THE PROTEOLYTIC ACTIVITY OF TAKA-DIASTASE FROM *ASPERGILLUS ORYZAE* ON CASEIN

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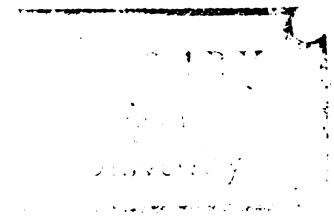

THE PROTEOLYTIC ACTIVITY OF TAKA-DIASTASE FROM *ASPERGILLUS ORYZAE* ON CASEIN

By

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A THESIS

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TABLE OF CONTENTS

I. INTRODUCTION

The enzyme catalyzed hydrolysis of proteins has been studied since the early 1900's. The objectives of such work have included those of seeking information on the structure of proteins and the nature of the proteolytic reaction.

The following observation was made in this laboratory. Taka-Diastase when introduced into a clear, neutral solution of casein, produced after several hours at room temperature, a white, turbid, cloudiness. It had the appearance of milk! This transformation could be construed as the result of the influence of proteolytic enzymes in Taka-Diastase.

It is not known that any systematic studies have been reported on the factors influencing the activity of this particular combination of substrate and enzyme. Hence, the following investigation was undertaken to contribute information concerning such factors, as well as, the physical and chemical nature of the transformation.

II. HISTORICAL

A. The Source of Proteolytic Enzyme.

Taka-Diastase is prepared from a culture of the mold Aspergillus oryzae according to the process patented by Takamine (1923). The mold is grown on wheat bran, the moldy bran is extracted with water, and alcohol is then added to a final concentration of 70 % by volume. The precipitate thus obtained is dried and marketed under the name Taka-Diastase. This product has been found to consist of a number of enzymes which can be demonstrated by their catalytic action on various substrates. Tauber (1949) lists at least twenty-three enzyme systems as being present.

Recently, attempts have been made to isolate a crystalline proteolytic enzyme from Taka-Diastase. Crewther and Lennox (1950) using a combined alcohol and salt precipitation method, obtained such a crystalline fraction. Its proteolytic activity on gelatin was comparable to that of crystalline trypsin. The preparation, though crystalline, was not pure. It could be demonstrated that solutions of the crystals contained at least 2 proteolytic enzymes; one reduced the viscosity of gelatin, and the other acted on the lower molecular weight components of gelatin. Esterase activity was also associated with the crystals. Whether this indicated the presence of a true esterase or the unspecific action of a peptidases was not determined.

Gillespie, Jermym and Woods (1952) employed paper chromotgraphy and paper electrophoresis to achieve a partial separation of the proteolytic enzymes present in Aspergillus oryzae cultures. These workers obtained four main fractions and at least three subsidiary components. The four main fractions exhibited a number of activities among which were two proteinase activities on gelatin.

Astrup and Alkjaersig (1952) have attempted to classify enzymes by the effect of cationic and anionic detergents and natural inhibitors on the proteolytic activity of a number of enzymes. They observed that all the enzymes studied were inhibited by blood serum and mentioned that the proteolytic activity from Aspergillus oryzae in fibrinolysis was inhibited by laurylamine and activated by laurylsulfonate and cetyl-pyridinium chloride.

B. The Substrate Casein.

The nature and properties of casein have been well summarized by Sutermeister and Browne(1939) and since that time by McMeekin and Polis (1950).

Casein, a phosphoprotein, was long considered to be a "pure" protein. However, it became apparent, particularly from the studies of Linderstrøm-Lang (1925), (1929) and others that a modification of this view was necessary. The electrophoretic investigations of Mellander (1939) demonstrated that casein is composed of, at least, three independently migrating components, which he designated α-, β-

and γ-casein in order of their decreasing mobilities. Warner (1944) discussed previous methods for fractionating casein and devised an improved chemical method for separating electrophoretically homogenous α- and β-casein. Gordon, Semmet, Cable and Morris (1949) published on a thorough analysis of the amino acid composition of α- and β-casein, prepared according to Warner. The α-fraction was found to contain a greater number of amino acids with polar side groups whereas the β-component possessed the more non-polar type. In addition, the ratio of phosphorus to nitrogen in the α-component was appreciably greater than that of β-casein.

Warner and Polis (1945) made a viscosimetric study on a proteolytic enzyme contained within casein which previously had been called "Galactase." They stated that the viscosity change occurring at an optimum pH of about 8.5 could be attributed to an enzyme catalyzing the slow hydrolysis of casein in solution. It was also noted that the enzyme within casein could be inactivated by heating a solution to 80° C. for ten minutes.

C. Proteolytic Activity of Taka-Diastase from Aspergillus Oryzae.

Since the terminology used by enzyme chemists has varied, the term proteolytic activity as used herein means: any catalytic action of an enzyme that causes a detectable physical or chemical change which leads to or results in

hydrolysis of the intact protein.

Vines (1910) found that Taka-Diastase had a marked proteolytic effect upon fibrin and Witte peptone at 37° C. Upon fractionating the crude enzyme material with 50 % ethyl alcohol, there resulted an extract that catalyzed the hydrolysis of fibrin, which was termed as having "ereptase" activity. The alcohol insoluble residue, when extracted with water, showed activity upon peptone only and was characterized as having "peptase" activity. The results were arrived at by use of a tryptophane color test on filtrates of the digests.

Wohlgemuth (1912) carried out similar studies and included skim milk as substrate for investigating rennin activity contained in Taka-Diastase. In this connection it was observed that at 38° C. milk clotted. Using the Gross-Fuld method of analyzing (described by Tauber (1949)) other protein digests at 38° C., he concluded that 1 gram of Taka-Diastase was equivalent to 100 c.c. of human or canine pancreatic juice. In general proteolytic action upon 5-6 % protein substrates was, like that of trypsin, strongly inhibited by blood serum of dog and horse. In contrast, an activation resulted upon digestion of peptone substrates and was greatest in neutral or slightly alkaline media.

Szanto (1912) made a comparative study of the effect of weak and strong acids, bases, and neutral salts on proteolytic activity, in which Taka-Diastase was used as one source of enzyme. It was concluded that compared to trypsin

and pepsin, Taka-Diastase activity was: 1- not materially influenced by neutral salts, 2- more sensitive to mineral acids (e.g. in their presence it was irreversibly inactivated), 3- less sensitive to organic acids, 4- not destroyed by alkaline treatment but diminished, and 5- generally not affected by carbohydrates, except slightly by fructose. These conclusions were based upon digesting 1 % casein at 38-9° C. for 1 hour and measuring the activity by the aforementioned Gross-Fuld method.

Okada (1916) incubated 4 % Witte-peptone and 10 % Taka-Diastase solutions at 37-39° C. and found an optimum pH 5.6. The result was based upon Sørensen (1908) formol titration analysis of the digest.

Oshima and Church (1923) reported that the optimum pH for Taka-Diastase digestion of 0.5 % casein depended on the method of following activity. If casein disappearance was measured by the Gross-Fuld method, the optimum was pH 8.0. On the other hand, liberation of amino nitrogen as determined by Van Slyke analysis exhibited an optimum at pH 6.2.

Digesting 5 % gelatin or fibrin or skimmed milk with 1 % Taka-Diastase at 30° C., Nishikawa (1927) confirmed the observations of Wohlgemuth that trypsin and rennin type enzymes were present. He stated that the optimum pH for rennin activity was 5.2-6.7 and that metal ions such as Zn^{++}, Cu^{++}, and Hg^{++} inhibited the tryptic-like activity of Taka-Diastase on gelatin. Activity was measured by the alcohol titration method of Willstätter and Persiel (1925).

Kawakami (1929) stated that at digestion temperature of 65° C., Taka-Diastase was inactivated as shown by no increase in alkali or formol numbers. It was noted that during the first 1 to 2 hours, the digestion was most rapid at 55° C., and after 6 hours had nearly ceased. At 45° C. the change was very slow with reaction still in progress after 80-90 hours. No inhibitory effect could be detected by ultra-violet light irradiation of the enzyme in solution if kept cooled during treatment.

Berger, Johnson and Peterson (1937) tested the proteolytic activity of a number of molds among which was included Aspergillus oryzae. Digesting 4 % gelatin at 40° C. and measuring activity by the Linderstrøm-Lang (1927) acetone titration method, they concluded the enzyme had an optimum pH of approximately 7. Similarly, from synthetic polypeptides there was found amino-peptidase, carboxypeptidase and dipeptidase activity.

By extracting powdered mycelia from Aspergillus oryzae with water for 4 hours at 40° C., Otani (1940) found in the extract the presence of papain-type, rennin-type, and peptidase enzymes.

Lichtenstein (1947) demonstrated that dialyzed solutions of Taka-Diastase were capable of catalytically hydrolyzing casein, gelatin, and a number of synthetic polypeptides. These results confirmed the findings of Berger, Johnson and Peterson (1937).

III. EXPERIMENTAL

A. Equipment Used:

Thermostat .-The constant temperature bath was equipped with a reservoir bottle to automatically maintain a constant level of water. The thermoregulator (H-B Instrument Co., Inc.) controlled the temperature at 29.9 ± 0.1°C.

Glassware.-All pipettes and volumetric glassware were the Kimble Glass brand. A one milliliter burette, used for alcoholic titrations, made by Kimble Glass Co., was graduated to 0.01 ml. and could be read to ± 0.002 ml.

Timers.-The reaction periods were timed with either a Meylan stopwatch or a Precision Scientific Co. timer, calibrated to tenths of a second.

Viscometer.-Its flow time for 10 ml. of water at 29.9°C. was found to be 75 seconds.

Polarimeter.-A Precision Polarimeter, Model 169, manufactured by O. C. Rudolph and Sons, provided with a sodium vapor lamp as light source, was employed.

Refractometer.-The Abbe' refractometer used in these measurements was manufactured by Carl Zeiss, Germany.

Turbidimeter.-A Model 400, Hellige-Diller Photo-electric Nephelometer was used. The instrument was calibrated for percent transmittance or optical density units.

pH Meter.-A Beckman model G, glass electrode, pH meter was used in making pH measurements.

Dialyzer.-All dialysis were carried out with a rotating external liquid dialyzer constructed in this laboratory.

Semi-micro Kjeldahl Apparatus.-The 100 ml. digestion flasks and distillation apparatus as modified in this laboratory was used in the determination of total protein and non-protein nitrogen.

B. Materials and Solutions:

The Enzyme Source.-Taka-Diastase (marketed by Parke, Davis & Co.) a yellow, amorphous, non-hygroscopic powder analyzed for 1.51 % N. It was readily soluble in water and produced a clear yellow-brown solution.

The Casein Preparation.- Casein was prepared by the directions of Cohn and Hendry (1943). The white product contained 15.6 % N and 7.52 % moisture (determined by drying overnight in an oven at 105° C.). Electrophoretic analysis revealed that the material was comparable to that of Warner (1944) both in number of components and their mobility. No calcium ion could be detected in the outside liquid of a 6 % casein solution which was dialyzed against redistilled water.

Fresh Casein Stock Solution.-Six grams of air dry casein was weighed into a 100 ml. volumetric flask. Seventy-five ml. of glass redistilled water was introduced in small portions until a smooth paste formed. To this, 20 ml. of 0.2 N NaOH was added gradually, with shaking, until a clear solution of pH 7 was obtained. The liquid was made to

volume, filtered and a crystal of thymol added as preservative. The stock solution prepared in this manner was stored in the refrigerator for immediate use.

The immediate use of the stock solution is imperative. It was observed by electrophoretic analysis that the protein in solution underwent a change if allowed to stand for a period of a week or more. This was particularly true if the solution was allowed to come to a temperature other than that of cold storage (5° C.).

Taka-Diastase Stock Solutions.-The pre-determined amount of dry powder was weighed on an analytical balance, then it was dissolved in redistilled water and filtered clear. The final concentration was expressed in terms of milligrams per milliliter. The solutions were stored in a refrigerator when not in use. Fresh solutions were prepared each day prior to use.

0.05 N Alcoholic KOH.-3.75 grams (Baker) potassium hydroxide (KOH) was dissolved in 62.5 ml. of redistilled water, and diluted to 1 liter with 95 % ethanol. After separating from precipitated carbonates the reagent was standardized against 0.1052 N HCl with methyl red indicator.

Thymolphthalein Indicator Solution.-The indicator solution for the Willstätter and Waldschmidt-Leitz (1921) titration was prepared by diluting 6 ml. of 0.5 % thymolphthalein in 95 % ethanol to 100 ml. with absolute ethanol.

0.1 N Standard HCl and NaOH.-These solutions were prepared according to accepted standard procedures in

quantitative analytical chemistry.

Kjeldahl Reagents and Solutions.-These were prepared, with minor modifications, as described by Clark (1943).

Buffers.- Buffer solutions were prepared according to the tables in Gortner (1949).

Activator and Inhibitor Reagents.-All chemicals used to prepare these solutions were from EKCo., white label, A.C.S. specifications or C.P. grade reagents. Any not so labelled are indicated.

C. Experimental Methods:

General Digestion Procedure.-An appropriate volume (usually 3 ml.) of 6 % casein was pipetted into one arm (10 ml. capacity) of a bifurcated test tube, and into the other arm was placed an appropriate amount of enzyme solution. Usually the volumes of substrate and enzyme solution were chosen so as to give upon mixing a resulting digest concentration of enzyme in terms of mg./ml. digest. The reaction vessel containing the unmixed solutions was placed in the thermostat for 20 minutes prior to mixing. Digestion was commenced by tilting the two-branched tube back and forth 10 times. The time of initial contact was taken as zero digestion time and noted by starting the stop watch. Suitable aliquots of digestion mixture (usually 1 ml.) were removed and quenched at specified intervals for the types of analysis to be described.

Controls using enzyme solution previously heated in

a sealed tube to 100° C. for 1 hour were subjected to digestion in the same manner.

1. The Influence of Enzyme Concentration.-A series of digestions were carried out where the initial concentration of casein was always 3 %. Taka-Diastase concentrations were taken at 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 mg./ml. of digest for each run, respectively. Several types of analysis were periodically performed upon digest aliquots and are described as follows:

The Alcoholic-KOH Titration for Total Acidity Change.-One ml. digest aliquots were removed at intervals and total acidity was immediately titrated in the manner described by Willstätter and Waldschmidt-Leitz (1921). This method is a modification of Foreman's (1920) original alcoholic sodium hydroxide titration. To arrest the digestion, the aliquots were pipetted directly into 2.5 ml. of an absolute alcohol-indicator mixture already contained in a 25 x 100 mm. test tube. This sample was then titrated to a distinct blue color; 7.5 ml. of absolute ethanol was added and the sample again was titrated to the appearance of a blue color in the solution. During titration the solution was kept well mixed with the aid of a motor driven glass rod stirrer. The titer obtained from the aliquot taken immediately after mixing was first recorded. This initial titer was subtracted from subsequent titers and gave the increment in ml. ($\triangle$ml.) of standard alcoholic KOH titrated per ml. of digest. These results representing increase in

titratable acidity or liberation of acid groups are the values reported in the subsequent tables as indicated.

It should be emphasized that the digest aliquots were titrated immediately. It was observed that the alcohol-indicator mixture did not completely quench the reaction if it stood around for several hours. When a number of simultaneous digestions were performed, they were started at 15 minute intervals, allowing ample time for immediate titration. The results are reported in Table I and represented in Figure 1.

Change in pH or Hydrogen Ion Activity.-The prescribed digestion mixtures were started in an open glass cup adequate to receive the electrodes of a pH meter with its temperature adjustment set for 30° C. Measurements were made directly on the digestion mixture clamped in the thermostat. The resulting changes in hydrogen ion activity are reported in Table II and shown in Figure 2.

Non-Protein Nitrogen (N.P.N.) Formed.-After starting digestion, aliquots were periodically pipetted into an equal volume of 20 % (w/v) Trichloroacetic Acid (T.C.A.). These samples were shaken occasionally during a period of 1 hour and were then filtered through Whatman #40 filter paper. Total nitrogen was determined on the filtrates as described by Clark (1943). Blanks were run on reagents with the substrate alone. The rate of the increase in non-protein nitrogen with digestion can be seen in Table III and Figure 3.

Optical Density for Change in Turbidity.-Five ml. digests were prepared by the previously described procedure and placed into 6-inch test tubes and kept in the thermostat between turbidity readings. At the specified time intervals the tubes were momentarily placed in the turbidmeter for optical density readings. The readings obtained during digestion are shown in Table IV and plotted in Figure 4.

2. Influence of Substrate Concentration.-A series of digestions were conducted where substrate concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 percent were prepared by proper dilution of the 6 % stock casein solution. Kjeldahl total nitrogen analysis were made upon the digests to more exactly define the substrate concentration. The digest concentration of Taka-Diastase was always 2.0 mg./ml. One ml. aliquots were titrated with alcoholic KOH according to the preceding directions. Activity measured by increase in titratable acidity is given in Table V and illustrated in Figure 5.

3. Influence of pH on Activity.-The substrate solutions in one branch of the digestion tube were adjusted to the desired pH, using 0.1 N HCl or NaOH. The volume required was predetermined from a titration vs pH curve obtained from a 3 ml. sample of 6 % casein. One ml. of enzyme solution containing 12 mg. of Taka-Diastase was added to the other arm of the bifurcated tube and diluted with redistilled water to a volume, which when mixed with the substrate, would give a casein concentration of 3 % and enzyme

concentration of 2 mg./ml. with respect to the digest. The initial pH of the digest was determined with the Beckman pH meter and 1 ml. aliquots removed for titration. At subsequent digestion times pH readings on the digest were taken 30 seconds before the aliquot was removed and titrated. The results are reported in Table VI and demonstrated in Figure 6.

4. Influence of Temperature.-To observe the effect of temperature upon activity, the following procedure was adopted. Thermostatically controlled digestion was carried out at approximately 10° temperature intervals up to 60° C. One ml. samples were removed and titrated with alcoholic KOH. The digest concentration in all cases was 3 % with respect to casein and 2 mg. per ml. with respect to Taka-Diastase. The increase in titratable acidity was recorded at specified digestion times and data from these experiments are recorded in Table VII and represented in Figure 7.

5. Influence of Added Electrolyte.-The previously described digestion procedure was employed, with the following modification. From a 1 N solution of sodium chloride (Merck), appropriate aliquots were added to the substrate before mixing. When enzyme and substrate were mixed, the resulting digest contained 3 % casein, 2 mg./ml. of Taka-Diastase, and according to the salt added was 0.05, 0.10, 0.15, and 0.20 molar respectively. After equilibrating to temperature and mixing, 1 ml. digest alquots were titrated with alcoholic KOH. The results demonstrating the effect

of added ionic strength to the medium are given in Table VIII and Figure 8.

6. Activators and Inhibitors of the Enzyme.-Stock solutions (0.1 M) of the compounds listed in Table IX were prepared. The solutions were appropriately diluted to 10^{-3} M and allowed to react with 8 mg./ml. of Taka-Diastase for exactly 1 hour in the thermostat. Twenty minutes before the hour was up an equal volume (2 ml.) of 6 % casein was introduced into the other side arm of the bifurcated vessel used. At the end of the hour, the contents were mixed for digestion and aliquots of digest were removed at the beginning and after 1 hour of digestion. A similar digest without enzyme treatment was used as control. The activation or inhibition result was arbitrarily taken as the increase or decrease in titratable acidity over the control, and is expressed as percent in Table IX.

Hydrogen Ion Treatment of the Enzyme.-To study the effect of added hydrogen ion upon the enzyme, 5 ml. of solution containing 40 mg. of Taka-Diastase, pH 6.85 was adjusted to pH 3 with 0.6 ml. of 0.1 N HCl. The solution was placed in a constant temperature bath at 30° C. for exactly 1 hour, after which 0.6 ml. of 0.1 N NaOH was added to restore the original pH. 1.24 ml. of this solution was taken and diluted to 2 ml. and mixed with an equal volume of 6 % casein. Thus the digest was 3 % with respect to casein and contained 2 mg./ml. of enzyme material. Aliquots for alcoholic titration were removed at the beginning and

after 1 hour of digestion. A control was run on an untreated sample. The results are listed in Table IX.

Hydroxyl ion Treatment of Enzyme.-The effect of hydroxyl ion upon the enzyme was determined in a similar manner. Five ml. of enzyme solution containing 40 mg. Taka-Diastase was adjusted to pH 11 with 0.8 ml. of 0.1 N NaOH. At the end of the hour the pH was restored to 6.85 with 0.8 ml. of 0.1 N HCl and 1.32 ml. of this solution was diluted to 2 ml. The sample was assayed for activity as previously described. The results are shown in Table IX.

Ultra-violet Irradiation of Enzyme Solution.-Three 10 ml. volumes of Taka-Diastase solution (4 mg./ml.), each contained on watch glass covers, were placed one at a time beneath a Kupper-Hewitt ultra-violet lamp. The samples were exposed at 40, 50, 60 cm. distant from the light source. A 2 ml. aliquot of each irradiated sample was digested with 2 ml. of 6 % casein according to the prescribed procedure. The increase in titratable acidity after 1 hour of digestion was measured and compared to a control. See Table IX.

Dialysis of the Enzyme Solution.-Fifteen ml. of a Taka-Diastase solution (8 mg./ml.) was dialyzed against four 50 ml. changes of redistilled water. Total dialysis time was 14 hours. Two ml. of 3 % casein was digested with 2 ml. of the dialyzed enzyme solution. The increase in titratable acidity after 1 hour of digestion was compared to a control. See Table IX.

7. Physico-Chemical Changes During Digestion.

Viscosity Changes.-The samples were prepared by the general digestion procedure. The digest concentration was 3 % with respect to casein and 2 mg./ml. with respect to enzyme. Immediately upon mixing, 10 ml. of the digest was pipetted into the Ostwald viscometer. Flow times were determined as rapidly as possible during the early stages of digestion. For results see Table X and Figure 9.

Polarimeteric Changes.-A digest was made 3 % with respect to casein and 2 mg./ml. with respect to enzyme. It was immediately transferred into a 100 mm., jacketed, polarimeter tube. The jacket was maintained at 30° C. by circulating water through from a water bath. Readings were taken as rapidly as possible until turbidity obscured the readings. The instrument may be read to ±0.002 angular degrees. For results see Table XI.

Refractometeric Changes.-A 3 % casein digest containing 2 mg./ml. of enzyme was prepared. Immediately after mixing and at subsequent intervals samples were taken and read on the refractometer at room temperature. For results see Table XII.

8. Electrophoretic Analysis.-Two ml. samples of 3 % casein were pipetted into 4 ml. of phosphate buffer pH 7.0 and ionic strength 0.1 with respect to phosphate plus 0.05 M NaCl. The sample was dialyzed against 100 ml. of buffer for 1 hour, against 100 ml. of fresh buffer for 2 hours, and finally for 14 hours against 300 ml. of new buffer. The

sample was then subjected to electrophoretic analysis with a Perkin-Elmer, Model 38, Tiselius Electrophoresis apparatus.

TABLE I.

The Effect of ENZYME CONCENTRATION Upon Activity. Taka-Diastase and 3 % Casein. 1. Increase in Ml. 0.0483 N Alcoholic KOH Titrated per Ml. of Digest.

Digestion[1] Time, min.	Taka-Diastase Concentration, mg./ml. digest 0.5 gave Δml.[2]	1.0 gave Δml.	1.5 gave Δml.	2.0 gave Δml.	3.0 gave Δml.	4.0 gave Δml.
15	0.001	0.008	0.011	0.015	0.023	0.033
30	0.007	0.017	0.026	0.039	0.050	0.062
45	0.012	0.029	0.039	0.053	0.071	0.083
60	0.024	0.038	0.055	0.073	0.086	0.105
75	0.034	0.052	0.066	0.082	0.098	0.111
90	0.041	0.058	0.072	0.086	0.104	0.146
105	0.045	0.065	0.076	0.091	0.115	0.175
120	0.050	0.068	0.081	0.098	0.137	0.200
135	0.053	0.073	0.092	0.112	0.165	0.225
150	0.054	0.083	0.109	0.135	0.181	0.244
165	0.056	0.094	0.125	0.157	0.203	0.252
180	0.062	0.112	0.150	0.183	0.224	0.263
195	0.070	0.129	0.163	0.210	0.233	0.271
210	0.082	0.143	0.182	0.214	0.246	0.277
225	0.098	0.161	0.195	0.227	0.254	0.282
240	0.116	0.167	0.200	0.234	0.260	0.285

[1]Initial pH 7.0.

[2]All values in this table, except in column 6, represent the average of at least 2 runs.

TABLE II.

The Effect of Taka-Diastase Concentration Upon Activity With 3 % Casein. 2. Net Change in Free Hydrogen Ion Activity.

Digestion Time, min.	Taka-Diastase Concentration, mg./ml. Digest					
	0.5 gave pH[1]	1.0 gave pH	1.5 gave pH	2.0 gave pH	3.0 gave pH	4.0 gave pH
0	7.10	7.10	7.10	7.05	7.02	7.00
15	7.05	7.05	7.03	6.98	6.94	6.90
30	7.00	6.99	6.96	6.92	6.88	6.83
45	6.99	6.94	6.92	6.89	6.83	6.78
60	6.98	6.91	6.90	6.88	6.80	6.73
75	6.98	6.90	6.88	6.86	6.78	6.70
90	6.96	6.90	6.86	6.83	6.75	6.69
105	6.94	6.87	6.82	6.80	6.72	6.66
120	6.91	6.85	6.79	6.76	6.70	6.64
135	6.90	6.83	6.77	6.74	6.68	6.64
150	6.89	6.81	6.75	6.72	6.68	6.60
165	6.89	6.81	6.73	6.70	6.68	6.60
180	6.89	6.80	6.72	6.70	6.68	6.60
195	6.89	6.80	6.72	6.70	6.65	6.59
210	6.89	6.80	6.70	6.69	6.62	6.57
225	6.89	6.79	6.69	6.67	6.60	6.55
240	6.89	6.77	6.68	6.67	6.59	6.55

[1]Beckman Model G pH meter set at 30° C.

TABLE III.

The Effect of Taka-Diastase Concentration Upon Activity With 3 % Casein. 3. The Formation of Non-Protein Nitrogen (NPN).

Digestion[1] Time, min.	Digest Aliquot, ml.	T.C.A. Filtr. Aliquot ml.	0.0212 N HCl Titer ml.	NPN/ml. of digest, mg.[2]	Increase NPN/ml. digest, mg.
Taka-Diastase Concentration of 1.0 mg./ml. digest					
0	10	14	3.32	0.137	-
30	10	14	5.36	0.244	0.087
60	5	6	3.20	0.309	0.172
90	5	6	3.81	0.375	0.238
120	3	3	2.18	0.416	0.279
150	3	3	2.64	0.507	0.370
180	2	1	1.04	0.570	0.433
210	2	1	1.15	0.635	0.498
240	2	1	1.19	0.659	0.522
Concentration 2.0 mg/ml. digest					
0	10	12	3.22	0.149	-
30	10	14	7.50	0.313	0.164
60	5	5	4.08	0.470	0.321
90	5	5	5.12	0.594	0.445
120	3	3	3.77	0.722	0.573
150	3	3	4.40	0.847	0.698
180	2	2	3.48	0.997	0.848
210	2	2	3.78	1.090	0.941
240	2	2	4.06	1.170	1.021
Concentration 4.0 mg./ml. digest					
0	10	14	4.09	0.170	-
30	10	14	11.14	0.469	0.299
60	5	6	7.43	0.726	0.556
90	5	6	9.91	0.971	0.880
120	3	3	6.14	1.21	1.040
150	3	3	7.05	1.38	1.210
180	2	1	2.86	1.67	1.500
210	2	1	3.07	1.78	1.610
240	2	1	3.29	1.84	1.670

[1] Initial pH 7.0.

[2] $\frac{\text{Milliequivalent of HCl} \times 14}{\frac{\text{Filtrate aliquot}}{2 \times \text{digest aliquot}}} \times \text{digest aliquot}$ = mg. of N.P.N./ml. of digest

TABLE IV.

The Effect of Taka-Diastase Concentration Upon Activity With 3 % Casein. 4. The Change in Optical Density.[1]

Digestion[2] Time, min.	Taka-Diastase Concentration, mg./ml. Digest gave d.u.[3] 0.5	1.0	1.5	2.0	3.0	4.0
0	0.0	0.0	0.0	0.0	0.0	0.0
15	0.7	0.9	1.0	1.1	1.2	1.5
30	1.4	1.6	1.9	2.0	2.1	2.3
45	1.5	2.0	2.3	2.8	2.9	3.3
60	2.0	2.2	2.6	3.2	3.7	4.3
75	2.1	2.5	2.8	3.6	4.4	6.0
90	2.4	2.6	3.0	3.9	4.6	7.9
105	2.8	2.9	3.2	4.3	5.2	11.2
120	2.8	3.0	3.6	4.7	5.9	16.9
135	2.9	3.2	3.9	5.3	7.1	25.8
150	2.9	3.4	4.2	5.9	9.0	-
165	3.0	3.7	4.6	6.7	12.5	-
180	3.0	4.0	5.0	7.9	18.0	-
195	3.2	4.2	5.6	9.7	26.0	-
210	3.4	4.5	6.5	12.8	-	-
225	3.5	5.0	7.9	16.5	-	-
240	3.5	5.5	9.8	25.4	-	-
255	3.6	6.1	13.0	-	-	-
270	3.8	7.0	17.2	-	-	-
285	4.0	9.0	24.7	-	-	-
300	4.6	12.0	-	-	-	-
315	5.0	16.3	-	-	-	-
330	6.8	23.8	-	-	-	-
345	8.5	-	-	-	-	-
360	11.2	-	-	-	-	-
375	14.8	-	-	-	-	-
390	21.7	-	-	-	-	-

[1] Measured by Hellige-Diller, Model 400, Photoelectric Colorimeter.

[2] Initial pH 7.0.

[3] Increase in density units after zero setting.

TABLE V.

The Effect of SUBSTRATE (Casein) CONCENTRATION Upon Activity Using 2.0 mg. Taka-Diastase per Ml. of Digest. The Increase in Ml. of 0.0483 N Alcoholic KOH Titrated per Ml. of Digest.

Digestion[1] Time, min.	Concentration of Casein, % as gr./ 100 ml. 1.1 gave Δ ml.	2.2 gave Δ ml.	3.1 gave Δ ml.	4.3 gave Δ ml.	5.4 gave Δ ml.
15	-	0.010	0.015	0.025	0.035
30	0.015	0.023	0.039	0.055	0.062
45	-	0.036	0.053	0.070	0.094
60	0.027	0.048	0.073	0.087	0.109
90	-	0.056	0.087	0.107	0.140
120	0.039	0.065	0.098	0.124	0.155
150	-	0.078	0.135	0.153	0.195
180	0.046	0.099	0.183	0.190	0.240
240	0.065	0.126	0.234	0.254	0.316

[1]Initial pH 7.0.

TABLE VI.

The EFFECT OF pH on the Activity of 2 mg. of Taka-Diastase per Ml. of Digest on 3 % Casein.

Initial pH of Digest	Increase in ml. 0.0483 N alc. KOH/ml. digest At 60 min., pH	Δml.	At 120 min., pH	Δml.	At 240 min., pH	Δml.
6.31	6.28	0.046	6.26	0.082	6.22	0.150
6.50	6.44	0.086	6.38	0.124	6.30	0.200
6.70	6.64	0.109	6.60	0.140	6.52	0.242
6.96	6.89	0.080	6.79	0.114	6.70	0.230
7.05	6.88	0.073	6.76	0.103	6.67	0.225
7.50	7.44	0.035	7.37	0.062	7.28	0.126
8.10	8.07	0.013	8.01	0.038	7.98	0.062

TABLE VII.

The Effect of Digestion TEMPERATURE Upon the Activity of 2.0 mg. of Taka-Diastase per Ml. of Digest on 3 % Casein.

Digestion Temperature[1], C.	Increase in ml. 0.0483 N alcoholic KOH/ml. digest after digesting: 30 min., Δ ml.	60 min., Δ ml.	120 min., Δ ml.	240 min., Δ ml.
1.2	0.0	0.003	0.003	0.008
5.8	0.0	0.003	0.005	0.008
10.2	0.0	0.008	0.014	0.023
20.0	0.018	0.040	0.072	0.100
29.9	0.039	0.073	0.098	0.232
40.2	0.070	0.100	0.130	0.260
50.1	0.110	0.118	0.122	0.122
60.3	0.060	0.070	0.070	0.072

[1]Initial pH 6.8 to 7.0.

TABLE VIII.

The Effect of Added ELECTROLYTE Upon Activity of 2 mg. of Taka-Diastase per Ml. of Digest[1] with 3 % Casein.

Conc. NaCl, moles	Change in ml. 0.0483 N alcoholic KOH/ml digest, at 30 min., Δ ml.	at 60 min., Δ ml.	at 120 min., Δ ml.	at 240 min. Δ ml.
0.00	0.040	0.072	0.098	0.233
0.05	0.019	0.051	0.072	0.130
0.10	-0.030	0.030	0.055	0.108
0.15	-0.080	-0.002	0.024	0.062
0.20	-0.122	-0.083	-0.067	-0.009

[1]Initial pH varied from 6.8 to 7.0.

TABLE IX.

The Effect of ACTIVATORS AND INHIBITORS Reacting in 10^{-3} M Concentration on 4.0 mg. Taka-Diastase per Ml. for 1 Hour and Then Mixed With an Equi-volume of 6 % Casein.

Cations	**Source and Brand**	**Result**[2]	
Mg^{++}	$MgSO_4.7H_2O$ (Merck)	None	
Zn^{++}	$ZnSO_4.7H_2O$ (B&A)	None	
Ag^{+}	$AgNO_3$ (Schaar)	None	
Cu^{++}	$CuSO_4$ (Baker)	Inhibits	84 %
Mn^{++}	$MnSO_4.H_2O$ (Baker)	Activates	17 %
Co^{++}	$Co(NO_3)_2.6H_2O$ (B&A)	Activates	29 %
Ca^{++}	$CaCl_2.6H_2O$ (Fischer)	Activates	34 %
Ni^{++}	$Ni(OOCCH_3)_2.4H_2O$ (Baker)	None	
Hg^{++}	$HgCl_2$ (Kahlbaum)	Inhibits	98 %
H^{+}[3]	0.1 N HCl (B&A)	Inhibits	98 %
Anions			
CN^-	NaCN (Merck)	None	
F^-	NaF (Baker)	Inhibits	42 %
OH^-[4]	0.1 N NaOH (B&A)	Inhibits	30 %
Carbonyl Reagents			
$NaHSO_3$	Sodium bisulfite (Merck)	None	
$NH_2OH.HCl$	Hydroxylamine.HCl (EKCo)	None	
Oxidizing Agent			
H_2O_2	Superoxol (Merck)	None	
Sulfhydryl Reagent			
ICH_2COOH	Iodoacetic acid (EKCo)	None	
Amino and Carboxyl			
Ninhydrin	Triketohydrindene hydrate (EKCo)	None	
Detergent			
Sodium Lauryl Sulfonate (EKCo)		None	
Physical Agents	**Exposure Distance**		
Ultra-violet irradiation	(60 cm., 15 min.	Inhibits	30 %
	(50 cm., 15 min.	Inhibits	40 %
	(40 cm., 15 min.	Inhibits	70 %
Dialysis	For 14 hrs.	Inhibits	50 %
Heat	At 105° C., 1 hr.	Inhibits	100 %

[1]All digests had initial pH 6.8-7.0.
[2]Based upon increase in ml. 0.0503 N alc. KOH titrated/ml. digest after 1 hr., compared with non-treated control.
[3]Brought to pH 3 for 1 hr. and neutralized before digesting.
[4]Brought to pH 3 for 1 hr. and neutralized before digesting.

TABLE X.

The Change in VISCOSITY When 2.0 mg. Taka-Diastase per ml. of Digest is Acting Upon 3 % Casein.

Digestion Time, min.	Flow Time[1], sec.	Relative Viscosity[2]	Digestion Time, min.	Flow Time, sec.	Relative Viscosity
2.3	147.1	0.96	1.65	149.6	1.09
7.1	144.0	0.92	4.65	145.4	0.94
10.0	137.9	0.84	7.51	140.4	0.87
13.1	134.8	0.80	10.3	136.8	0.82
16.1	131.2	0.75	13.1	134.6	0.79
18.9	129.0	0.72	16.1	131.0	0.75
21.7	127.0	0.69	18.7	129.2	0.72
24.3	125.0	0.67	22.3	127.2	0.70
26.8	124.0	0.65	23.9	125.4	0.67
29.5	122.0	0.63	26.5	124.6	0.66
32.2	121.0	0.61	29.0	123.2	0.64
34.7	119.8	0.60	31.5	121.2	0.62
37.2	119.2	0.59	34.8	120.2	0.60
39.8	117.4	0.57	36.5	119.0	0.59
42.8	116.6	0.56	41.5	117.4	0.57
44.8	115.6	0.54	46.1	116.0	-.55
49.0	114.2	0.52	50.7	114.4	0.53
117.0	103.8	0.38	55.4	113.8	0.52
120.0	103.2	0.38	59.8	112.4	0.50
164.0	100.4	0.34	79.0	110.3	0.47
			90.2	107.5	-.43
			110.0	105.2	0.40
			135.0	103.0	0.37
			180.0	100.2	-.34
			198.0	99.2	0.32
			225	98.2	0.31
			240	97.4	0.30

[1]Measured in 75 sec., Ostwald Viscometer at 29.9° C; initial pH 7.0.

[2]Values from: (Flow time of digest/Flow time water) -1)

TABLE XI.

The Change in OPTICAL ROTATION When 2.0 mg. Taka-Diastase per Ml. Digest is Reacting with 3 % Casein.

Digestion[1] Time, min.	Reading, angular degrees	Digestion Time, min.	Reading, angular degrees
3.5	-2.962	17.9	-3.268
4.5	-2.920	18.5	-2.982
5.0	-2.970	19.4	-3.012
5.4	-2.930	20.0	-2.982
6.0	-2.910	25.0	-2.992
6.5	-2.952	30.7	-2.964
7.0	-2.938	34.7	-3.000
7.4	-2.940	40.0	-2.994
7.8	-2.946	45.0	-2.936
8.5	-3.016	50.0	-2.972
9.0	-2.992	55.4	-3.022
9.4	-3.000	60.0	-2.934
10.0	-2.992	70.7	-2.934
10.9	-2.992	79.2	-2.998
11.5	-2.998	89.4	-2.980
12.0	-2.998	99.4	-2.971
13.0	-2.990	109.4	-2.958
15.2	-3.296	117.4	-2.981
15.8	-3.194	131.0	-2.935
16.5	-3.218	149.4	-2.970
17.2	-3.222		

[1] Initial pH 7.0.

TABLE XII

The Change in INDEX OF REFRACTION when 2.0 mg. Taka-Diastase per Ml. Digest is Reacting on 3 % of Casein.

Digestion Time, min.	Refractive Index n^{25}	Digestion Time, min.	Refractive Index n^{25}
1.0	1.3384	17.0	1.3388
3.0	1.3390	19.0	1.3388
4.0	1.3382	21.0	1.3390
5.0	1.3390	26.0	1.3388
6.0	1.3390	31.0	1.3390
7.0	1.3390	41.0	1.3390
8.0	1.3390	60.0	1.3380
9.0	1.3388	90.0	1.3380
10.0	1.3380	120.0	1.3375
11.0	1.3390	154.0	1.3380
12.0	1.3390	213.0	1.3380
13.0	1.3390	280.0	1.3379
14.0	1.3392	320.0	1.3379
15.0	1.3392	1,440	1.3385

[1]Initial pH 7.0.

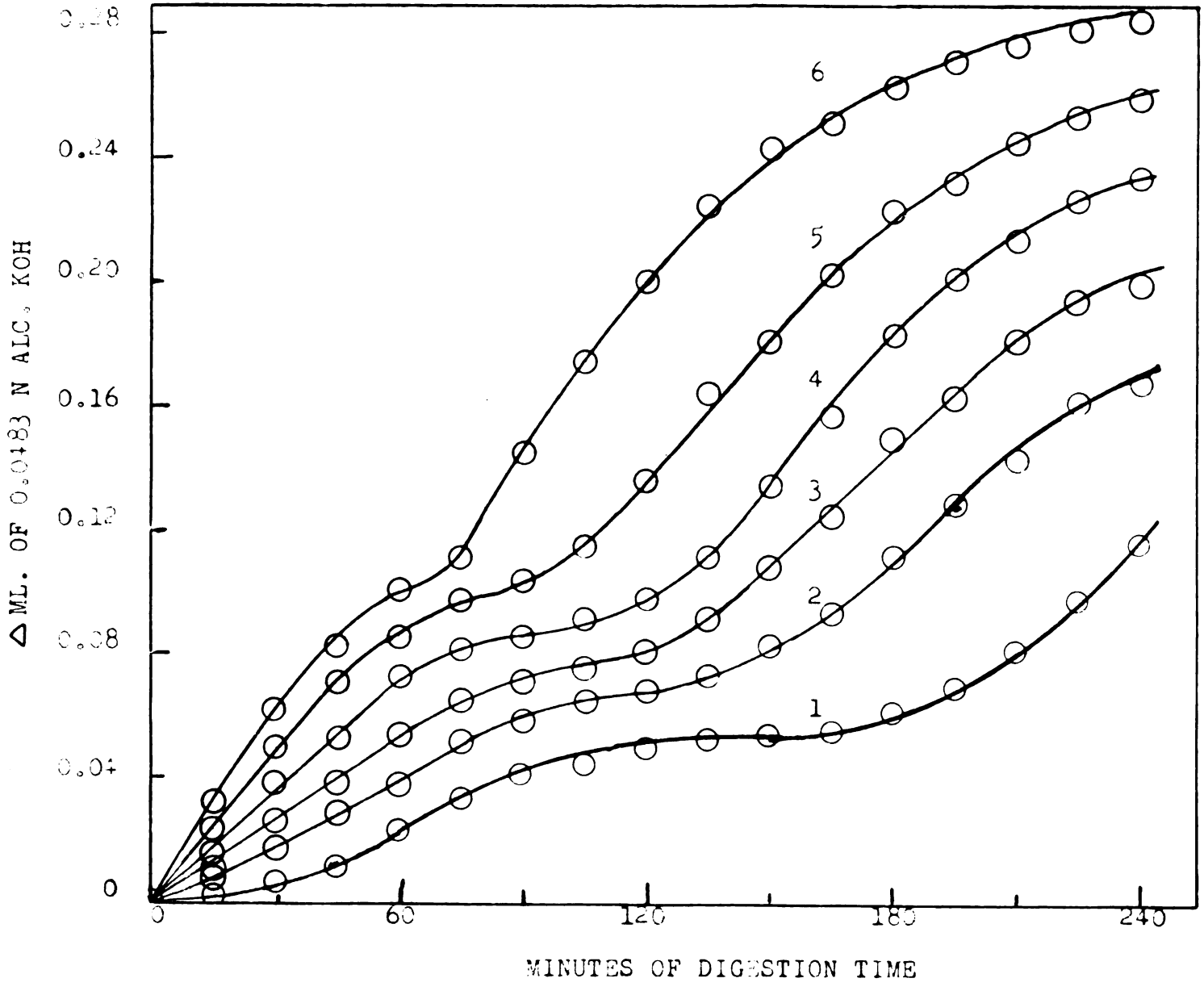

FIGURE 1.

THE LIBERATION OF TOTAL FREE ACID GROUPS.

CURVES:
(1) 0.5,
(2) 1.0,
(3) 1.5,
(4) 2.0,
(5) 3.0,
(6) 4.0
mg. TAKA-DIASTASE/ML. OF DIGEST, respectively

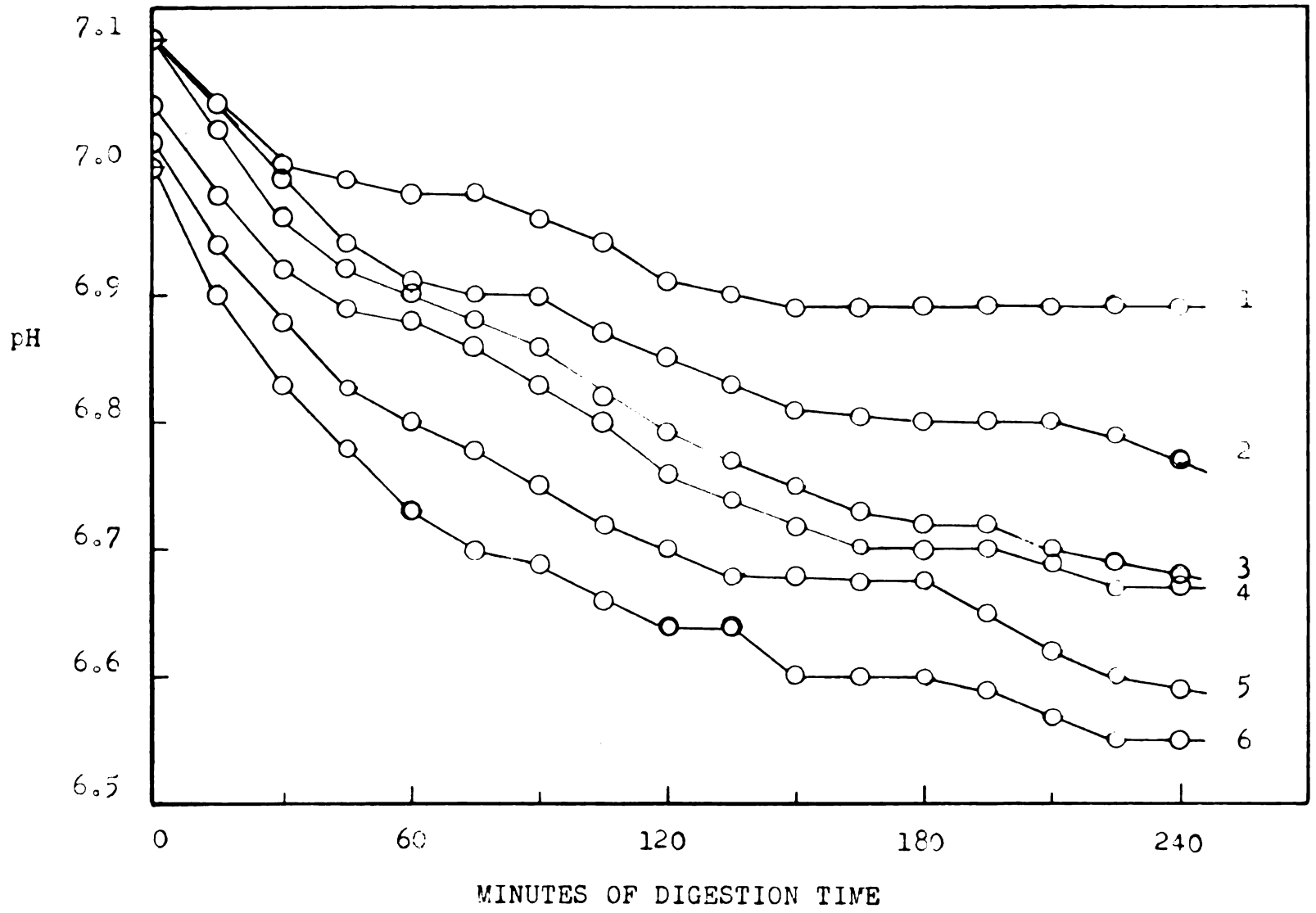

FIGURE 2.

THE CHANGE IN FREE HYDROGEN ION CONCENTRATION.

CURVES:
(1) 0.5,
(2) 1.0,
(3) 1.5,
(4) 2.0,
(5) 3.0,
(6) 4.0
mg. TAKA-DIASTASE/ml. of Digest, respectively

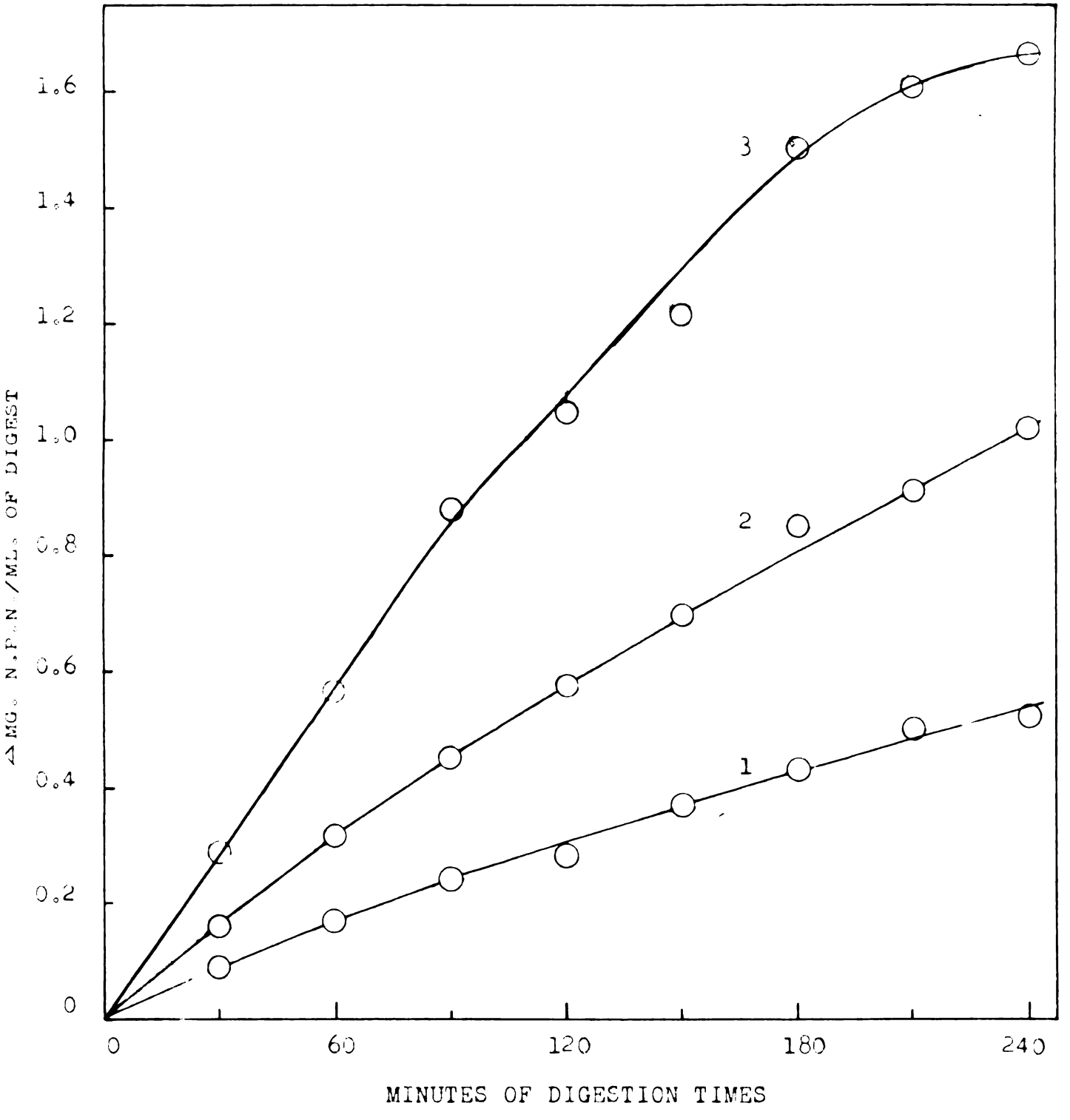

FIGURE 3. THE FORMATION OF NON-PROTEIN NITROGEN. CURVES: (1) 1.0, (2) 2.0 and (3) 4.0 mg. TAKA-DIASTASE/ml. of digest, respectively.

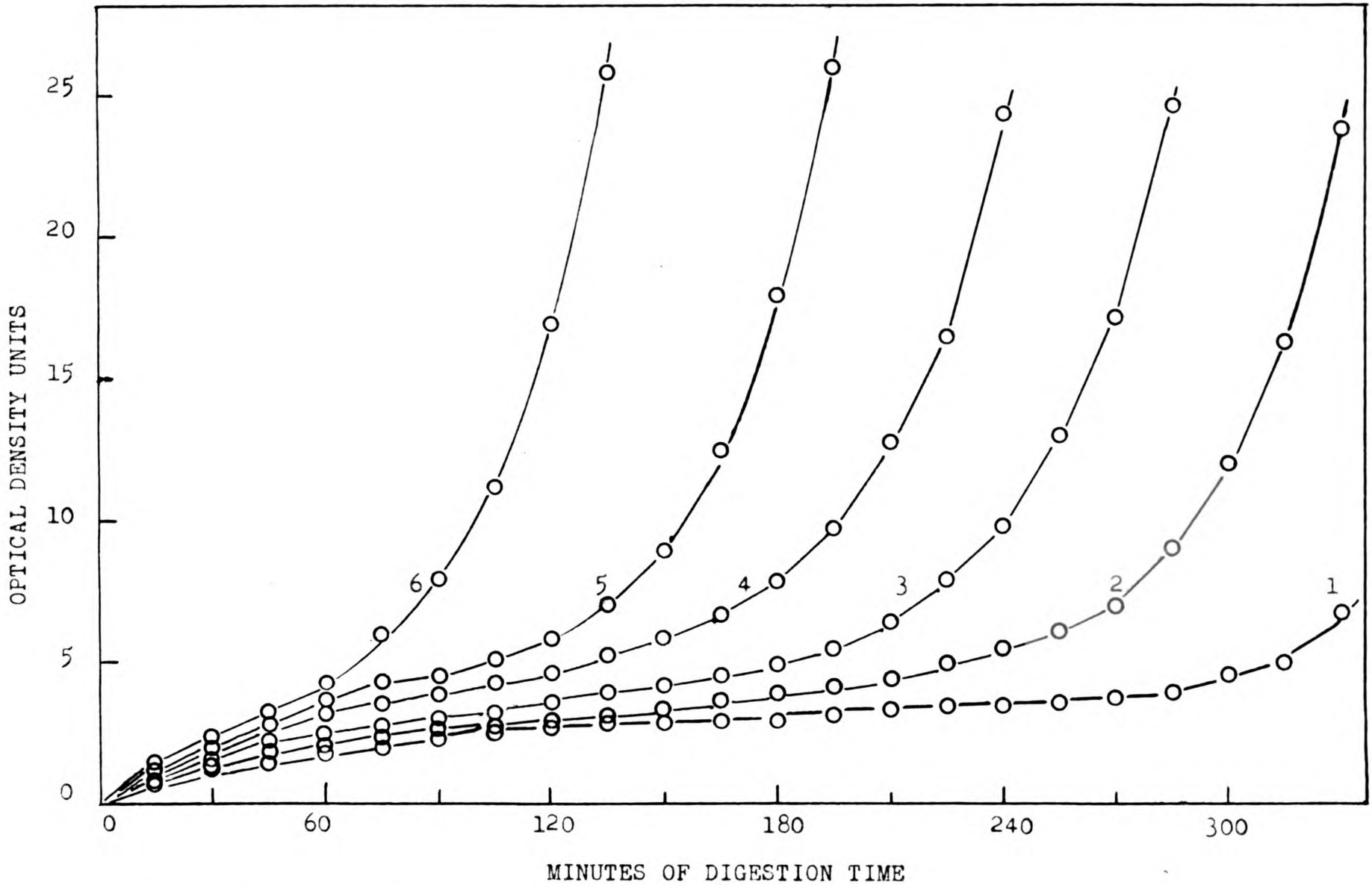

FIGURE 4. TURBIDITY FORMATION. CURVES: (1) 0.5, (2) 1.0, (3) 1.5, (4) 2.0, (5) 3.0, and (6) 4.0 mg. TAKA-DIASTASE per ml. of digest, respectively.

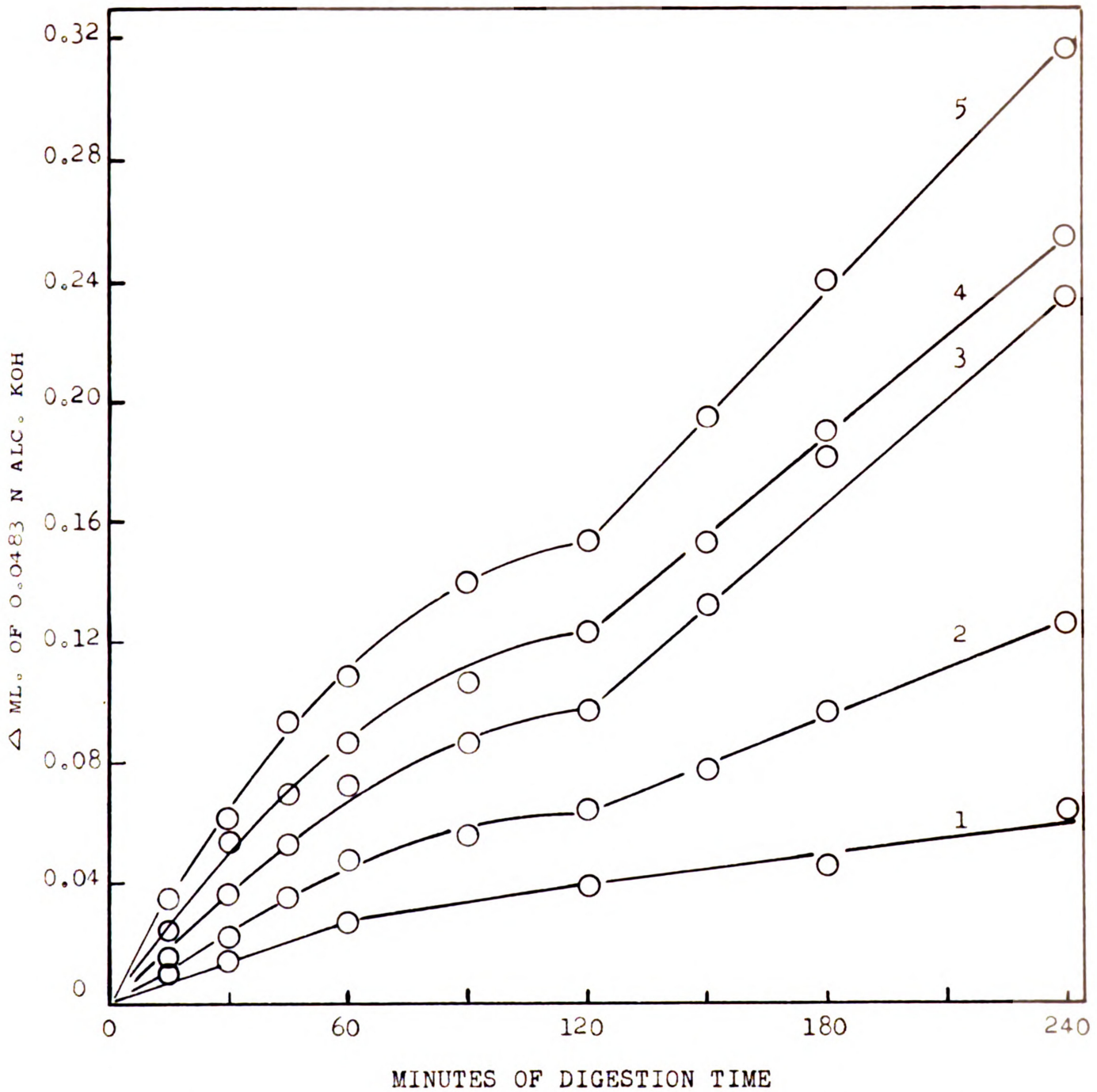

FIGURE 5. EFFECT OF SUBSTRATE (CASEIN) CONCENTRATION. CURVES: (1).(2),(3),(4) and (5) represent 1.1, 2.2, 3.1, 4.3 and 5.3 percent (w/v) casein.

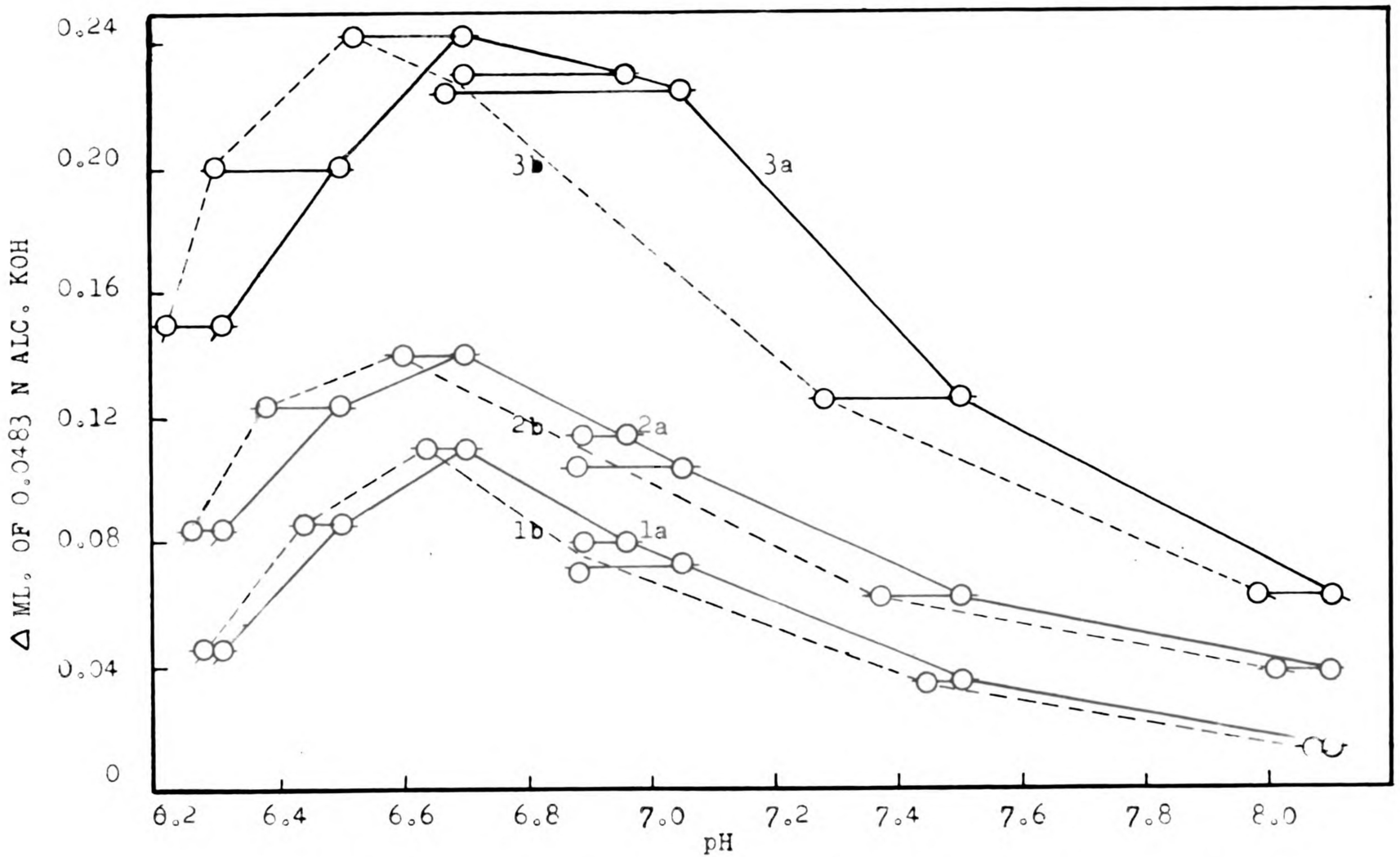

FIGURE 6. OPTIMUM pH, INITIAL AND FINAL. CURVES: (1), (2) and (3) are activities at 1, 2 and 4 hours of digestion, respectively.

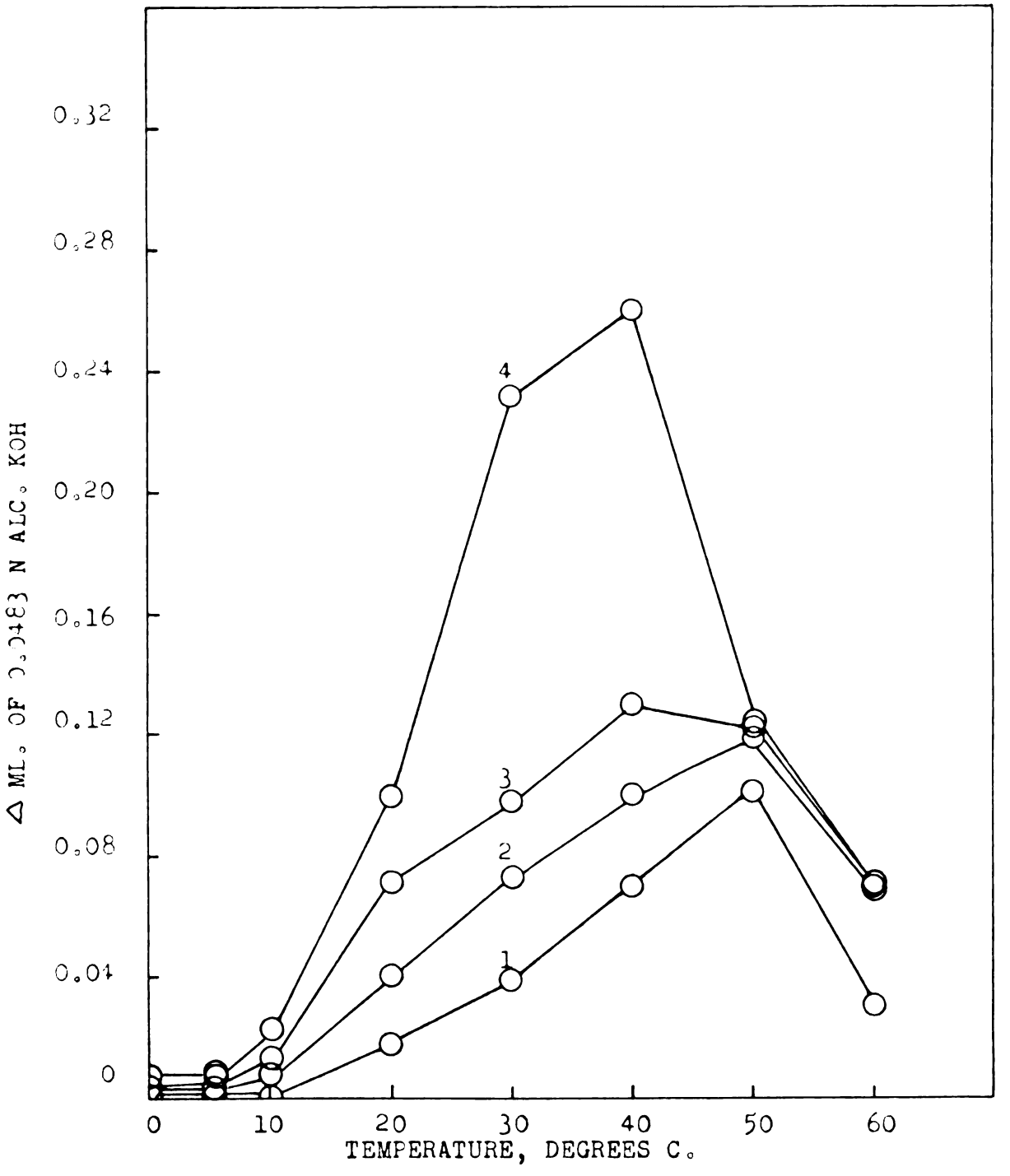

FIGURE 7. OPTIMUM TEMPERATURES. CURVES: (1), (2), (3) and (4) are activities at 0.5, 1, 2 and 4 hours of digestion, respectively.

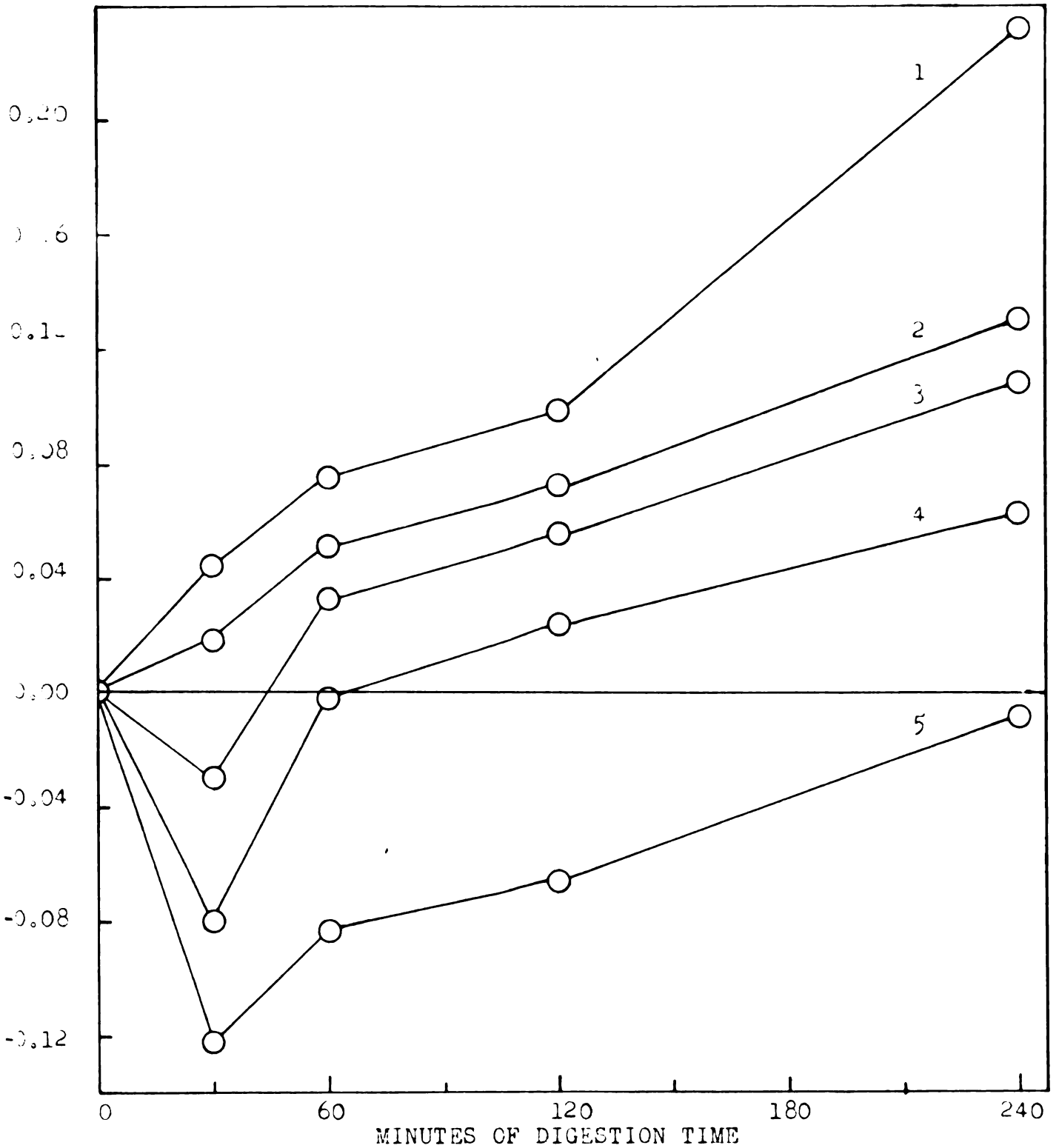

FIGURE 8. EFFECT OF ADDED ELECTROLYTE. CURVES: (1), (2), (3), (4), and (5) represent 0, 0.05, 0.10, 0.15 and 0.20 M NaCl added.

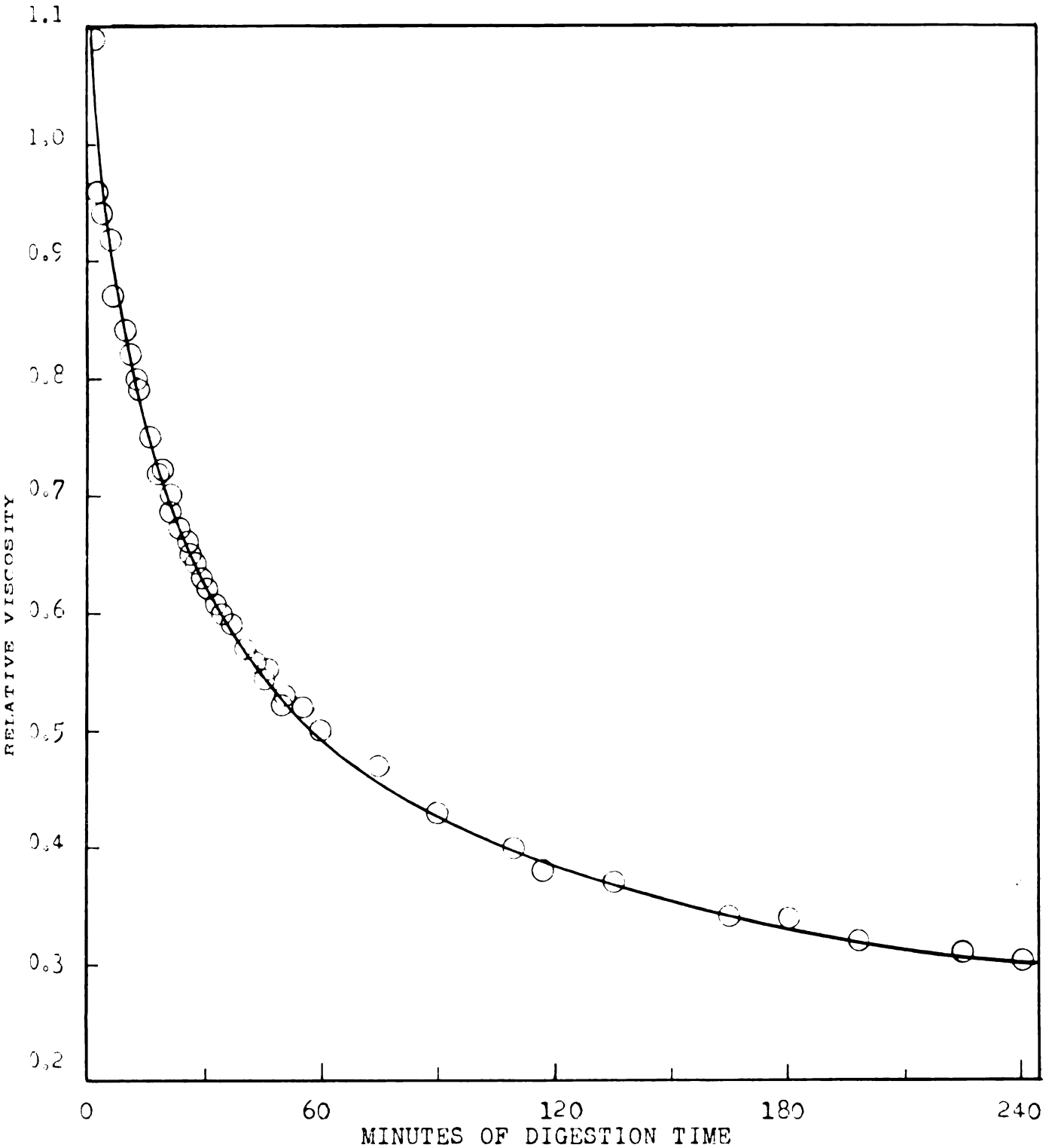

FIGURE 9. THE CHANGE IN VISCOSITY. The values are from a composite of 2 determinations.

IV. DISCUSSION

This discussion will be an attempt to evaluate the data and results by considering the scope, significance and limitations of the experimental methods employed. In such manner it is proposed to present information about the following factors that influence the proteolytic activity of Taka-Diastase on casein: 1- enzyme concentration, 2- substrate concentration, 3- optimum pH, 4- optimum temperature, 5- ionic strength, and 6- activators and inhibitors. Additional physio-chemical information about the over all reaction and remarks concerning the nature of the reaction will be presented.

A. The Influence of Varying Enzyme Concentration.

Change in Total Acidity.-The increment obtained by alcoholic KOH titration may be considered a measure of the increase in total acidity, due to formation of hydrogen ion donating groups. The theoretical considerations involved in this analytical method have been discussed by Richardson (1934) and others. Such acidic groups are presumed to arise from protein hydrolysis of predominantly peptide bonds. But this may not be the sole source of acid groups liberated from native proteins. There may be an additional contribution of phosphoric acid residues formed as a result of phosphoamide and/or phosphoester hydrolysis. Proteolytic enzymes are known to catalyze the hydrolysis of such types of bonds, as evidenced by the work of Holter and Li (1950) and Smoke,

Schwert and Neurath (1948) on synthetic peptides. However, one would expect that the data of this measurement is principally the result of peptide bond cleavage, particularly in the more advanced stages of the reaction.

Referring to the results in Table I and Figure 1, the following observations are apparent. The total acid groups liberated depends upon the enzyme concentration. All curves seem to support the hypothesis of a two stage reaction. But, as enzyme concentration is increased, the transition from the first to the second is less apparent and approaches a condition where it would become indistinguishable. On the basis of these experiments digestions run at constant enzyme concentration were chosen as 2 mg. of Taka-Diastase per ml. of digest. This was the enzyme concentration best representing the two stage nature of the reaction.

Change in Active Acidity as Measured Potentiometrically.- The net change in hydrogen ion activity as brought about by protein hydrolysis would be due to the type of acid groups liberated. Carboxylic acid groups from peptide bond cleavage being of the weak type, would not contribute as many free hydrogen ions as the stronger phosphoric acid groups. Further, proton accepting groups present could associate with the liberated free hydrogen ions to maintain their respective equilibria. Thus a partial buffering effect could be expected to be involved in the net change. This may explain the buffering effect apparent in Figure 2.

All digestions were unbuffered, in spite of the usual practice to do so. Sumner and Myrbäck (1950) point out that buffers may exert specific as well as a general ionic strength effect on enzymatically catalyzed reactions. There are cases known where common buffer substances acted as substrates, especially when crude enzyme preparations were used. Hence it could be difficult to distinguish between the influence of free hydrogen ions formed from buffer and that of the substrate. Buffer components may also interfere with analytical methods employed for following enzymatic activity. Consequently, the initial pH was always checked either colorimetrically or potentiometrically at the beginning of all digestions. All results are recorded with the initial pH defined. The data of Table II and Figure 2 demonstrates an increase in active acidity during the reaction and dependency on the enzyme concentration.

The Liberation of Non-Protein Nitrogen.-Non-protein nitrogen is that nitrogen of hydrolysis products not precipitated by 10 % trichloroacetic acid. The upper limits of molecular weight of such products is not well defined. In general, it has been considered to consist of mainly low-molecular weight polypeptides and free amino acids. Such measurements would be indicative of the extent to which these types of products are liberated. It can serve to demonstrate the fraction of the total protein nitrogen that has undergone extensive hydrolysis. The results in Table III and Figure 3 show linear dependency between such activity and enzyme

concentration during the early stages of the reaction. At the highest enzyme concentration under the defined conditions, 6.1 % of the total nitrogen was thus liberated.

Turbidity.-One of the preliminary observations leading to this study was that Taka-Diastase produced a cloudy solution when digested with casein. It was desired to evaluate this observation on a quantitative basis. Therefore, a method of analysis that would register any change in the transmissibility of light through the solution was devised. Before digestion the solution was a clear, slightly amber colored liquid. This would indicate that the substrate was well dispersed and stabilized by hydration and charge.

Whereas when proteolytic action set in and had proceeded for a period of time, a turbidity developed. This might be interpreted as being the result of altered charge and hydration capacity of the disperse phase due to proteolytic activity. The data of Table IV and Figure 4 show a direct relationship between enzyme concentration and the period of time for maximum change in optical density. It seems that there is an induction period proportional to the enzyme concentration. This would suggest that a preliminary or a series of reactions must have taken place before the cloudiness set in.

Comparison and Information from Results.-In enzymatically catalyzed reactions the usual behavior anticipated has been summarized by Somers (1950). He states that in the presence of a sufficiently large amount of substrate, such

that the rate of reaction is independent of the substrate concentration, the rate of reaction may be changed by changing the concentration of enzyme present.

In all these studies, it may be concluded that the data discussed in this section fulfills the above condition. The relationship, at least in the early stages of the reaction, holds true. Table I and Figure 1 show that the relationship does exist. In addition the rate curves in this figure show that the digestive process is not a single stage reaction, but is indicative of at least two stages. It seems that each stage initially is independent of the substrate concentration. The slow transition from one stage to the other at the lowest enzyme concentration indicates the possibility that there is not sufficient new substrate to completely saturate the enzyme of the second reaction. This transition effect varies from 1 to 2 hours of digestion in the enzyme concentration ranges studied.

If the non-protein nitrogen curves are compared with those of titration, there does not seem to be any distinct correlation between the activity plots of Figures 1 and 3. In Figure 3 no transition is apparent between 1 and 2 hours of digestion, but dependency upon enzyme concentration is exhibited. The liberation of non-protein nitrogen may not necessarily follow that of total acidity, inasmuch as measurements of two different entities are involved.

The net change in hydrogen ion activity curves of Figure 2 exhibit no intercorrelation with those of

non-protein nitrogen or total acidity. Dependency upon enzyme concentration is evident.

The optical density curves of Figure 3 demonstrate dependency upon enzyme concentration. If one inspects the data, the digestion time at which the turbidity approaches maximum rate of change corresponds favorably with the time at which transition step is noted in the titration curves. Such a relationship is not apparent with the non-protein nitrogen or hydrogen ion liberation.

B. The Influence of Variable Substrate Concentration.

This factor was studied by measuring the increase in alcoholic KOH titrated or total acidity. The significance of the titration has been discussed under the previous section on the influence of enzyme concentration. It is usually found that the reaction rate is a linear function of the initial substrate concentration. At relatively higher substrate concentrations less or no influence is exerted on the rate, since the enzyme becomes sufficiently saturated with substrate. In addition a substrate concentration may be reached where the rate may decrease.

Figure 5 demonstrates the dependency of acid group liberation rate upon substrate concentration. It is apparent that the substrate concentration at which the rate would become uninfluenced was not attained. A correlation between Figure 1 where enzyme concentration was varied and Figure 5 where the substrate concentration was varied is apparent.

The break in the curve of the latter figure, which appears after 2 hours of digestion, is quite pronounced since the points that would more clearly define the transition were not measured. However, a correlation in favor of a 2-stage reaction is observed between the two graphs.

C. The Optimum (Initial) pH.

Most all enzymes exhibit a maximum activity at some pH value, known as the pH optimum. In some cases this may be at a rather sharp maximum and means that the enzyme shows its greatest activity over a restricted pH range. In other cases a rather broad mixiumum zone is exhibited indicating less sensitivity to hydrogen ions. But pH optima cited for an enzyme may depend on other factors. The optimum may be influenced by the concentration and nature of the substrate temperature and concentration of enzyme. Oshima and Church (1923) pointed out that the method of measurement as well, exerts an influence on the result.

From Figure 6, the reaction appears to take place most favorably in slightly acidic media, with an optimal (initial) pH range 6.6-6.8. This agrees closely with the pH value of 6.7, reported by Nishikawa (1927). He stated that this value was also the upper limit of the optimum pH observed for rennin activity. The value pH 7.0, reported by Berger, Johnson and Peterson (1937), for Taka-Diastase activity on gelatin also agrees. As mentioned the substrate and method of assay may influence this factor. By the conditions of

these experiments, the optimum value appears to be between 6.6-6.8. The increase in titer at 1, 2 and 4 hours of digestion is plotted against initial pH on curve (a) of Figure 6 and similarly final pH's at the time of measurement is plotted on curve (b) of Figure 6.

The optimum pH range broadens as measurements on longer digestion periods are made. The direction seems to be towards a less acid solution for the longer times of digestion. A similar effect was reported about urease by Van Slyke and Zacharias (1914). They concluded that the optimum pH value was a result of two reactions: one which could be attributed to the addition of neutral salts and the other by the concentration of substrate, and that optimum pH was a compromise between their respective optima.

D. The Optimum Temperature.

Optimum temperature, like optimum pH, is influenced by concentration and nature of substrate, the method employed, and the time of digestion. It also varies widely with the enzyme being studied and source of the enzyme.

Below certain characteristic values, temperature influences the rate of enzyme-catalyzed reactions in much the same way it does comparable non-catalyzed reactions. That is, an increase in temperature increases the effect of catalysis. It is generally known that most enzymes being thermolabile are inactivated at elevated temperatures. As a result, the point of balance between the accelerating

influence of temperature and the inactivating influence of heat is designated as the optimum temperature.

Figure 8 indicates a shift from an optimum of 50° C. in the initial stages of digestion toward a lower optimum around 40° C. in the later stages.

Since the substrate used by Kawakami (1929) is not mentioned in the literature available, no significant comparison of results can be made with this work.

E. The Effect of Added Ionic Strength by Neutral Salt.

Ionic strength influences charge, hydration and solubility of protein dispersions. This could show alteration of the rate of reaction either as a direct result or as an influence upon the mode of activity measurement. The influence of such an effect was tested by increasing the ionic strength of the digestion medium. The results are tabulated in Table IX and shown in Figure 9.

The effect of added salt (NaCl) appreciably decreases the activity at all salt concentrations as compared to the control. Szanto (1912) reported that the presence of 0.1 M neutral salts did not exert appreciable influence on the activity of Taka-Diastase in a 1 % casein solution. The results were based upon an isolectric precipitation of undigested casein. The apparent contradiction may be due to the methods of assay and thus a strict comparison of results may not be valid.

From Figure 9, the NaCl concentrations of 0.1 M and above show greatest activity change over all stages of the

digestion. The titers for digests with 0.2 M NaCl never reached the initial value showing the anomaly of a negative increment. Among various considerations, the salt effect on the indicator might account for this, but other observations led to a more tenable explanation. At all salt concentrations, the digest solubility was altered as evidenced by a rapid turbidity formation. No precipitation occurred during the digestion period, but a distinct difference was noted when the samples were titrated. This was most evident at the higher salt concentrations. On adding digest aliquots to the alcohol-indicator mixture, a heavy white floculation appeared. This was not completely redissolved when the final end point was reached. Such behavior was not evident at 0.5 M salt concentration or in the control which contained no added salt. Hence the anomalous character of the initial part of the curves, 1, 2 and 3 of Figure 9, may be due to such circumstances. In the later stages of digestion when titrating aliquots this floculation behavior disappeared.

F. Activators and Inhibitors.

Massart (1950) points out that activator and inhibitor reagents when allowed to react with the enzyme and then testing its catalytic power may give information about the 'active centers" which were associated with the normal enzymatic activity. Experiments were designed to provide, if possible, some information concerning the type of groups in the enzyme that are associated with its activity. The results are in Table IX.

Cations.-The strong inhibition exerted by reacting Cu^{++}, and Hg^{++} with the enzyme compares with work of Nishikawa (1927), who reported that tryptic action upon gelatin was inhibited by Cu^{++}, Hg^{++} and Zn^{++}. Perhaps the 2 ions form weak dissociating complexes with substrate binding groups of the enzyme. Table IX shows no inhibition by Zn^{++}. The activating effect of Mn^{++}, Co^{++}, and Ca may be due to supplementing a cofactor requirement or to an unspecific cation effect. Enzymes which are metallo-proteins respond similarly.

The strong irreversible inactivation caused by hydrogen ion and the partial inactivation with hydroxyl ion confirm the report of Szanto (1912) about the behavior of strong acids and bases.

Anions.- The inhibition by flouride ion may possibly be due to its high complexing ability with cations. Since Ca , Mn and Co exert an activating effect, the flouride ion may complex these metallic ions of a metallo-protein enzyme.

Carbonyl Reagents.-Since the carbonyl reagents tested do not influence the activity, it would seem that carbonyl centers of the enzyme sensitive to these are not essential for activity.

Oxidizing Agents and Sulfhydryl Reagents.-If the enzyme depended on sulfhydryl groups for activity, inactivation should be expected by oxidizing agents or sulfhydryl reagents. From Table IX it would appear that sulfhydryl groups may not be necessary for activity, since both hydrogen

peroxide and iodoacetic acid exerted no noticeable influence on activity.

Amino Group.-Ninhydrin can react with free ∝-amino, ∝-carboxyl groups. Any effect of ninhydrin upon such was not detected in these experiments.

Detergents.-Denaturation or other chemical effects of sodium lauryl sulfonate on Taka-Diastase was not evident. This is contrary to that reported by Astrup and Alkjaersig (1952), but their observations were applied to its fibrinolytic activity.

Physical Agents.-Inhibition by ultra-violet irradiation might be expected to be due to a photochemical reaction on the enzyme. But Kawakami (1929) stated that it might be due to a heat effect unless the solutions being irradiated were cooled. The results reported in Table IX were obtained upon solutions that had not been cooled during irradiation, but indicate that inactivation decreased as the samples were further removed from the light source. Under the conditions of the experiments it seemed logical that the results were as representative of irradiation effects as that of heating. Nevertheless a more careful examination should be made.

Dialysis.-Since an estimation of the dilution resulting upon dialysis of the enzyme solution was not made, it is not possible to state with certainty that the loss in activity was due entirely to a removal of a co-factor or essential prosthetic group. However the cation activating

effect lends substantiation to such a conclusion.

Heat.-Heating Taka-Diastase solutions at 100° C. for 1 hour was found to completely inactivate the proteolytic enzyme. This property of thermo lability was employed in preparing inactive enzyme solutions for control experiments.

G. Physico-Chemical Studies on the Nature of the Reaction.

Viscosity Change.-The colloidal nature of a protein solution lends itself to the possibility of studying viscosity changes due to reactions leading to or resulting in hydrolysis. This change should be quite sensitive to physical and chemical effects upon the state of aggregation, nature of associated complexes or any physical alteration of the initial condition of the substrate. Figure X demonstrates a rather striking initial decrease in viscosity, and subsequent independency of further reaction. This supplements information on nature of the initial stages of the digestion previously discussed from titration data. The drop in viscosity almost parallels the first stage of the reaction.

Optical Rotation Change.-It might be anticipated that a change in protein structure which would lead to or result in hydrolysis could be directly followed by change in optical rotation.

The results in Table X did not meet this expectation, at least in the manner the method was employed. The specific rotation for casein as calculated from the initial rotation

observed is $[\alpha]_D^{30} = -98.6$ and agrees favorably with $[\alpha]_D^{30} = -95.2$ reported by Long (1905). Among others, Winnick and Greenberg (1941) applied optical rotation change to follow protein hydrolysis. But in all cases, the rotation was determined upon filtrates of trichloroacetic acid after precipitation of the unreacted protein. The experiments should be repeated along these lines. It is possible that t the rotations reported in this work were a result of compensating effects induced by the products formed. But the fact remains that there was a polarimetric change in the presence of the enzyme; however, it did not yield a significant trend. Perhaps higher substrate concentration would have improved the results.

Refractive Index Change.-As light passes from one media to another it is refracted. If any change takes place in the composition of media, it is conceivable that this would manifest a change in refractive index. The results in Table XII do not demonstrate a significant change. Again, this may be due to an inadequate substrate concentration or other conditions requiring further investigation.

Effect of Age on the Substrate.-When a casein stock solution (up to 5 weeks old) was not carefully refrigerated when not in use, the following observations were made. Initial titer of alcoholic KOH on digest aliquots varied from one day to another. The electrophoretic patterns were altered. Duplication of Proteolytic activity measurement was not possible from one period to the next. The acid or

base binding capacity was shifting. The pH of the stock solution itself was shifting towards a more acidic condition.

To test the stability of a stock casein solution carefully maintained under refrigerator conditions, electrophoretic analyses were run weekly from date of preparation through 6 weeks. The patterns showed no alteration. It is quite likely the proteolytic enzyme within casein as mentioned by Warner and Polis (1945) brought about the above noted gradual change.

H. Remarks on the Mode of Reaction:

The increase in free hydrogen ions, viscosity decrease, liberation of acidic groups, formation of non-protein nitrogen, increased optical density, change in optical rotation, change in refractive index, all indicate that during digestion the substrate was undergoing physical and chemical alteration that resulted in hydrolysis.

The liberation of acidic groups is representative of principally peptide bond cleavage, but in addition may include phosphoamide or phosphoester linkages. The curves of Figure 1 suggest two stages for this type of change. Perhaps stage 1 reaction may be associated with an initial attack upon one of the fractions of casein, followed by a reaction of stage 2 upon the remaining components or products of the initial reaction. The change in viscosity during the first hour of reaction shown by Figure 9 corresponds favorably with stage 1 of curve 3 in Figure 1. It

suggests that the initial stage is the most significant hydrolytic step affecting viscosity.

The rapid increase in turbidity corresponds with the digestion time of stage two. Figure 3 shows that turbidity becomes most pronounced after 5 units of optical density have been attained. This occurs between 1 to 2 hours of digestion, depending upon enzyme concentration or appears at the end of stage 1 and the beginning of stage 2. Turbidity suggests change in solubility of an altered component of casein or a remaining unattacked fraction with more hydrophobic properties. The fact that only up to 1 % non-protein nitrogen was formed during stage 1 lends support to this contention. At present it is not possible to ascertain whether the products or the insolubility of one of the components of casein was contributing to the turbidity.

Non-protein nitrogen may be the result of over-all hydrolytic changes in the media, since its formation rate showed no deviation at the times of stage 1 and stage 2 of reaction.

Direct correlation could not be made between the change in hydrogen ion activity and the preceding observations. This may be the result of buffering in the medium which arises from the products or remaining substrate. It would seem that the more strongly acidic phosphoric acid groups were being liberated during the initial stage, since the hydrogen ion activity increased most rapidly at this time.

The shifting of the pH optimum, to a less acidic condition, and the shift in the optimum temperature requirements during the second stage of reaction may indicate the presence of more than one proteolytic enzyme. That is, the initial reaction may involve an enzyme system different from that in operation later on.

The foregoing remarks point out that further investigations on casein fractions as substrates, using the crystalline proteolytic enzymes described by Crewther and Lennox (1950), would be highly desirable. However, the data presented here gives some indication of the circumstances lending to turbidity formation.

V. SUMMARY

1. The liberation of acidic groups, turbidity formation, hydrogen ions and non-protein nitrogen are dependent on the concentration of Taka-Diastase during casein digestion.

2. Acidic groups are liberated in a two stage reaction. This is most pronounced with 3 % casein digests containing 2 mg./ml. of Taka-Diastase. Transition from one step to the other takes place between 1 to 2 hours of digestion.

3. The onset of rapid turbidity formation corresponds with stage 2 of acidic group liberation.

4. The maximum drop in viscosity during the first hour of digestion parallels the first stage of acid group liberation in the reaction.

5. The formation of non-protein nitrogen shows a dependency upon enzyme concentration, but gives no parallel correlation with a 2 stage liberation of acidic groups.

6. At all substrate concentrations up to 5 % the rate of reaction was dependent on the concentration of substrate.

7. An optimum initial pH 6.6-6.8 was found for 1 to 2 hours of digestion. A broader optimum range extending to pH 7.0 was observed with 2 to 4 hours of digestion.

8. The optimum temperature was found to be 50° C. for 1 to 2 hours of digestion. A shift down towards 40° C. was observed for 4 hours of digestion.

9. Sodium chloride added to the digest decreased

activity at all concentrations. Titration anomalies were observed at added salt concentration of 1.0, 1.5, 2.0 Molar.

10. Digests exhibited no significant change in optical rotation or refractive index during the first 4 hours.

11. All chemical reagents tested as activators or inhibitors were added in 1 x 10^{-3} M concentration for 1 hour of reaction with the enzyme. The effect upon digestion with casein was tested by measuring the increase in titratable acidity after 1 hour and showed these results:

a. Co^{++}, Mn^{++}, Ca^{++} accelerated the rate of reaction, 29, 17, and 34 % respectively.

b. Cu^{++}, Hg^{++}, F^{-}, inhibited the reaction rate, 84, 98, 42 % respectively.

c. Reaction with HCl at pH 3 for 1 hour and restoring to the original pH, gave irreversible (98 %) inactivation. Similar treatment with NaOH to pH 11.0 resulted in 30 % inactivation.

d. All other chemicals tried had no noticeable effect on the enzyme activity.

e. Depending upon distance, ultra-violet irradiation for 15 minutes resulted in up to 70 % inhibition.

f. Dialysis for 14 hours against 20 volumes redistilled water with 4 changes resulted in 50 % inactivation.

g. Heating the enzyme solution at 100° C. in a sealed tube for 1 hour produced irreversible inactivation.

12. Some effects of age on casein stock solutions are presented and warn of the necessity for fresh solutions and low temperature storage.

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THE PROTEOLYTIC ACTIVITY OF TAKA-DIASTASE FROM *ASPERGILLUS ORYZAE* ON CASEIN

By

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AN ABSTRACT

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ABSTRACT

It was observed that a clear, neutral solution of casein became turbid, within several hours in the presence of Taka-Diastase.

This study was undertaken to determine the optimal conditions and the nature of the reaction involved in the formation of these turbid solutions.

The rate of liberation of acidic groups, as determined by alcoholic potassium hydroxide titration, was the principle method of measuring activity. The influence of enzyme concentration upon the liberation of acidic groups, non-protein-nitrogen, free hydrogen ions, as well as the rate of turbidity formation, was measured by titration, micro-Kjeldahl analysis, pH measurements and change in optical rotation.

The rate at which acidic groups were liberated at different substrate concentrations was determined by titration.

The influence of initial pH, temperature and added neutral salt upon the rate of acidic group liberation was studied. The digestion mixtures used for these measurements were 3 % with respect to casein and contained 2 mg. of Taka-Diastase per ml. of digest.

A number of possible activators and inhibitors of the enzyme were tested. Their effect was compared with the rate of acid group liberation of untreated enzyme. Viscosity, optical rotation and index of refraction measurements were made on digests containing 3 % casein, and 2 mg./ml. of

Taka-Diastase. Only viscosity measurements gave significant results.

The results of these experiments are as follows: The liberation of acidic groups, turbidity formation, free hydrogen ions, and non-protein-nitrogen during casein digestion are dependent upon the concentration of Taka-Diastase. Acidic groups are liberated in a two-stage reaction. The time at which the most rapid change in turbidity is observed corresponds with the second stage of acid group liberation.

The rapid drop in viscosity during the first hour of digestion parallels the first stage of acidic group liberation.

Liberation of non-protein-nitrogen and changes in hydrogen ion activity exhibit a dependency upon enzyme concentration. But, no direct correlation can be drawn between their rate of change and that of acidic groups and turbidity.

For digestion mixtures containing 3 % casein and 2 mg./ml. of Taka-Diastase, the optimum pH appears to be 6.6-6.8 for 1 to 2 hours of digestion.

Under the same digest conditions, initial pH 7.0, the optimum temperature for 1 to 2 hours of digestion appears to be 50° C.

At all salt concentrations tested, the rate of liberations of acidic groups was decreased.

In concentrations of 1×10^{-3} Molar, Co^{++}, Mn^{++}, Ca^{++} accelerate the rate of casein digestion. Cu^{++}, Hg^{++}, F^{-},

dialysis and ultraviolet irradiation inhibit the liberation of acidic groups. If the enzyme solution is kept at pH 3.0 for for 1 hour the enzyme solution is irreversibly inactivated. Similar treatment at pH 11 results in 30 % inactivation. If the enzyme solution is heated at 100° C for one hour, total inactivation occurs.