1. Alais, Ch. von., Mocquot, G., Nitschmann, Hs., und Zahler, P. Das Lab und sein Wirkung auf das Casein der Milch, Helv. Chim. Acta, 36, 1955 (1953).

2. Alberty, R. A. Electrophoretic Properties of the Proteins and Amino Acids. Neurath, H. and Bailey, K., Editors. The Proteins, Academic Press, Inc., Publishers, New York, N. Y. Vol. I Part A. 509 (1953).

3. Alexander, F. Zur Kenntniss des Caseins und seiner Peptischen Spaltungs-Produkte, Z. Physiol. Chem. 25, 411 (1898).

4. Balls, A. K. and Jansen, E. F. Stoichiometric Inhibition of Chymotrypsin. Advances in Enzymology, Vol. XIII, 321 (1952).

5. Beck, J. The Hydrolysis of Casein by Enzymes with Special Reference to the Distribution of Amino Acids in the Hydrolysate. Univ. Microfilms Pub. 859, Microfilms Abstracts No. 2,9 (1947).

6. Bellof, A. and Anfinsen, C. B. The Products of Proteolysis of Some Purified Proteins, J. Biol. Chem., 176, 863 (1948).

7. Biffi, Virchow's Arch. 152,130. Quoted from Rimington and Kay (reference No. 79, follows).

8. Berenblum, I. and Chain, E. Studies on the Colorimetric Determination of Phosphates, Biochem. J., 32, 286 (1938).

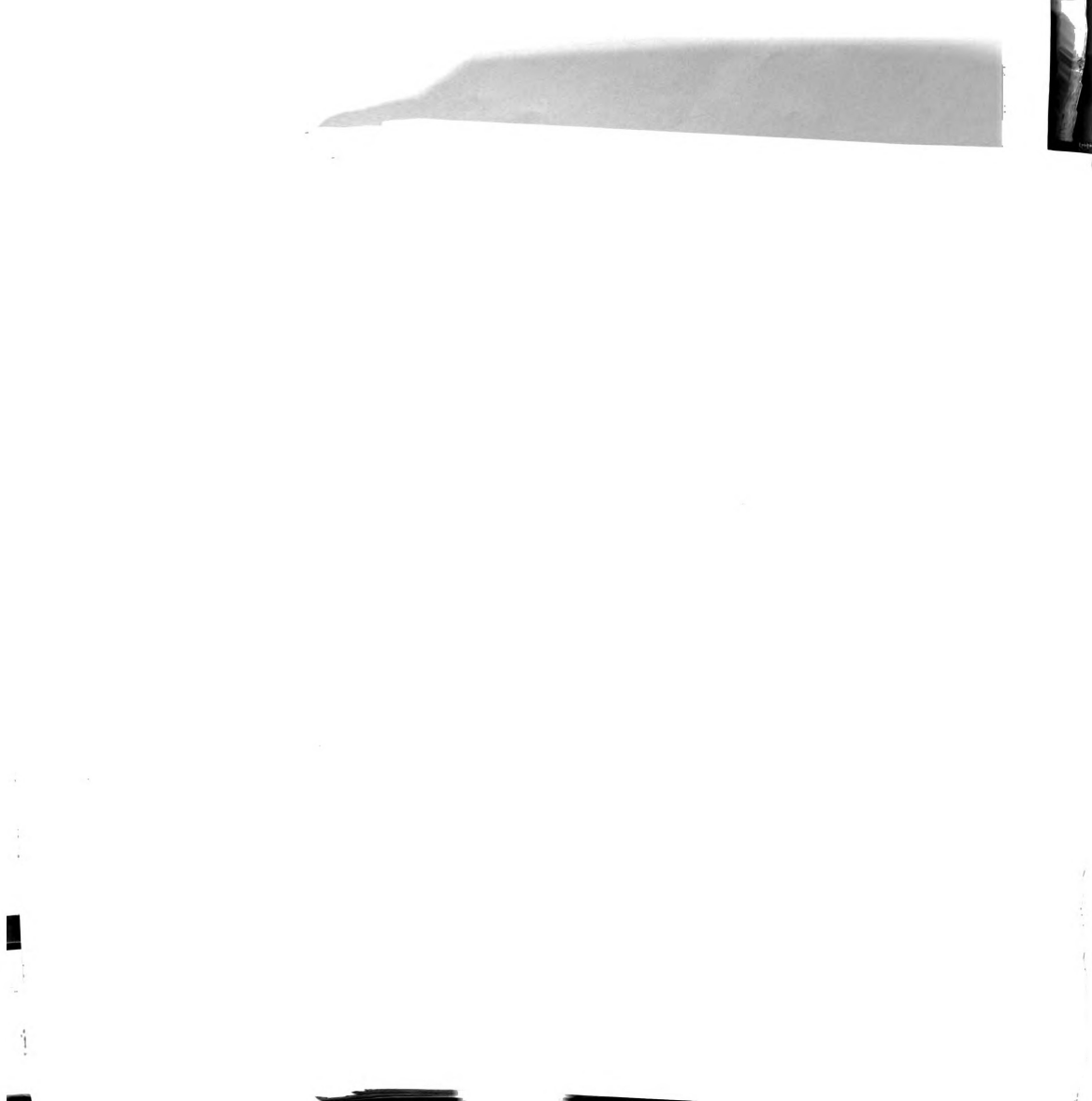
9. Cherbuliez, E. and Meyer, F. Recherches Sur la caseine, Helv. Chim. Acta, 16, 600 (1933).

10. Cherbuliez, E. and Schneider, M. L. Recherches Sur la Caseine, La Caseine n'est pas un Corps Homogene. Etude de son Fractionnement par le Chlorure d'ammonium, Helv. Chim. Acta. 15, 579 (1932).

11. Cherbuliez, E. and Jeannert, Recherches Sur la Caseine. Sur le Fractionnement de la Caseine et de la Paracaseine au Chlorure d'ammonium, Helv. Chim. Acta, 22, 952 (1939).

12. Cherbuliez, E. and Baudet, P. Recherches Sur la Caseine V. Sur les Constituants de la Caseine, Helv. Chim. Acta, 33, 398 (1950).

13. Christensen, L. R. The Action of Proteolytic Enzymes on Casein Proteins, Arch. Biochem. and Biophys., 53, 128 (1954).



14. Cohen, J. A., Oosterbaan, R. A., Warringa, J. and Janz, H. S. Chemical Structure of the Reactive Groups of Esterases, Discussions Faraday Soc., No. 20, 144 (1955).

15. Consden, R., Gordon, A. H., and Martin, A. J. P. Qualitative Analysis of Proteins: A Partition Chromatographic Method Using Paper, Biochem. J. 38, 224 (1944).

16. Damodaran, M. and Ramachandran, B. V. Enzymatic Proteolysis. Amino Acids of Casein Phosphopeptone, Biochem. J., 35, 122 (1941).

17. Dent, C. E. A Study of the Behavior of Some Sixty Amino Acids and Other Ninhydrin-Reacting Substances on Phenol-Collidine Filter-Paper Chromatogrames, with note as to the occurrence of some of them in Biological Fluids, Biochem. J., 43, 168 (1948).

18. Denton, A. E. and Elvehjem, C. A. Enzymatic Liberation of Amino Acids from Different Proteins, J. Nutrition, 49, 221 (1953).

19. Djang, S. S. T. The Isolation, Fractionation, and Electrophoretic Characterization of the Globulins of Mung Bean, Ph. D. Thesis, Michigan State College, pp. 75-80 (1951).

20. Dunn, M. S. Preparation of Casein. Carter, H. E., Editor. Biochemical Preparations, John Wiley & Sons, Inc., New York, Vol. I 22 (1949).

21. Eldred, N. R. and Rodney, G. The Effect of Proteolytic Enzymes on Raw and Heated Casein, J. Biol. Chem., 162, 261 (1946).

22. Farber, L. and Wynne, A. M. Studies on Pancreatic Proteinase, Biochem. J., 29, 2323 (1935).

23. Fiske, C. H. and Subbarow, Y. The Colorimetric Determination of Phosphorus, J. Biol. Chem., 66, 375 (1925).

24. Fiske, C. H. and Subbarow, Y. Phosphocreatine, J. Biol. Chem., 81, 629 (1929).

24a. Foreman, F. W. Rapid Volumetric Method for the Estimation of Amino Acids, Organic Acids and Organic Bases. Biochem. J., 14, 451 (1920).

25. Gergely, G., Gouvea, M. A. and Karibian, D. Fragmentation of Myosin by Chymotrypsin, J. Biol. Chem. 212, 165 (1955).

26. Gortner, R. A. Outlines of Biochemistry, 3rd ed., John Wiley & Sons, Inc., New York, pp. 67 (1949).

27. Green, N. M. and Neurath, H. Proteolytic Enzymes. Neurath, H. and Bailey, K. editors. The Proteins, Academic Press, Inc. Publishers, New York, Vol. II Part B., 1173-1174 (1954).

28. Groh, J., Kardos, E., Denes, K. and Serenyi, V. Uber die Fraktionierung des Caseins, Z. Physiol. Chem., 266, 32 (1934).

29. Groves, M. L., Hipp, N. J. and McMeekin, T. L. Phosphopeptones Obtained from Alpha and Beta Caseins by Partial Hydrolysis with Pepsin, Abstracts of Papers, 131st National Meeting American Chemical Society, Atlantic City, N. J., Sept. 16-21, 33c (1956).

30. Gutfreund, H. and Sturtevant, J. M. Mechanism of the Reaction of Chymotrypsin with p-nitrophenyl Acetate, Biochem. J., 63, 656 (1956).

31. Hammarsten, O. Ueber den Gehalt Des Caseins an Schwefel Und Uber die Bestimmung des Schwefels in Proteinsustanzen, Z. Phsiol. Chem., 9, 273 (1885).

32. Hammarsten, O. and Hedin, S. G. Textbook of Physiological Chemistry, ed 7. Mandel, J. A., Translator John Wiley & Sons, Inc., New York, 652 (1914).

33. Harris, J. I. Effect of Urea on Trypsin and Alpha Chymotrypsin. Nature, 177, 471 (1956).

34. Hawk, P. B., Oser, B. L. and Summerson, W. H. Practical Physiological Chemistry, ed 13, The Blakiston Company, Inc., New York, pp. 629-635 (1954).

35. Hipp, N. J., Grove, M. L., Custer, J. H., and McMeekin, T. L. Separation of Gamma Casein, J. Am. Chem. Soc., 72, 4928 (1950).

36. Hipp, N. J., Groves, M. L., McMeekin, T. L. Separation of Alpha Casein from Whole Casein. U. S. Patent No. 2,572,026, Oct. 23, 1951.

37. Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. Separation of Alpha, Beta, and Gamma Caseins. J. Dairy Sci., 35, 272-81 (1952).

38. Hipp, N. J., Groves, M. L., McMeekin, T. L. Separation of Alpha, Beta, and Gamma Caseins from Whole Casein. U. S. Patent No. 2,702,800, Feb. 22, 1955; CA, 71999 (1955).

39. Hoover, S. R. and Kokes, E. L. C. Effect of pH on Proteolysis by Papain, J. Biol. Chem., 167, 199 (1947).

40. Horwitt, M. K. The First Stage of Casein Hydrolysis by Chymotrypsin and Trypsin, J. Biol. Chem., 156, 421 (1944).

41. Hugaard, G. and Roberts, R. M. Heats of Organic Reactions XIV. The Digestion of Beta Lactoglobulin by Pepsin, J. Am. Chem. Soc., 64, 2664 (1942).

42. Jacobsen, C. F. The Activation of Chymotrypsinogen, Compt. rend. Lab. Carlsberg, 25, No. 14, 325 (1947).

43. Janddorf, B. J. and Michel, H. O. Proteolytic Enzymes, Annual Review of Biochem., 26, 101 (1957).

44. Jansen, E. F., Nutting, M. D. F., Jang, R. and Ball, A. K. Inhibition of the Proteinase and Esterase Activity of Trypsin and Chymotrypsin by Di-isopropylfluorophosphate: Crystallization of the Inhibited Chymotrypsin, J. Biol. Chem., 179, 189 (1949).

45. Kossel, (1891). Quoted from Rimington and Kay (ref. 79).

46. Krehl and Matthes, Arch. Exp. Path. Pharm., 36, 439 (1895). Quoted from Rimington and Kay (ref. 79).

47. Kubachi, V., Brown, K. D. and Laskowski, M. Electrophoresis and Solubility of Chymotrypsinogen B and Chymotrypsin B, J. Biol. Chem., 180, 73 (1949).

48. Kunitz, M. and Northrop, J. H. Crystalline Chymotrypsin and Chymotrypsinogen. 1. Isolation, Crystallization and General Properties of a New Proteolytic Enzyme and its Precursor, J. Gen. Physiol., 18, 435 (1935) and 459 (1935).

49. Levene, P. A. and Hill, D. W. On a dipeptide Phosphoric Acid Isolated from Casein, J. Biol. Chem., 101, 711 (1933).

50. Li, H. C., Papkoff, H., Fønss-Bech, P. and Condliffe, P. G. Action of Chymotrypsin on Hypophyseal Growth Hormone, J. Biol. Chem. 218, 41 (1956).

51. Linderstrøm-Lang, K. and Kodama, S. On the Solubility of Casein in Hydrochloric Acid, Compt. rend., Lab. Carlsberg, 16, No. 1 (1927).

52. Linderstrøm-Lang, K. On the Fractionation of Casein, Compt. rend., Lab. Carlsberg, 17, No. 9 (1929).

53. Linderstrøm-Lang, K. Compt. rend., Lab. Carlsberg, 17, No. 4 (1927) Quoted from Jacobsen (ref. 42).

54. Lillevik, H. A. and Sandstrom, W. M. The Origin of the Humin Formed by the Acid Hydrolysis of Proteins IX. Hydrolysis in the Presence of Djenkolic and of Thiozolidine-4-carboxylic Acids, J. Am. Chem. Soc., 63, 1028 (1941).

55. Lipmann, F. Uber die Bindung der Phosphorsaure in phosphorproteinen, Biochem. Z., 262, 3 (1933).

56. Lubavin. Ergeb. Physiol. 2, 193 (1871). Quoted from Rimington and Kay (ref. 79).

57. Lundsteen, E. Uber das pH-Optimum der Chymosinaktiven Enzyme. (Chymotrypsin, Pepsin, Chymosin), Biochem. Z., 295, 191 (1938).

58. MacDougall, F. H. Physical Chemistry, The Macmillan Company, New York, pp. 269 (1936).

59. Martin, J. B. and Dotty, D. M. Determination of Inorganic Phosphorus, Anal. Chem., 21, 965 (1949).

60. Massey, V. and Hartley, B. S. Active Centre of Chymotrypsin I & II Biochem. et Biophys. Acta, 21, 58 (1956) and 21, 361 (1956).

61. Mattenheimer, von H., Nitschmann Hs., and Zahler, P. Das Lab und seine Wirkung auf das Casein der Milch. IV. Uber die Phosphatasewirkung des Labes, Helv. Chim. Acta, 35, 1971 (1952).

61a. Mattenheimer, von H. and Nitschmann, Hs. Das Lab und seine Wirkung auf das Casein der Milch. VIII, Helv. Chim. Acta, 38, 687 (1955).

62. Mcglory, D. M., Olsen, N. S. and Field, L. A Pressor Material Produced by the Action of Pepsin on Casein, J. Biol. Chem., 219, 299 (1955).

63. McMeekin, T. L., Groves, M. L., Hipp, N. J. The Separation of a New Component of Casein. Abstracts of Papers, 132nd National Meeting, American Chemical Society, Miami, Florida, April 7-12, 65c (1957).

64. Mellander, O. Electrophoretische Untersuchung von Casein, Biochem. Z., 300, 240 (1939).

65. Moraczewski, v. W. Ver daung Producte des Caseins und ihr Phosphorgehalt, Z. Physiol. Chem. 20, 28 (1895).

66. Mulder, G. J. Ann. der Pharm., 28, 73 (1838); McMeekin, T. L. Milk Proteins. Neurath, H. and Bailey, K., Editors. The Proteins, Academic Press, Inc., Publishers, New York, Vol. 2 Part A, 395 (1954).

67. Nielsen, H. C. Molecular Weight Studies on Acid-Precipitated, Calcium Precipitated, Alpha, and Beta Caseins by Osmotic Pressure Measurements in 6.66 M Urea, Unpublished Ph. D. thesis, Michigan State University, 1957, 113 numb. leaves.

68. Nicholson, B. J. The Liberation of Phosphorus from Casein During Proteolysis by Taka-Diastase, Unpublished M. S. thesis, Michigan State University, 1955, 54 numb. leaves.

69. Nitschmann, Hs. and Lehmann, W. Experimentia, 3, 153 (1947); McMeekin, T. L. and Polis, D. Milk Proteins, Advances in Protein Chemistry, 5, 201 (1949).

69a. Nitschmann, Hs. and Bohren, H. U. Das Lab und Wirkung auf das Casein der Milch. X). Eine Methode zur direkten Bestimmung der Geschwindigkeit der Primarreaktion der Labgerinnung der Milch, Helv. Chim. Acta, 38, 1953 (1955).

70. Norberg, B. Phosphoprotein Phosphatase in the Rat, Acta Chem. Scand., 4, 1206 (1950).

71. Northrop, J. H., Kunitz, M. and Herriott, R. M. Crystalline Enzymes, 2nd ed., Columbia University Press, New York, 98 (1948).

72. Osborne, T. B. and Wakeman, A. J. Some New Constituents of Milk, J. Biol. Chem., 33, 243 (1918).

73. Perlmann, G. E. The Nature of Phosphorus Linkages in Phosphoproteins, Advances in Protein Chemistry, 10, 1 (1955).

74. Perlmann, G. E. Enzymatic Dephosphorylation of Casein, J. Am. Chem. Soc., 74, 3191 (1952).

75. Peterson, R. F., Harrington, B. J., and McMeekin, T. L. Separation of Primary Products Formed from Beta Casein by the Action of Trypsin, Abstracts of Papers, 126th, National Meeting of the American Chemical Society, New York, Sept. 12-27, 89c (1954).

76. Plimmer, R. H. A. and Bayliss, W. M. The Separation of Phosphorus from Caseinogen by the Action of Enzymes and Alkali, J. Physiol., 33, 439 (1906).

77. Posternak, T. and Pollaczek, H. De la Protection Contre l'hydrolyse Enxymatique Exercee par les Groupes Phosphorles. Etude de la Degradation Enzymatique d'un Peptide et d'un Polyose Phosphoryle, Helv. Chem. Acta, 24, 921 (1941).

78. Riesen, W. H., Hankes, L. V., Henderson, L. M. and Elvehjem, C. A. Liberation of Amino Acids from Raw and Heated Casein, J. Biol. Chem., 176, 467 (1948).

79. Rimington, C. R. and Kay, H. D. Some Phosphorus Compounds of Milk. The Liberation of Phosphorus from Caseingen by Enzymes and other Agents, Biochem. J., 20, 777 (1926).

80. Salkowski and Hahn. Pfluger's Arch. 59 225 (1895). Quoted from Rimington and Kay (ref. 79).

81. Schwert, G. W., Neurath, H., Kaufman, S. and Snoke, J. E. The Specific Estrase Activity of Trypsin, J. Biol. Chem., 172, 221 (1948).

82. Schwert, G. W. The Molecular Size and Shape of the Pancreatic Proteases, J. Biol. Chem., 190, 799 (1951).

83. Sebelien, J. Uber das Verhalten des bei der Pepsindigestion des Caseins abgespaltenen Pseudonucleins, Z. Physiol. Chem., 20, 443 (1895).

84. Sizer, W. I. Essential Groups of Crystalline Chymotrypsin, J. Biol. Chem., 160, 547 (1945).

85. Slotta, K. H. and Primosigh, J. Amino Acid Composition of Crotoxin, Nature, 168, 696 (1951).

86. Sullivan, R. A., Dowey, W. E., Stanton, E. K., Wagoner, E. V. and Hansell, M. J. Liberation of Amino Acids and Active Peptides from Heated, Raw, and Vitamin free Casein by Trypsic Digestion, Federation Proceedings, 9, 236 (1950).

87. Sundararajan, T. A. and Sarma, P. S. Preparation and Amino Acid Composition of Enzymically Dephosphorylated Casein, Biochem. J. 65, 261 (1957).

88. Tiselius, A. and Eriksson-Quensel, I. B. Peptic Digestion of Egg Albumin, Biochem. J. 33, 1752 (1939).

89. Von Tavel, P. and Signer, R. Counter Current Distribution in Protein Chemistry, Advances in Protein Chemistry, 11, 296 (1956).

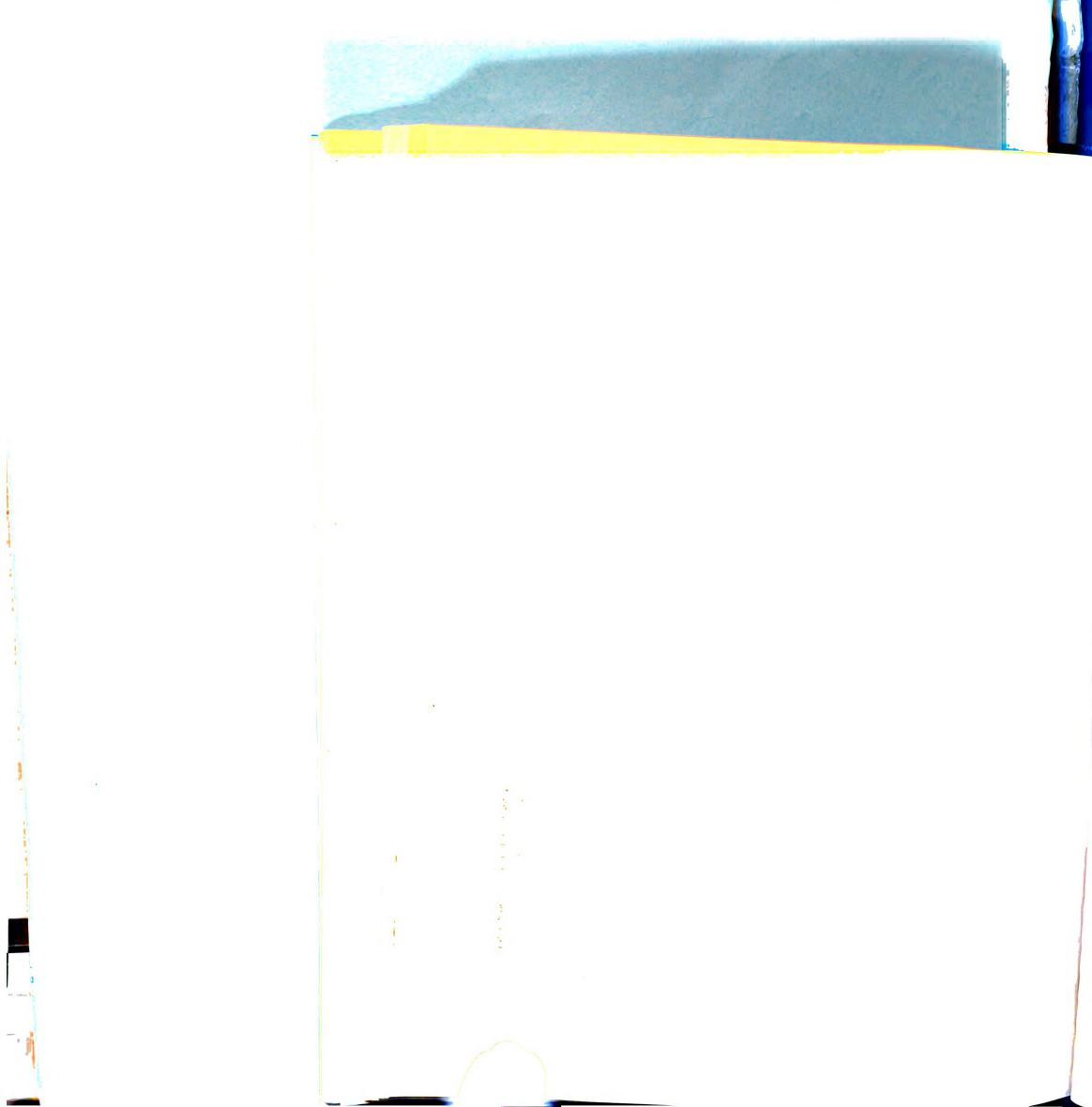
90. Vernon, H. M. Pancreatic Zymogens and Pro-zymogens. J. Physiol. 28, 448 (1902).

91. Warner, R. C. Separation of Alpha and Beta Casein, J. Am. Chem. Soc. 66, 1723 (1944).

92. Waugh, D. F. Process for Producing a Water-Soluble Casein, U. S. Patent No. 2,744, 891, May 9, 1956.

93. Waugh, D. F. and Hippel, P. H. von. Kappa Casein and the Stabilization of Casein Micelles, J. Am. Chem. Soc., 78, 4576 (1956).

94. Weil, L., James, S. and Buchert, A. R. Photooxidation of Crystalline Chymotrypsin in the Presence of Methylene Blue, Arch. Biochem. Biophys., 46, 266 (1953).



95. Willstatter, R. and Waldschmidt-Leitz, E. Alkalimeterische Bestimmung von Aminosauer und Peptiden, Ber, 34 B: 2988 (1921).

96. Winnik, T. General Characteristics of the Partial Hydrolysis Products from the Action of Peoteolytic Enzymes on Casein, J. Biol. Chem., 152, 465 (1944).

97. Wood, H. N. and Balls, A. K. Enzymatic Oxidation of Alpha Chymotrypsin, J. Biol. Chem., 213, 297 (1955).

APPENDIX I

TABLE I

RESULTS OBTAINED FOR ANALYSIS OF INORGANIC PHOSPHORUS IN TRICHLOROACETIC ACID FILTRATES OF CASEIN DIGESTS BY THE PROCEDURE OF MARTIN AND DOTY (59)

Casein Digestion Time (minutes)	Phosphate Found µg.P/ml.	Phosphate Found µg.P/ml. (added 4.0 ug.P/ml.)	
		Observed	Calculated
0	1.8	-	-
15	1.2	4.4	5.2
30	1.1	3.0	5.1
60	1.8	3.6	5.8
120	0.4	2.8	4.4

TABLE II

RESULTS OBTAINED FOR ANALYSIS OF INORGANIC PHOSPHORUS IN TRICHLOROACETIC ACID FILTRATES OF CASEIN DIGESTS BY THE RECOMMENDED PROCEDURE (i.e. used for the investigations)

Casein Digestion Time (minutes)	Phosphate Found µg.P/ml.	Phosphate Found µg.P/ml. (added 4.0 ug.P/ml.)	
		Observed	Calculated
45	9.20	13.10	13.20
120	10.10	14.00	14.10
	2nd Run, Different Enzyme Concentration		
30	10.80	14.9	14.80
120	11.00	15.20	15.00
	Beta Casein		
30	1.85	5.90	5.85
60	2.10	6.20	6.10