THE LIBERATION OF PHOSPHORUS FROM CASEIN DURING PROTEOLYSIS BY TAKA-DIASTASE

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By

Bobbie Jean Nicholson

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VITA

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AN ABSTRACT

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ABSTRACT

Proteolytic enzymes from microbiological sources such as B. *subtilus* or A. *oryzae* bring about the digestion of casein with accompanying formation of a milky appearing solution. Such is observed when Taka-Diastase is added to a clear, neutral solution of casein. This study was concerned with the nature of phosphorus liberated during casein proteolysis by Taka-Diastase. The quantities of inorganic and 1N trichloroacetic acid soluble phosphorus released were determined. Proteolytic activity was simultaneously followed by increase in optical density at 280 mu of the acid soluble hydrolysate and by change in its specific conductivity. Both rate of specific conductivity increase and inorganic phosphorus liberation indicated a two step reaction. The first step preceded onset of milkiness whereas the second followed. Increases in optical density and total acid soluble phosphorus when compared with digestion time were linear. Proteolysis and all forms of phosphorus liberated showed identical dependency upon concentrations of enzyme, substrate, and hydrogen ion. Therefore these variables are believed to be directly associated with one and the same proteinase.

These experimental results suggested that the initial reaction(s) preceding milkiness involved the disappearance of β-casein. Furthermore that this was also caused by a proteolytic enzyme catalyzed hydrolysis of the phosphodiester and some peptide bonds of this

fraction. Also, at a much slower rate there was some hydrolysis of monophosphate groups to produce inorganic phosphate ions.

The reaction(s) following milkiness was regarded as proteolytically catalyzed hydrolysis of the phosphorus and nitrogen bonds of the α-casein fraction. The use of a specific phosphodiesterase, i.e. snake venom from the diamond back rattler (Crotalus adamanteus), on casein brought about the reaction(s) of step one. The disappearance of β-casein only, following incubation, was demonstrated by electrophoretic analysis.

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I. INTRODUCTION

The proteolytic hydrolysis of casein has been investigated with various enzyme preparations for the purpose of gaining information about protein structure, mechanism of reaction, or the role of the protein in nutrition.

Some sources of enzyme such as from cultures of Bacillus subtilus (38) or Aspergillus oryzae (35) bring about the formation of a milky appearing digest after addition to a clear neutral solution of casein. Studies on the proteolytic changes with respect to nitrogen products during this transformation have been made by Lieser (35) and the results seem to indicate that a two step reaction is involved. Electrophoretic analysis during the reaction shows that β-casein disappears prior to milkiness and subsequently α-casein is attacked (37).

Since casein is one of the most prevalent phosphoproteins and due to the scarcity of previous study, it seemed logical to investigate the nature of phosphorus liberation during proteolysis.

II. HISTORICAL

A. The Occurrence of Phosphoproteins

Phosphoproteins are found in abundance in embryonic tissues and in the food for the young. The phosphoproteins of milk and eggs have been the most extensively studied. Vitellin, ovalbumin, and phosvitin are the outstanding phosphoproteins of eggs. The nature and properties of these proteins have been summarized in several books (62), (84), (28). Casein, found in milk, has received the most attention of all the phosphoproteins. Reviews have been written by Sutermeister and Browne (18), McMeekin (45), and also by McMeekin and Polis (45).

Phosphorus in Casein and Its Fractions — Casein has been prepared by several different methods. Hammarsten (24) employed acetic acid for isoelectric precipitation and his purified product contained 0.85 percent phosphorus. The casein prepared by Van Slyke and Bosworth (86) by using ammonium hydroxide for redissolving and ammonium oxalate to remove calcium contained only 0.71 percent phosphorus. Zoller (3) reported that whole casein prepared by using a mixture of five percent hydrochloric and nitric acids contained 0.78 percent phosphorus.

Casein was long considered a pure protein. In 1927 Linderstrøm-Lang and Kodama (39) by extraction and precipitation methods demonstrated that casein is a mixture of several proteins. Later Linderstrom-Lang (40) succeeded in separating casein into three different fractions that differed in phosphorus content. The percentages were 0.96, 0.52, and 0.10 respectively. Cherbuliez and co-workers (4), (5), (6) principally with the aid of five percent ammonium chloride and acetone precipitations prepared a number of casein fractions and subfractions of phosphorus content ranging from 0.55 to 2.32 percent. Groh (22) by the use of urea, phenol, and alcoholic ammonia succeeded in separating ten casein fractions of phosphorus content ranging from 0.65-0.90 percent.

Mellander (51) found three peaks when he electrophoretically analyzed whole casein of 0.86 percent phosphorus. He named them α-, β-and γ- casein based on their decreasing mobilities. Warner (85) was the first to follow the fractionation of casein by electrophoretic analysis. From unfractionated casein of 0.86 percent phosphorus, he prepared the α- and β-fractions with a phosphorus content of 1.0 and 0.61 percent respectively. Gordon and co-workers (20) affirmed the same phosphorus content in α- and β-caseins. In 1950 Hipp and co-workers (29) determined the phosphorus content of γ-casein as 0.11 percent. They also prepared α- and β-casein by a new alcohol method that analyzed 0.98 percent and 0.55 percent phosphorus respectively. The phosphorus content of γ-casein closely resembled that of the alcohol soluble protein of casein separated by Osborne and Wakeman (57) in 1918. By dry grinding of casein Cohen (8) in 1943 produced a water soluble fraction of 0.68 percent phosphorus and an insoluble fraction of 0.85 percent.

Phosphopeptones of Casein and Fractions -- Posternak (66) in 1926 isolated a phosphopeptone from a two to three day tryptic digest that analysed 5.86 percent phosphorus. About the same time Rimington and Kay (72) isolated a phosphopeptone in the same manner that contained 3.8 percent phosphorus. In 1927 Posternak (66) analyzed the phosphopeptone structure that he had isolated the previous year and stated that it contained three moles of isoleucine, three moles of serine, three moles of glutamic acid, and six moles of aspartic acid.

Lipmann (41) in 1933 showed for the first time that phosphorus was attached to the hydroxyl group of serine in casein. His phosphopeptone prepared by tryptic hydrolysis of casein contained 3.34 percent phosphorus. Levene and Hill (36) were the first workers to show what amino acids might be attached to serine phosphate. They isolated a dipeptide composed of serine phosphate and glutamic acid and proposed the structure to be either phosphoseryl-glutamic acid or glutamyl-serine phosphate.

Three groups of workers published further findings during the year of 1941. Lowndes, Macara, and Plimmer (42) isolated an octapeptide from casein tryptic hydrolysate containing two moles of glutamic acid, two moles of phosphoserine (5.67 percent phosphorus) one mole of dicarboxylic acid (probably aspartic), and three moles of unknown simple amino acid (probably isoleucine). Rimington (68), (69), (70), (71), from his work on tryptic phosphopeptones, suggested that the essential phosphorus linkage in casein seemed to be between phosphoric acid and serine. Serine appeared to be united in peptide linkage with other amino acids, predominately glutamic and possibly isoleucine. He found the phosphopeptone with 7.4 percent phosphurous to be composed of five moles of glutamic acid, four moles of serine and three moles of phosphoric acid. Damodaran and Ramachandran (9) prepared a phosphopeptone by peptic followed by tryptic hydrolysis. The barium salt of the phosphopeptone isolated analysed 4.3 percent phosphorus.

Mellander (47) isolated a trypsin resistant phosphopeptone as the barium salt from human milk casein. He later (48) stated that a large part of the phosphorus in human casein is converted by intestinal proteolytic enzymes to phosphopeptones of 4.9 percent phosphorus content. Mellander (49) also determined that human milk whole casein contains from 0.25-0.42 percent phosphorus.

Nicolet and Shinn (54) by casein tryptic digestions and fractional precipitation obtained what they believed to be a relatively pure octapeptide. The amino acids present were one mole of serine, two moles of phosphoserine, two moles of isoleucine, two moles of glutamic acid, and one mole not identified. When the peptone was treated with a phosphatase, seryl-glutamic acid was liberated. This was the first indication of the phosphoseryl-glutamic acid residue in casein.

Crystalline phosphoserine of 15.80 percent phosphorus content was first obtained and compared with a synthetic sample by Agren, De Verdier and Glomset (1) in 1951. The following year De Verdier (10), similarily, isolated and compared phosphothreonine from casein digests. He also published the same year (11) the finding that bovine casein contains 6.3 percent phosphoserine and 4.9 percent phosphothreonine.

The only work reported on phosphopeptones of separated casein fractions has been that of Peterson, Harrington, and McMeekin (64) in 1954. They isolated a phosphopeptone from the tryptic digest of β-casein that contained 3.0 percent phosphorus. Perlmann (59) reported that relatively short polypeptides were formed when β-casein was digested with a phosphodiesterase followed by phosphomonoesterase. α-casein (63) gave similar products when it was pretreated with a phosphodiesterase followed by a combination of phosphomonoesterase and a pyrophosphatase.

B. Enzyme Studies Related to Phosphorus Linkages in Casein

Proteolytic Enzymes and Phosphorus Liberation -- In 1895 Sebelien found casein to be completely solubilized by trypsin but made no investigations with regard to the nature of the phosphorus liberated. However, in 1898 Biffi, a student of Salkowski, extended this experiment by precipitating about 27 percent of the solubilized phosphorus with magnesia mixture and presumed the rest to be organic phosphorus (65).

Bayliss in 1904 stated that early tryptic action on casein produced a great increase of electrical conductivity and with Plimmer (65), a year later, reported on the rates of separation of phosphorus from casein by trypsin, pepsin, papain, and alkali. By utilizing tannic acid as protein precipitant, they concluded that the total acid soluble phosphate was split off in a way similar to that of the acid soluble nitrogen and corresponded in the early stages to the electrical conductivity increase. Pepsin was much slower and never completely solubilized the casein phosphorus. Papain reacting in neutral media produced results similar to trypsin.

Rimington and Kay (72) extended their studies to distinguish organic from inorganic phosphorus solubilized during proteolysis. No inorganic phosphorus was found using pepsin, but with high amounts of trypsin all was eventually converted. During three to five hour digestions at pH 8.4 and 37°C. total phosphorus was liberated at a rate comparable to amino-nitrogen production. The organic phosphorus released approached a maximum slowly and then diminished. Inorganic phosphorus produced during this time was considerably slower than any of the other forms.

Mattenheimer, Nitchmann, and Zahler (44) tested the possible phospho-linkage splitting power of rennin. Crystalline rennin did not show any phosphorus liberation activity, unless it was activated with thermostable ultra-filtrate of crude rennin or milk.

<u>The Effect of Phosphatases on Whole Casein</u> -- Phosphatases are enzymes that catalyze the hydrolysis of phosphorus compounds (34). With respect to organic phosphorus compounds (73) the phosphatases may be specific as to whether the linkage is in phosphomonoesterase, phosphodiester, phosphoamide, pyrophosphate, or acyl phosphate

combination. Further subclassification is based upon optimum pH, and/or need of metal ion activation. However, other phosphatases are quite specific for one or more substrates only. Phosphoprotein phosphatases (not classified by any authority) are thusly specific for substrates such as casein, vitellin, and phosvitin. They do not seem to be active on singly bonded organophosphorus compounds.

The alkaline phosphomonoesterases (optimum pH 8.6-9.4) from bone, kidney, ox spleen, and intestine have not shown pohsphatase activity on unfractionated casein (23), (75). But in 1946 Harris isolated an enzyme from bull frog eggs (*Rana catesbiana*) and leopard frog eggs (*Rana pipiens*) that liberated at pH 5 inorganic phosphorus from casein, vitellin, or heated egg yolk. Glycerophosphate and monophenyl phosphate treated in the same manner gave no such reaction. Since this enzyme had not been previously described, he called it phosphoprotein phosphatase.

In 1947 Axelrod (2) discovered an enzyme in the unfractionated juice of citrus fruits (oranges, lemons, and grapefruit) that released inorganic phosphate from casein at a pH of 6.0. In an attempt to classify the enzyme, he treated α-glycerophosphate, β-glycerophosphate, and p-nitro phenylphosphate in the same manner. The first three substrates yielded some inorganic phosphate, but the last one did not. Magnesium ion showed erratic activation. He decided that the enzyme which produced inorganic phosphate was an acid phosphomonoesterase. He also found ribonuclease, acyl phosphatase, and apyrase activity in these fruit juices.

It was reported in 1949 by Feinstein and Volk (13) that an enzyme in rat spleen split inorganic phosphate from casein. Magnesium ion activated the enzyme whose maximum activity was at pH 6.0. The enzyme would not produce inorganic phosphate from phosphoserine under the same conditions. Inhibition with sodium fluoride produced the reduction of inorganic phosphate but the appearance of acid-soluble nitrogen was unaffected, and there was no accumulation of organic phosphorus. They concluded that there was an enzyme acting upon protein phosphorus not requiring proteolytic activity.

Norberg (56) failed to confirm the results of Feinstein and Volk. In 1950 he set out to demonstrate the existence of an independent phosphoprotein phosphatase enzyme and to find its distribution in the different organs. He isolated an enzyme from rat spleen that at pH 5.8 released inorganic phosphate from casein and phosvitin. He found high activity also in brain, adrenals, sex glands, and kidney. Low activity was found in heart, skeletal muscles, red bone marrow, and erythrocytes. However, he was unable to demonstrate this activity on either casein-phosphopeptone or phenyl phosphate under the same conditions. He compared heat lability, pH sensitivity, optimum pH, and metal activation behavior of this enzyme with that of acid phosphomonoesterase, neutral pyrophosphatase, adenyl phosphatase and glucose-6-phosphatase. He found these properties were not comparable with any of the other enzymes and concluded that he had isolated a phosphoprotein phosphatase.

Foote and Kind (16) isolated an enzyme from chick embryo in 1953 that was active at pH 5.8 in releasing inorganic phosphate from casein and phosvitin. However, it was inactive against β-glycerophosphate, fructose diphosphate, phosphoglyceric acid, adenosine triphosphate, serine phosphate, and sodium pyrophosphate. Hence they regarded the enzyme as a phosphoprotein phosphatase.

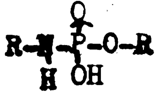
Two enzyme preparations were described by Mattenheimer (43) in 1953, one was from rat liver and the other was from hog stomach. The rat liver preparation proved to be the most active on β-glycerophosphate, less active on whole or α-casein and inactive on phosphopeptone; therefore, it was regarded as a phosphomonoesterase. The hog stomach extract was not active on any of the bond type substrates but caused phosphate liberation from whole casein, α-casein, casein phosphopeptone; hence it was regarded as a phosphoprotein phosphatase. Furthermore, addition of catheptic enzyme extract did not enhance phosphoprotein phosphatase activity; consequently it had no relation to nor dependency upon the latter.

In 1954 Sundararajan and Sarma (76) showed that an enzyme prepared from ox spleen would liberate at pH 6.0 inorganic phosphate from casein, phosvitin, and vitellin. When attempting to classify their enzyme, they found that only a little inorganic phosphate was released after incubation with glycerophosphate and none from casein phosphopeptone. They believed this preparation was not an acid phosphomonoesterase. In another report the same year (77) they announced a preparation of rat spleen, free of acid phosphomonoesterase. At pH 5.8 equal amounts of inorganic phosphate from whole casein, α-casein, and β-casein were hydrolyzed. It was also active upon phosvitin, but not upon glycerophosphate. Since it split phosphorus from -casein, it was eliminated from the list of phosphomonoesterases.

Two phosphatase enzymes, one from potato and the other from calf spleen, were reported by Thoai, Roche, and Pin in 1954 (81). The potato preparation at a pH of 6.0 split inorganic phosphate from whole casein, α-casein, phosvitin, ovalbumin, and a casein phosphopeptide. It had no effect on casein-phosphopeptone or diphenyl phosphate. So they concluded that the enzyme was a non-specific phosphomonoesterase, not magnesium ion activated. The enzyme from calf spleen had its optimum pH at 5.5. It catalyzed the hydrolysis of inorganic phosphate from whole casein, α-casein, phosvitin, ovalbumin, and casein-phosphopeptone. It had no effect on casein phosphopeptide or β-glycerophosphate. It did however split phenol from diphenyl phosphate. The authors decided that the calf spleen enzyme was an acid phosphodiesterase.

The Effect of Phosphatases on Casein Fractions -- Gertrude Perlmann has rather extensively investigated the effect of phosphatase enzymes on the separated fractions of casein. Her results have helped to explain why in certain instances phosphatases heretofore mentioned have not released inorganic phosphate from whole casein.

In 1952 Perlmann (61) reported that α-casein was about 42 percent dephosphorylated in the pH range of 5.6-6.6 by prostate phosphatase. The α-casein when thus dephosphorylated decreased in solubility and showed several new components electrophoretically. Prostate phosphatase had no effect on β-casein, but upon 24 hour exposure to whole casein 12 percent inorganic phosphorus was freed. When α-casein and β-casein were remixed so that β-casein exceeded 30 percent, enzyme inhibition developed and increased proportionately.

At the Symposium on Phosphorus Metabolism in 1952 Perlmann (62) revealed that intestinal phosphatase usually regarded as an alkaline phosphatase, actually released phosphorus from the phosphoamide linkage  $R-\underset{H}{N}-\overset{O}{\underset{OH}{P}}-O-R$ at either pH 5.3 or 9.0, but only slightly at pH 7.0. This behavior was found with such substrates as N(p-chlorophenyl) amidophosphate, ovalbumin, and α-casein. With α-casein digests at pH from 5.3 to 10.0 (optimums at 6.1 and 8.4) 40.0 percent of the phosphorus was readily liberated.

With respect to β-casein Perlmann (59) demonstrated that prostate phosphatase (an acid, non-magnesium ion activated phosphomonoesterase of optimum pH 5.8 to 6.0) did not liberate any phosphorus. Neither was inorganic phosphate found when β-casein was treated at pH 8.2 with purified phosphodiesterase from rattle snake venom (*Crotalus adamanteus*). However when β-casein was pretreated with the snake venom phosphodiesterase at pH 8.2 followed by prostate phosphatase at pH 5.6, from 54.0-72.0 percent of the phosphorus in β-casein was found converted to inorganic phosphate. This led Perlmann to conclude that β-casein contained phosphorus linked principally in the diester form.

Perlmann (60) in 1954 pointed out that α-casein contains a certain number of phosphate groups with ionizable hydroxyls which contribute to the net charge and thus to protein electrophoretic molbility. Such groups are readily attacked by the suitable phosphatases. These conditions are not fulfilled with β-casein. Dephosphorylation of this protein fraction results in the formation of trichloroacetic acid soluble nitrogen (N. P. N.) products.

In another publication in 1954 Perlmann (63) showed that 40 percent of α-casein phosphorus was removed by prostate phosphatase at pH 5.8 to 6.0, but that none was given by purified phosphodiesterase from rattle snake venom at pH 8.5. However when α-casein was first pretreated with phosphodiesterase at pH 8.5, then adjusted to pH 6.0, and incubated with prostate plus intestinal phosphatase 78.0 percent of the total appeared as inorganic phosphate. Intestinal phosphatase caused reaction on the phosphoamide linkage. This result was considered as hydrolysis of the phosphodiester bonds followed by liberation of phosphorus from the resulting phosphomonoester groups. Yeast pyrophosphatase alone with Mg^{++} at pH 7.2 did not free any inorganic phosphorus from α-casein. But combination of yeast pyrophosphatase together with prostate phosphatase at pH 6.0 released 59.0 percent of the phosphorus in α-casein. This was regarded as action upon phosphoanhydride bonds followed by phosphomonoester cleavage. Perlmann reasoned from these results that 40.0 percent of the bonds in α-casein are phosphomonoester, 20.0 percent are pyrophosphate ester, and 40.0 percent are phosphoamide ester.

The Source of Proteolytic Enzyme — Taka-Diastase is prepared from a culture of the mold *Aspergillus oryzae* according to the patented process of Takamine (79). The mold culture grown on wheat bran is extracted with water. Taka-Diastase is precipitated from the water solution by addition of alcohol to 70 percent. The dried product has been reported to contain at least twenty-three enzyme systems (80). The isolation of proteolytic enzymes from the product has been reviewed by Lieser (35).

Some acid phosphatases have been reported to be in Taka-Diastase. Uzawa (82) showed in 1932 that Taka-Diastase contained a phosphomonoesterase of optimum pH of 3.2-4.0 on monophenyl phosphate. The same year he demonstrated (83) phosphodiesterase activity on diphenyl phosphate at pH 5.4 to 5.6. Neuberg and co-workers (52), (53), have demonstrated that Taka-Diastase also contains a pyrophosphatase active on trisodium pyrophosphate at pH 3.2-4.0.

III. EXPERIMENTAL

A. Equipment Used

Thermostat -- The constant temperature bath was equipped with a reservoir bottle to maintain automatically a constant level of water. The thermoregulator (Fisher-Serfass Electronic Relay) controlled the temperature at 29.9° ± 0.1° C.

Glassware -- All pipettes and glassware were the Kimble Glass brand.

Timer -- The reaction periods were timed with a Meylan stop-watch.

pH Meter -- A Beckman model H 2, glass electrode, line operated, pH meter was used in making pH measurements.

Dialysis -- All dialyses were carried out in visking tubing on a rotating external dialyzer constructed in this laboratory.

Spectrophotometers -- 1) A Beckman model B spectrophotometer was used for analyzing for total and inorganic phosphorus. The instrument was calibrated for percent transmittance or optical density units. 2) A Beckman model DU spectrophotometer was used for analysis of acid-soluble proteolytic products absorbing at 280 mu. The instrument was calibrated for percent transmittance or optical density units.

Semi-micro Kjeldahl Apparatus -- The 50 ml. digestion flasks and a rotating digestion rack (manufactured by American Instrument Co.), were used for digesting total phosphorus or nitrogen samples. The distillation apparatus was the modified type used in this laboratory.

Conductivity Bridge -- A model RC-1B conductivity bridge (manufactured by Industrial Instrument Co.) was used for resistance and conductivity measurements.

Conductivity Cell -- A two ml. capacity conductivity cell (Perkin-Elmer Corp.) was used for measuring resistance. The bulb section contained platinum cups which were connected through side arms to caps. The conductivity bridge was connected to these caps. The cell constant is 0.4893.

B. Materials and Solutions

The Enzyme Source -- Taka-Diastase is (marketed by Parke, Davis & Co.) a yellow, amorphous, non-hygroscopic powder analysed for 1.51 percent nitrogen, 0.21 percent phosphorus, and 0.10 percent moisture. It was readily soluble in water and produced a clear, yellow-brown solution.

The Casein Preparation -- Casein was made according to the directions of Dunn (12). The air dried product contained 4.8 percent moisture as determined by overnight drying at 105°C. It analysed for 15.5 percent nitrogen and 0.82 percent phosphorus (both expressed on a moisture free basis). Electrophoretic analysis demonstrated that the preparation was the same as that of Warner (85) both in number of components and their mobility.

Casein Stock Solution -- Six grams of the above air dried casein was weighed into a 100 ml. volumetric flask. Seventy-five ml. of water was introduced in small portions until a smooth paste formed. To this 20.0 ml. of 0.2 N. sodium hydroxide was gradually added, with shaking, until a clear solution of pH 7.0 was obtained. The liquid was made to volume, filtered, heated on a boiling water bath for fifteen minutes, and a crystal of thymol was added as a preservative. The solution was always stored in the cold room at 5.0°C.

Taka-Diastase Stock Solution -- The pre-determined amount of dry powder was weighed on an analytical balance, and dissolved in redistilled water. The final concentration was expressed in terms of milligrams per milliliter of digest. Fresh solutions were always prepared just prior to use.

Fiske-Subbarow Phosphorus Reagents -- These were prepared, with minor modifications, as described by Kabat and Mayer (31).

1.0 Normal Trichloroacetic Acid.-- One hundred sixty-three grams (Eastman) trichloroacetic acid was weighed out on analytical balance and transferred quantitatively to a 1.0 liter volumetric flask and diluted to the mark.

5.0 Normal Sodium Hydroxide -- One hundred grams (Fisher) sodium hydroxide was weighed out on an analytical balance, transferred quantitatively to a 500 ml. volumetric flask, and diluted to the mark.

0.5 Normal Sodium Hydroxide -- Twenty grams (Fisher) sodium hydroxide was weighed out on an analytical balance and transferred quantitatively to a 1.0 liter volumetric flask, and diluted to the mark.

10.0 percent Calcium Chloride -- Ten grams of (Mallinckrodt dihydrate) calcium chloride was dissolved in an ammonium chloride buffer (prepared as below), diluted to 100 ml., and saturated with (Fisher) calcium hydroxide. This reagent was prepared at least once a week, stored in a pyrex bottle, and filtered just before use.

Wash Reagent -- A one to five dilution with distilled water of the above 10.0 percent calcium chloride solution was made.

0.05 N Sulfuric Acid -- This was made by diluting 5.0 ml. of (Baker) 5.0 N sulfuric acid to 500 ml.

Ammonium Chloride Buffer pH 9.0 -- The buffer was prepared by dissolving 26.7 gm. (Fisher) ammonium chloride in 60.6 ml. of (Du Pont) concentrated ammonium hydroxide and diluting to 1.0 liter with distilled water.

Brom Thymol Blue -- The indicator for neutralization in inorganic phosphate analysis was prepared by dissolving 0.04 gm. brom thymol blue in 100.0 ml. of 95 percent ethanol.

C. Experimental Methods

General Digestion Procedure -- An appropriate volume of 6.0 percent casein was pipetted into one arm of a bifurcated test tube, and into the other arm was placed an equal volume of enzyme solution. When these were mixed the resulting digest concentration of enzyme was expressed in terms of mg./ml. of digest. The reaction vessel was placed in the thermostat 20 minutes before mixing. Digestion was begun by tilting the two-branched tube back and forth ten times. The time of initial contact was taken as zero digestion time and noted by starting the stop watch. Suitable aliquots were inactivated at specific intervals by pipetting them into two volumes of 1.0 N trichloroacetic acid. During a period of one-half hour these samples were shaken periodically and then filtered through Whatman #2 filter paper. The filtrates were analyzed for total acid soluble phosphorus, inorganic phosphorus, and acid-soluble proteolytic products as described below.

1. The Influence of Enzyme Concentration -- A series of digestions were performed with the initial concentration of casein always 3.0 percent. Amounts of Taka-Diastase producing 2.0, 4.0, and 8.0 mg/ml. of digest for each experiment was taken for study.

a) Analysis for Acid-Soluble Proteolytic Products -- The liberation of acid-soluble split products, other than phosphorus, was determined on the above trichloroacetic acid filtrates. The zero time sample was set for 100 percent transmission or zero optical density in a model DU Beckman Spectrophotometer and the succeeding time samples compared at 280 mu against the blank. The resulting changes in optical density are reported in Table II and shown in Figure 2.

b) Analysis for Inorganic Phosphate -- The inorganic phosphorus was determined by a procedure similar to that described by Norberg (56). Five milliliters (occasionally 3.0 ml.) of the protein-free filtrate was pipetted into a 15.0 ml. conical centrifuge tube. The aliquot was neutralized by dropwise addition of initially 5.0 N and finally 0.5 N sodium hydroxide to the green color of brom thymol blue indicator. One milliliter of 10.0 percent calcium chloride in 0.5 M ammonium chloride buffer at pH 9.0 and calcium hydroxide saturated was added as precipitating reagent. After 30 minutes the precipitate was centrifuged and washed with 5.0 ml. of a one to five dilution of the precipitating reagent. Finally the precipitate was suspended in 4.0 ml. of 0.05 N sulfuric acid and the inorganic phosphate determined by the method of Fiske and Subbarow (15). The milligrams of inorganic phosphorus was obtained from a standard phosphorus solution treated in the same manner. The results are recorded in Table II and shown in Figure 1.

c) Analysis for Total Acid Soluble Phosphate -- The procedure described here, with minor modifications, was described in Hawk, Oser, and Summerson (26). Five milliliters (occasionally 3.0 ml.) of the above protein-free filtrate was pipetted into a 50.0 ml. micro-Kjeldahl digestion flask. A glass bead (to prevent bumping) and 2.5 ml. of 5.0 N sulfuric acid were put into the flask and heated on the micro-Kjeldahl digestion rack until charring and fumes appeared. The samples were cooled 90 seconds, treated with a drop of 30.0 percent hydrogen peroxide, and heated again until the color disappeared. The peroxide treatment was repeated until the color was gone. Three milliters of distilled water was added to the cooled flasks and they were boiled momentarily. The flasks were cooled, rinsed into a 25.0 ml. volumetric flask and the total acid soluble phosphate determined by the Fiske and Subbarow Method (14). A blank and a phosphorus standard were run in the same manner. The results are shown in Table II and Figure 2.

2. Influence of Substrate Concentration -- A series of incubations were conducted where substrate concentrations of 1.5, 3.0, 4.5, and 6.0 percent casein were prepared from stock solution. The digest concentration of Taka-Diastase was always 4.0 mg./ml. The acid-soluble proteolytic products, inorganic phosphate, and total acid soluble phosphate were determined as described previously. Results are given in Table III and Figure 3.

3. Influence of pH -- A series of digestions was carried out after the pH of the casein stock solution had been adjusted to 6.6, 7.0, and 8.0. The digest concentration was always 3.0 percent for casein and 4 mg./ml. for Taka-Diastase. The acid-soluble proteolytic products, inorganic phosphate, and total acid soluble phosphate were determined as described above. The results are reported in Table IV and Figure 4.

4. Phosphodiesterase Pretreatment -- Ten milligrams of lyophilyzed venom* from the rattle snake (Cretalus adamanteus) was dissolved in

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*The receipt of a complimentary sample from the Ross Allen Reptile Institute, Silver Springs, Florida, is gratefully acknowledged.

10.0 ml. of 0.01 M. magnesium chloride solution. Three milliliters of this was added to 25.0 ml. of 6.0 percent casein at pH 7.35. After incubation for six hours, the pH had dropped to 6.95. Three milliliters were then removed and treated with 6.0 ml. of 1.0 N trichloroacetic acid for total and inorganic phosphate analysis upon the filtrate. At the same time a 20.0 ml. solution containing 200 mg. of Taka-Diastase was added to the remaining 25.0 ml. of digest. From then on the progress of phosphorus liberation and proteolysis was measured as previously described. The results are listed in Table V and shown in Figure 5.

5. Conductivity Change -- The resistance change during hydrolysis was recorded at 15 minute intervals on a 3.0 percent and 4 mg./ml. Taka-Diastase digest at pH 7.3. The specific conductance was calculated and is recorded in Table I and Figure I.

TABLE I

CHANGE IN CONDUCTIVITY DURING CASEIN PROTEOLYSIS

Digestion Time min.	Resistances ohms.	Specific Conductance* mhos.
0	388	1.26
15	386	1.27
30	379	1.28
45	378	1.29
60	378	1.29
75	374	1.31
90	372	1.32
105	369	1.33
120	367	1.33
135	367	1.33
150	367	1.33
** - - - - - - - - -	- - - - - - - - -	- - - - - - - - -
165	364	1.34
180	359	1.36
195	359	1.36
210	358	1.37
225	358	1.37
240	354	1.38

*Found from dividing the cell constant 0.4893 by values in column 2.

**- - - Point where opalescence began.

TABLE II

THE EFFECT OF ENZYME CONCENTRATION UPON PHOSPHORUS LIBERATION AND PROTEOLYSIS

Digestion Time, min.	2mg./ml.Taka-Diastase			4mg.ml.Taka-Diastase			8mg./ml.Taka-Diastase		
	P-Liberated		Proteolysis	P-Liberated		Proteolysis	P-Liberated		Proteolysis
	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.
0	0	0	0	0	0	0	0	0	0
15	3	2	over scale	6	4	over scale	13	5	0.288
30	5	3	0.009	9	5	0.068	18	7	0.562
60	8	6	0.137	17	9	0.286	*34	13	1.07
90	11	8	0.380	25	15	0.491	48	35	1.46
120	19	12	0.492	31	17	0.810	73	49	1.68
150	25	15	0.588	*35	22	1.05	78	60	1.95
180	31	24	0.705	43	42	1.22	84	66	
210	37	30	0.775	54	48	1.48	100	77	
240	41	33	0.964	61	54	1.60	105	85	

* - - - Point where milkiness began.

TABLE III

THE EFFECT OF SUBSTRATE CONCENTRATION UPON PHOSPHORUS LIBERATION AND PROTEOLYSIS

Digestion Time, min.	1.5 percent Casein			3.0 percent Casein			4.5 percent Casein			6.0 percent Casein	
	P-Liberated		Proteo-lysis	P-Liberated		Proteo-lysis	P-Liberated		Proteo-lysis	P-Liberated	
	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.
0	0	0	0	0	0	0	0	0	0	0	0
15	6	2	0.178	6	2	over scale	10	2	over scale	6	2
30	10	3	0.366	9	3	-0.068	16	3	-0.007	8	3
60	* 16	8	0.735	17	8	0.286	17	5	0.143	15	5
90	32	13	1.06	25	10	0.491	21	11	0.352	19	7
120	47	14	1.22	31	11	0.810	28	17	0.535	27	10
150	56	23	1.42	* 35	16	1.05	40	20	0.693	--	--
180	58	42	1.64	43	25	1.22	45	23	0.900	36	16
210	72	43	1.78	54	35	1.48	51	27	1.18	48	23
240	94	50	1.97	61	40	1.60	61	35	1.22	62	33

* _ _ _ Point where opalescence began.

TABLE IV

THE EFFECT OF pH UPON PHOSPHORUS LIBERATION AND PROTEOLYSIS

Digestion Time, min.	pH 6.6			pH 7.0			pH 8.0		
	P-Liberated		Proteolysis	P-Liberated		Proteolysis	P-Liberated		Proteolysis
	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.	Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	O.D.
0	0	0	0	0	0	0	0	0	0
15	12	4	-0.007	6	4	over scale	6	2	-0.014
30	13	5	0.092	9	5	-0.068	9	4	-0.026
60	15	9	0.312	17	9	0.286	11	7	-0.043
90	19	15	0.479	25	15	0.491	13	10	0.019
120	35	20	0.651	31	17	0.810	15	11	0.036
150	*36	26	0.871	*35	22	1.05	17	13	0.048
180	45	43	1.17	43	42	1.22	19	14	0.087
210	50	51	1.19	54	48	1.48	24	14	0.100
240	66	56	1.47	61	54	1.60	30	20	0.122

*_ _ _ _Point where opalescence began.

TABLE V

THE EFFECT OF CASEIN PRETREATMENT WITH PHOSPHODIESTERASE FOLLOWED BY TAKA-DIASTASE DIGESTION

	Digestion Time, min.	Phosphorus Liberated		Proteolysis O.D.
		Total $\Delta\mu$/ml.	Inorganic $\Delta\mu$/ml.	
1--	360*	70	18	0.197
2--	0	0	0	0
	15	10	11	0.143
	30	15	12	0.303
	- - -	- - -	- - -	- - -
	60	17	16	0.628
	90	35	22	0.936
	120	44	23	1.170
	150	57	36	1.400
	180	64	40	1.63
	210	72	45	1.80
	240	89	60	1.99

* pH decreased from initial 7.35 to final 6.95

*_ _ _ Point where opalescence began.

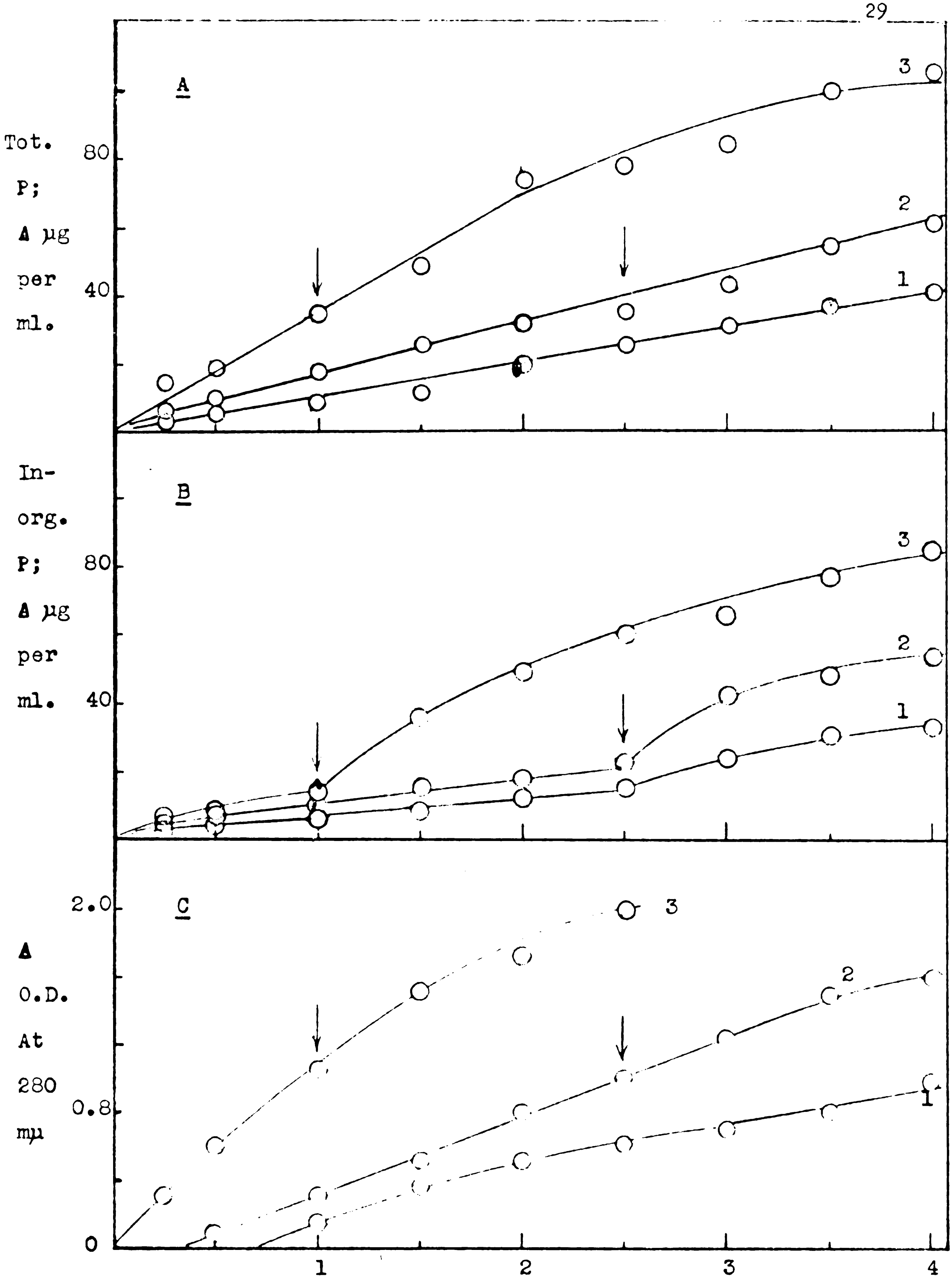


Figure 2. PHOSPHORUS LIBERATED. A total ac. sol. P; B inorg. P; C opt. dens. Curves 1,2,& 3 represent 2,4,& 8 mg. Taka-Diastase per ml. of digest.

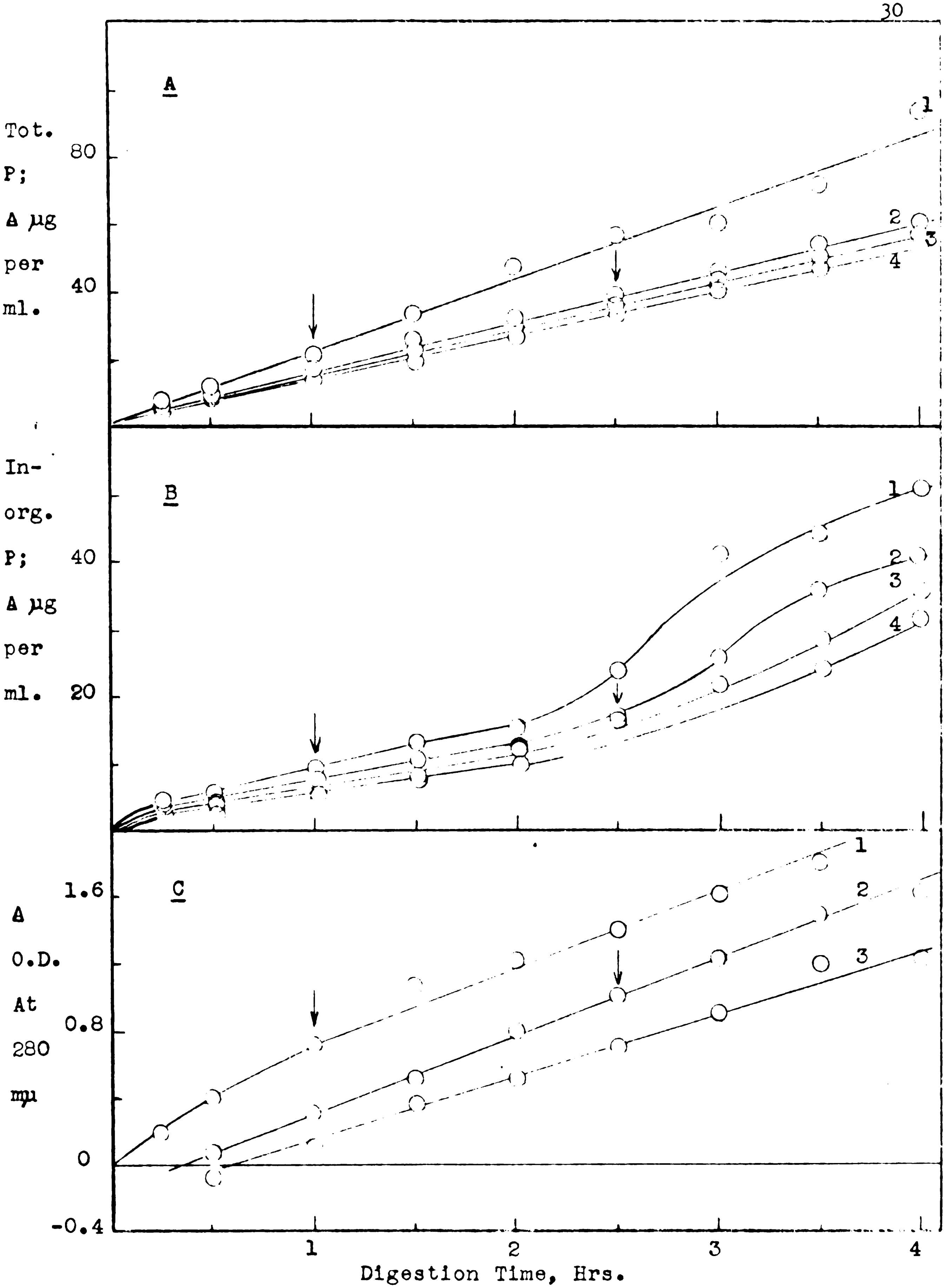


Figure 3. EFFECT OF SUBSTRATE (CASEIN) CONCENTRATION. A tot. ac. sol. P; B inorg. P; C opt. dens. Curves 1, 2, 3, & 4 represent 1½, 3, 4½, & 6 % w/v casein digests.

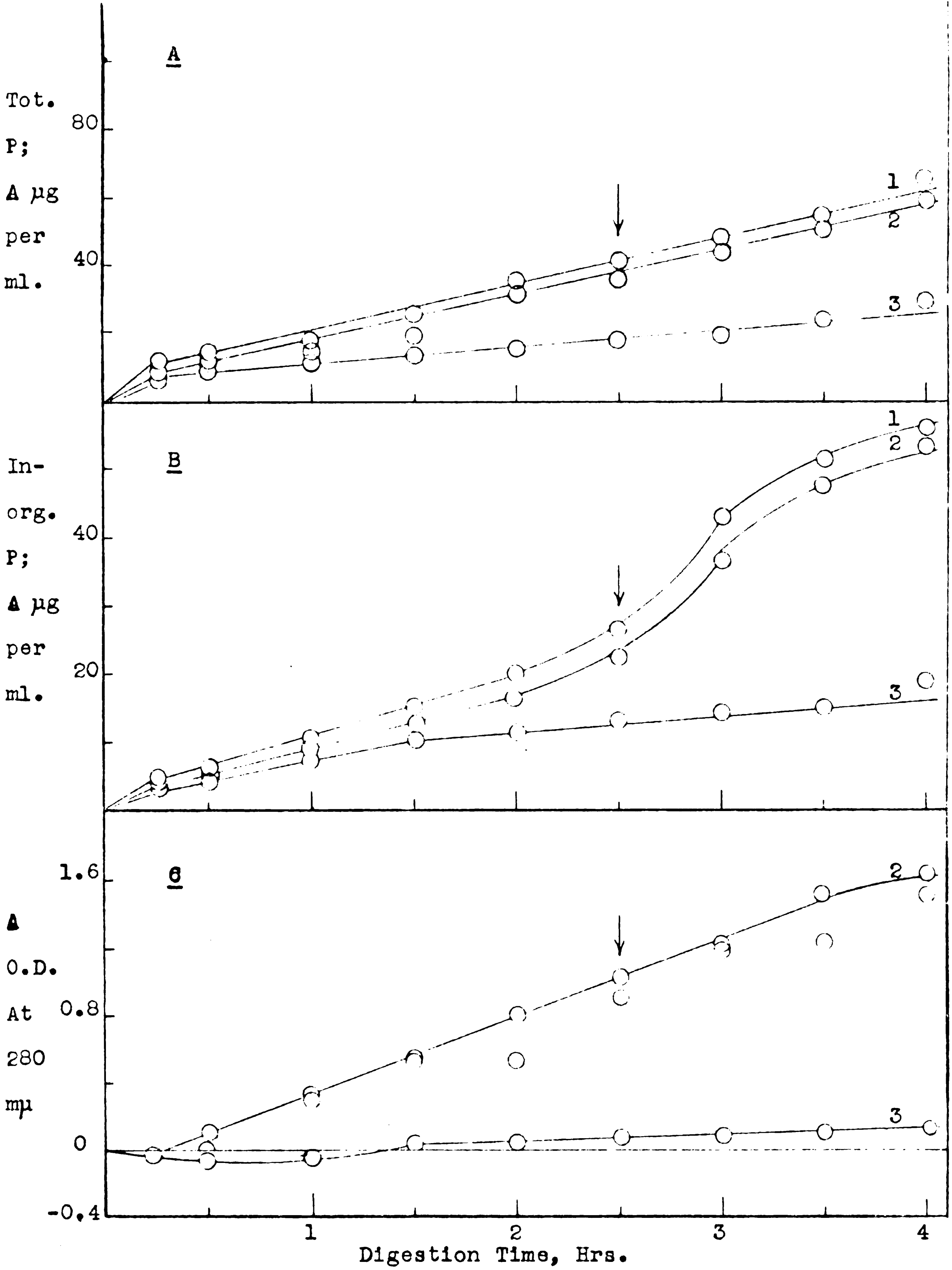


Figure 4. EFFECT OF pH. A tot. ac. sol. P; B inorg. P; C opt. dens. Curves 1, 2, & 3 represent initial digests of pH 6.6, 7.0, and 8.0, respectively.

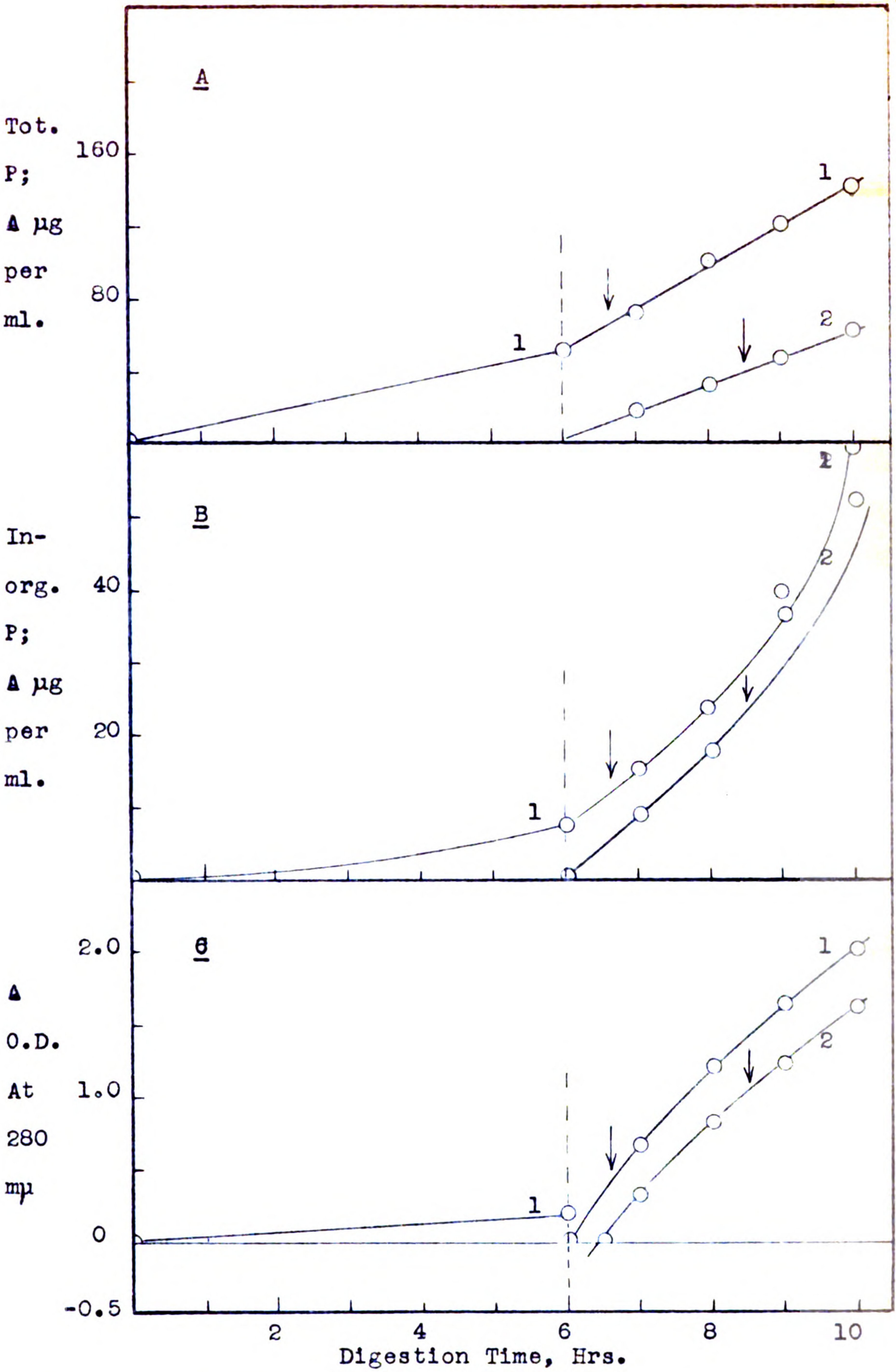


Figure 5. PHOSPHODIESTERASE PRETREATMENT. A tot. ac. sol. P; B inorg. P; C opt. dens. Curves: 1, snake venom incubation followed by Taka-Diastase; 2, control.

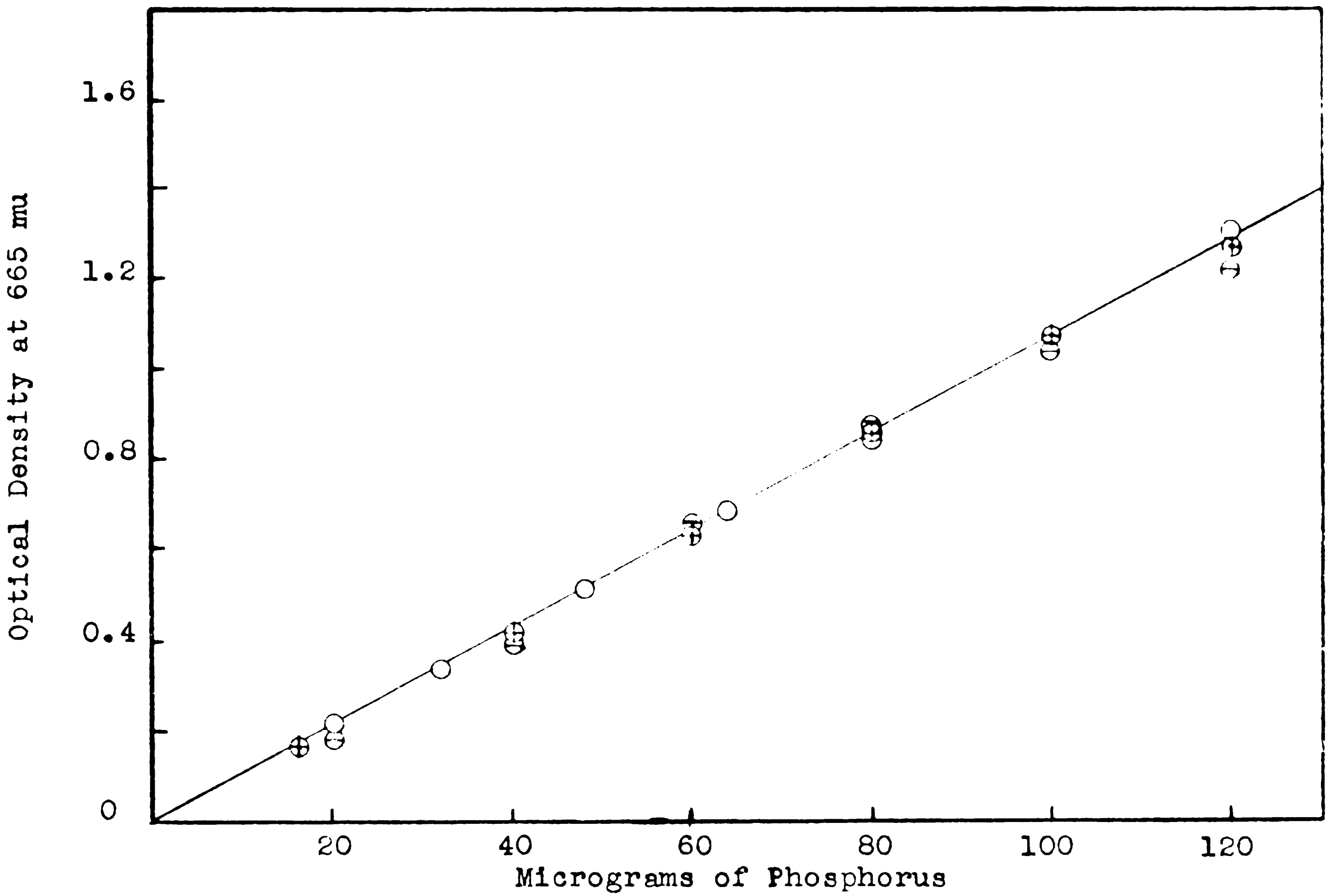


Figure 6. STANDARD PHOSPHORUS ANALYSIS CURVE. ○ Fiske-Subbarow method; ⊕ Norberg method with casein present; ⊖ Norberg method, casein absent.

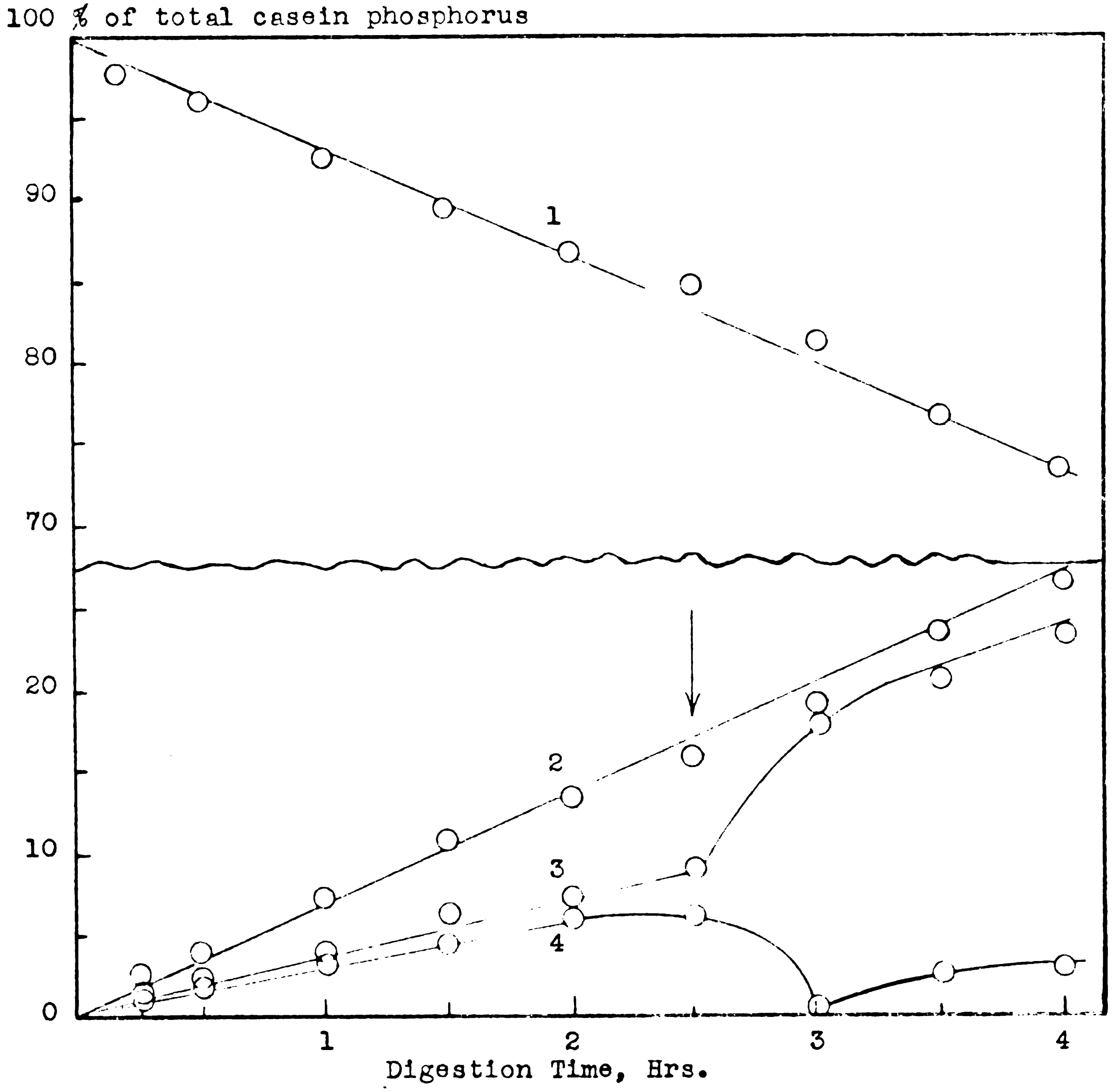
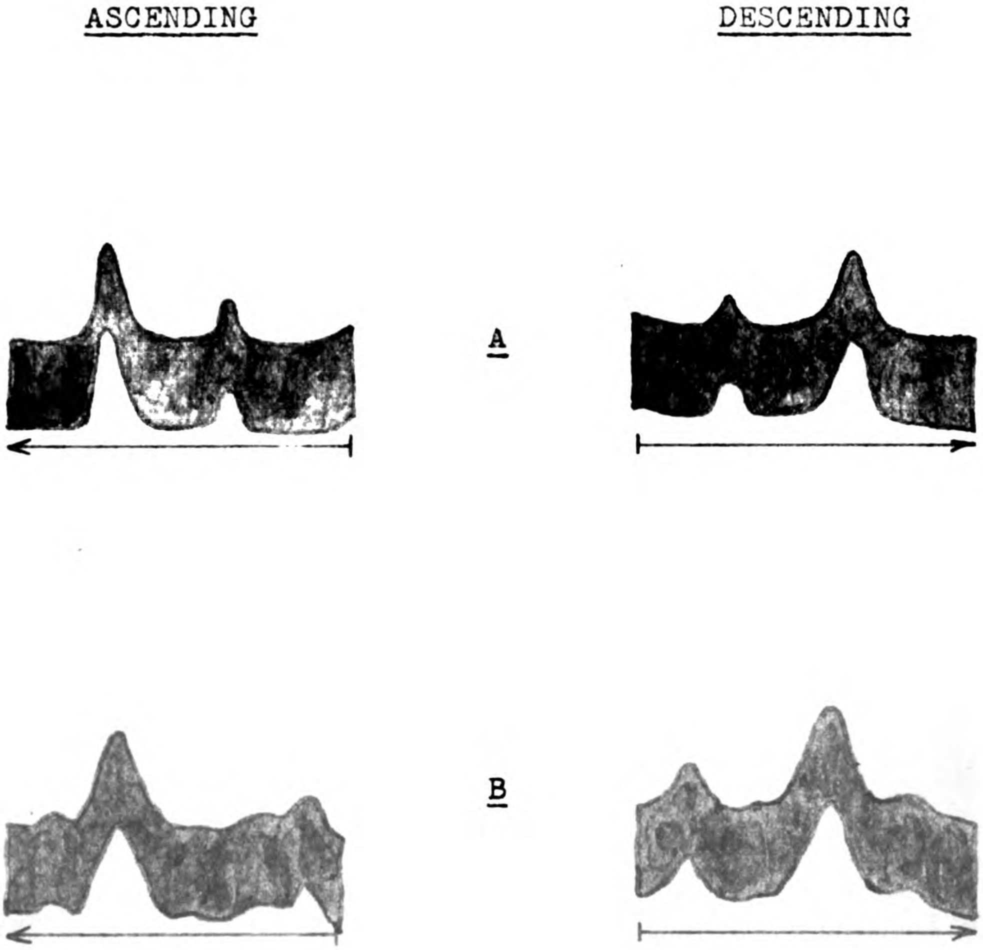


Figure 7. FORMS OF PHOSPHORUS DURING PROTEOLYSIS. Curves: 1, protein P; 2, tot. ac. sol. P; 3, inorg. P; and 4, ac. sol. org. P.

Figure 8. ELECTROPHORETIC PATTERNS (TRACED) OF: A CASEIN; B PHOSPHODIESTERASE TREATED CASEIN.



A in 1 % conc. in 0.1 M phosphate pH 7.0, 0.05 M NaCl, 6300 sec., pot. grad. 7.15 v./cm.

B in 1½ % conc., 0.1 M phosphate, pH 7.2, 0.05 M NaCl, 5610 sec., pot. grad. 9.14 v./cm.

IV. DISCUSSION

The following discussion will be concerned with the release of the various forms of phosphorus during proteolysis of casein by Taka-Diastase. The factors influencing the phases of investigation that will be considered are: A) Methods of phosphate analysis, B) General observations during phosphate release and proteolysis, C) Influence of enzyme concentration, D) Effect of substrate concentration, E) Optimum pH, F) Casein proteolysis in relation to phosphate liberation, and G) Test of reactions in step one with phosphodiesterase.

A. Methods of Phosphate Analysis

The Method for Inorganic Phosphate Analysis -- The method of Fiske and Subbarow (14) for inorganic phosphate determination was not directly applicable. When the trichloroacetic acid soluble phosphorus of a digest aliquot was treated with the molybdate reagent a white precipitate formed which contained most of the phosphorus. Other workers (63) have suggested that with such samples the small molecular weight organic phosphopeptides may be removed by preliminary adsorption upon Dowex 50 (4 percent cross-linked). Such was tried with Dowex 50 (12 percent cross-linked) available, but it did not alter the above observed interference. Trichloroacetic acid concentrations in the filtrate up to 16 percent were tried and no improvement was noted. Numerous modifications in treatment of the filtrate were tried and these included:

one hour time elapse before filtration, addition of 5.0 N sulfuric acid to lower the pH before filtration, addition of sodium hydroxide to dissolve the molybdenum precipitate in the filtrate, five minute heating of digest aliquot with trichloroacetic acid on boiling water bath before filtering (59), cooling in ice bath 15 minutes before filtering (13), use of sodium tungstate with trichloroacetic acid (77), and addition of acetone with trichloroacetic acid before filtration (30). All of these were unsuccessful in preventing or redissolving the unwanted precipitate caused by addition of molybdenum reagent to the trichloroacetic acid filtrate.

In 1950 Norberg (56) indicated having experienced similar difficulties. He introduced the pretreatment of trichloroacetic acid filtrates with a reagent consisting of calcium chloride dissolved in ammonium chloride buffer saturated with calcium hydroxide at pH 9.0. The reagent accomplished a successful separation of inorganic phosphate from organic phosphate as an insoluble apatite. To ascertain the reliability of this procedure various known combinations of inorganic phosphate with casein and also a standard phosphate series alone were tested. These results compared within experimental error with those of a direct Fiske-Subbarow analysis upon standard phosphate solutions. The results are shown in Figure 6. When it was found that the inorganic phosphate content of a digest aliquot exceeded the range of the standard curve a correspondingly smaller digest sample was taken.

Method for Total Acid Soluble Phosphate -- The method adopted for total acid soluble phosphate analysis is described essentially by Hawk, Oser, and Summerson (26). It was found that no color would develop when the quantity of trichloroacetic acid filtrate was increased and treated with proportional amounts of reagents unless the final volume was also correspondingly increased. For some determinations the liberated phosphorus was so low that it was desirable to take samples large enough to contain a more accurately measurable quantity of phosphorus. It was found quite satisfactory to do so provided the same amounts of reagents and final volume for color development were maintained.

B. General Observations

Proteolysis -- As noted previously (35) and in these experiments, when 3.0 percent casein was treated with Taka-Diastase (2-8 mg./ml.) at pH 7.0, opalescence commenced after one to two hours. Thirty minutes thereafter the digest took on the appearance of milk. Proteolytic activity measurements of this process by alcoholic potassium hydroxide titrations, rate of turbidity formation, viscosity change (35), and electrophoretic changes (37) have offered the hypothesis that a two stage reaction is involved.

In addition to the aforementioned measurements, specific conductivity changes during proteolysis support this hypothesis. The results are given in Table I and plotted in Figure 1. The increase in conductivity may be regarded as the result of the increase in charged species (18) upon hydrolysis of bonds and groups in proteins. Figure 1 shows a linear conductivity increase until the opalescence time is approached when for a period no change occurs until after milkiness has appeared. It may be noted (more details later) that simultaneous inorganic phosphate liberation followed the same pattern. It suggests that inorganic phosphate might well be determined by the much simpler resistance measurements.

Another standard method of proteolytic activity measurement is the determination of trichloroacetic acid soluble hydrolysis products by optical density or percent transmittance change at 280 mu in the Beckman ultraviolet Spectrophotometer (55). The progress of proteolysis was determined in this manner on all samples involving the measurement of total and inorganic phosphorus liberated during digestion. Hence all curves involving phosphorus liberation are accompanied by the corresponding plot of optical density increase at 280 mu. These proteolysis plots do not show two stage reaction as suggested in the previous experiments. Instead they correspond more closely to the non-protein nitrogen measurements made earlier (35). However there is another remarkable and yet unexplained phenomena. This is that during initial stages of casein proteolysis with lower enzyme concentrations there is a negative absorption of aromatic residues (such as tyrosine or tryptophan). Christensen (7) has observed the same with trypsin, chymotrypsin, and plasmin and found it to be only applicable to casein substrate.

Phosphorus Liberation During Proteolysis -- The following observations come from an inspection of Tables I, II, III, IV, V, and Figures 1, 2, 3, 4, and 5.

In practically all experiments the total acid soluble phosphorus released was produced at essentially a constant rate during proteolysis by Taka-Diastase. Hence the protein phosphorus (precipitated by trichloroacetic acid) retained was linearly related by negative slope to the digestion time.

As previously mentioned the inorganic phosphate liberated in most cases showed the two stage reaction being involved in casein proteolysis. The increase was linear until incipient opalescence and after onset of milkiness assumed a higher rate of liberation.

The organic phosphate liberated during proteolytic degradation of casein can be seen from Figure 5 to be initially liberated at a constant rate until onset of cloudiness when the value dropped to a minimum before resuming an increase again. This was in accord with the observations of Rimington and Kay (72) who stated that organic phosphate approached a maximum slowly and then diminished.

Comparison of Proteolysis and Phosphorus Liberated -- The formation of acid soluble phosphate and hydrolytic products of casein in most cases occurred at a constant rate. Rimington and Kay (72) found total phosphate and amino-nitrogen liberation to be linear functions during tryptic digestions. The inorganic phosphate liberation rate has already been compared with conductivity changes. Curves in Figures 2, 3 and 4 support the two step reaction hypothesis found by others (35).

C. Influence of Enzyme Concentration

One of the criteria for catalysis by enzymes is that generally initial activity is proportional to enzyme concentration in the presence of adequate substrate (21).

The proteolytic measurements of optical density change at 280 mu as shown in Table II and Figure 2 satisfy the above condition. There is of course the notable negative absorption at lower enzyme concentrations that awaits further explanation.

The total acid soluble phosphate released as shown in Table II and Figure 2 similarly supported the criteria of rate of release being essentially linearily proportional to the amount of Taka-Diastase used.

However the activity concerned with release of inorganic phosphate did not indicate the single linear dependency as the above. At whatever enzyme concentration tested the two stage release was evident but it became less apparent at the lower concentrations. The onset of cloudiness and eventual milkiness followed the identical dependency described above.

These observations seemed to suggest that phosphate liberation was apparently a result of the proteolytic degradation of casein by virtue of its parallel enzyme concentration dependency.

D. Effect of Substrate Concentration

In most enzyme catalyzed reactions the velocity increases, but not proportionately, when substrate concentration is made greater (27). When all enzyme molecules have become converted to enzyme-substrate complexes, maximum velocity is attained and becomes independent of substrate concentration. Occasionally a diminution in velocity may be caused by further increase in substrate concentration.

The results of this variable on proteolytic activity and phosphate liberation are reported in Table III and Figure 3. The data shows that when the ratio of substrate to enzyme concentration was constant the total and inorganic phosphate as well as the optical density changes had the same rate curves. This may be construed as evidence for the same enzyme being operative in phosphate liberation and proteolysis.

But the apparent observation of increased substrate concentration invoking an inhibitory effect with respect to phosphate liberation and proteolysis is inconclusive. Similar experiments by other workers are not known with respect to phosphate release but the proteolytic data is contradictory (27). This aspect of the investigation bears further extensive experimentation.

E. Optimum pH

In connection with the effect of hydrogen ion activity upon enzyme catalyzed reactions one should consider its influence on stability of the enzyme, on enzyme ionization, and also dissociation of the substrate (17).

The effect of pH on proteolysis and phosphorus liberation are tabulated in Table IV and plotted in Figure 4. A curve was not drawn through the optical density values at pH 6.6 since there was reason to suspect operational error in the instrument at the time the analysis was made. However the reliable values clearly substantiated previous optimum pH findings (17). An interesting additional observation is that the higher the pH of digestion the more prolonged was the period of negative absorption at 280 mu.

The phosphate measurements seem to vary in the same manner as the proteolytic. Hence if the influence of hydrogen ions upon enzyme is as stated above the results support the belief that the same enzyme is operative in both phosphate liberation and proteolysis.

Furthermore should one think that the previously described phosphoprotein phosphatases were co-active in this digestion, then certainly pH optimum would be from 5.8-6.0. Since Taka-Diastase activity in this range is actually hydrogen ion inhibited (17) this possibility seems remote when considered together with the findings reported in this study. All other phosphatases activity of Taka-Diastase have been reported to have their optimum pH at even lower values than 6.0.

F. Casein Proteolysis in Relation to Phosphate Liberation

Up to this point evidence regarding the manner of release of total acid soluble and inorganic phosphate together with proteolytic data has been examined. It seems that the liberation of inorganic phosphate together with formation of acid ionizing groups measured either conductometrically or titrimetrically supports the hypothesis of a two step reaction. The first step seems to precede milkiness formation whereas the second develops with onset of this digest appearance. By electrophoretic analysis it has been found that the β-fraction of the substrate is totally consumed during the first step and following milkiness the α-peak disappears. During initial proteolysis the formation of acid soluble organic phosphate is most predominant in comparison to the inorganic fraction of the total amount liberated. Following milkiness this relationship is reversed.

Hence the phosphate release so closely allied with formation of new ionic species or new acid groups can only logically suggest that groups or linkages involving phosphorus are associated with the proteolytic process. During step one where the organic form of phosphate release is predominant and where principally β-casein is involved, it suggests, as does also Perlmann's work (59), that the phosphodiester and some peptide bond cleavage is principally involved. Then following onset of cloudiness proteolytic degradation may principally be concerned with phosphomonoester and peptide bond hydrolysis of the α-casein fraction. Such would account for the greater rate of inorganic phosphate production, conductivity change and other proteolysis data.

G. Test of Reactions in Step One With Phosphodiesterase

If an enzyme specific for the nature of the reactions of step one were available, namely a phosphodiesterase with none or very little proteinase activity, the preceding speculation could be tested. Snake venom from the Diamond back rattler (*Crotalus adamanteus*) has been found to fulfill these specifications and was incubated at pH 7.4 for six hours with whole casein. The results in Table V and Figure 5 show what was predicted in the foregoing discussion. The pH of the digest decreased, organic phosphate release was predominant, production of new ionic species was slight, and optical density change demonstrated very little peptide bond hydrolysis. An electrophoretic pattern showed alteration in the β-peak only but unchanged α-fraction.

Then by addition of Taka-Diastase the onset of milkiness was advanced one hour as well as the increased production of inorganic phosphate, charged ionic species, and hydrogen ion liberating groups.

V. SUMMARY

1) A method for separating inorganic from organic phosphate in trichloroacetic acid filtrates of casein hydrolysates was developed.

2) The rate of liberation of inorganic and total acid soluble phosphate during proteolysis was studied.

3) Inorganic phosphorus followed a two step reaction rate. The first stage of slow liberation occurred before the onset of cloudiness and the second of fast liberation developed after milkiness appeared.

4) Total acid soluble phosphorus showed a constant rate of formation throughout the period of proteolysis.

5) Organic phosphorus was produced rapidly until the onset of cloudiness when the value dropped to a minimum before it resumed a slower increase.

6) The protein phosphorus decreased at a constant rate throughout the reaction.

7) Specific conductivity or measure of charged species developed as a two step reaction. The first step was before cloudiness and the second after milkiness.

8) Proteolysis products and phosphorus liberation showed identical dependency upon casein concentration.

9) The liberation of acid-soluble proteolytic products and all types of phosphorus compounds from casein were similarly proportional

to Taka-Diastase concentration.

10) Maximum activity for casein proteolysis and phosphorus liberation both occurred at pH 6.6.

11) Optical density increase at 280 mu or measure of tyrosine and tryptophan residues liberated by casein proteolysis was essentially constant at some initial periods.

12) The release of charged ionic species is related to formation of inorganic phosphorus products because both followed the two step type of reaction.

13) Optical density and total acid soluble phosphorus increases seem interdependent since they showed similar linear increase throughout the reaction.

14) The optimum pH of around 6.6-7.0 for maximum proteolysis and phosphorus release eliminated the possible participation of low pH active and stable phosphoprotein phosphatase or other phosphatases.

15) The negative absorption values sometimes had during initial proteolysis were affected by either concentration of enzyme, substrate, or hydrogen ions.

16) Total acid soluble phosphorus liberated during casein proteolysis compared closely with total acid soluble nitrogen formation previously reported (35).

17) All evidence to date suggests that the initial reaction preceding onset of milkiness was the disappearance of β-casein by catalyzed

hydrolysis of its phosphodiester bonds and peptide bond cleavage. Also simultaneously at reduced rate there was some hydrolysis to liberate inorganic phosphate ions.

18) Cloudiness itself is believed to be due to the relatively unchanged α-fraction.

19) The reaction following milkiness in the digest may be regarded as further hydrolysis now operative upon the phosphorus and nitrogen bonds of α casein.

20) A specific phosphodiesterase preparation of snake venom accomplished the same results as postulated to be in step one described in number 16 (above).

21) A phosphodiesterase pretreatment of casein demonstrated that only step two as described in numbers 17 and 18 (above) resulted when Taka-Diastase was added.

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