

## ENZYMIC DEGRADATION OF $\beta$ -CASEIN BY A SNAKE VENOM PREPARATION

E. B. KALAN AND MARIE TELKA

Eastern Regional Research Laboratory,<sup>1</sup> Philadelphia 18, Pennsylvania

### SUMMARY

The action of a snake venom preparation from *Crotalus adamanteus* on  $\beta$ -casein has been studied. The action resulted in the formation of a turbidity, which was measured with time in the spectrophotometer. The effects of temperature, metal ions, pH, and ionic nature of the medium have been observed. The reaction appears to be proteolytic in nature, being accompanied by an increase in ninhydrin-positive material and resulting in the formation of at least four different fractions from  $\beta$ -casein. The proteolytic activity always preceded the precipitate formation. The material precipitated during the reaction was essentially phosphorus-free and comprised 20-30% of the original  $\beta$ -casein.

During the course of a study on the nature of the phosphate linkages in casein fractions, it had been noted that when  $\beta$ -casein is incubated with a snake venom preparation having phosphodiesterase activity, turbidities developed (9). This phenomenon is accompanied by a shift in pH toward the acid side, as had been previously noted by Perlmann (14), who took this to mean the cleavage of phosphodiester bonds. However, this conclusion has been recently challenged (7, 9). The present communication reports some of the properties and characteristics of the reaction of  $\beta$ -casein with an enzyme preparation obtained from *Crotalus adamanteus*.

### MATERIALS AND METHODS

*$\beta$ -Casein.* The casein was prepared from whole casein by fractionation in aqueous urea solutions (6). The preparation used in these studies had a moisture content of 7.62% and a total phosphorus content of 0.61% on a moisture-free basis.

*Enzyme preparation.* The preparation used was obtained as a dry powder from Ross Allen's Reptile Institute, Silver Springs, Florida, and was fractionated by the method of Sinsheimer and Koerner (16). The precipitate formed with 0-40% acetone was found to be most active in causing turbidity with  $\beta$ -casein. This fraction, obtained in the first acetone cycle, was dissolved in water and used as the enzyme preparation. It also had phosphodiesterase activity. It contained 8 mg. protein/milliliter as determined by the micro-Kjeldahl procedure, using the factor 6.25. The enzyme solution retains full activity when stored at 4° C. for 3 mo.

*Measurement of turbidity.* The turbidity measurements were made in the Beckman Spectrophotometer (Model DU) at a wave length of 600 m $\mu$ . The reaction was carried out in cuvettes of 1-cm. path length at a constant temperature maintained by means of thermospacers.

Received for publication June 28, 1959.

<sup>1</sup> Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

*Measurement of proteolysis and pH.* The proteolysis was measured by the increase of ninhydrin-positive material of the entire reaction mixture as described previously (9). The modified ninhydrin method of Moore and Stein (12) was employed, and the results were quantitated by comparison with a standard leucine curve. It is necessary to keep in mind that the quantitative data are taken only as an approximation of the degree of proteolysis, for reasons previously stated (9). The pH determinations were made at room temperature before and after the reaction, using the glass electrode.

*Measurement of nitrogen and phosphorus.* Total nitrogen was determined by a modification of the micro-Kjeldahl method of Ma and Zuazaga (10), in which a mixture of  $K_2SO_4$  and  $HgO$  was used as catalyst during the digestion procedure. Inorganic orthophosphate was determined on trichloroacetic acid filtrates (final concentration—10% TCA) of protein solutions by the method of Fiske and Subbarow (5). Total phosphorus was determined as above after prior digestion with  $H_2SO_4$  (17).

#### EXPERIMENTAL PROCEDURE

The following types of experiments were carried out to study the properties and characteristics of the action on  $\beta$ -casein of the snake venom fraction noted above.

*Turbidimetric studies.* In these studies, the desired amount of enzyme was placed in the cuvette, which was then allowed to come to temperature, usually  $38^\circ C.$ , unless otherwise indicated, in the spectrophotometer. The remaining solution, including the casein and any other substances pertinent to the experiment, was placed in a tube immersed in a water bath at  $38^\circ C.$  At zero time, the contents of the tube was added to the cuvette to bring the final volume to 3.0 ml. The pH of the solution in the tube had been previously adjusted, and since the volume of the enzyme solution was, in general, less than 0.2 ml., there was very little change in pH. This was verified by independent mixing experiments. The first reading of the optical density was made 1 min. after mixing, and readings were continued at intervals until 30 min. had elapsed, as indicated in the accompanying figures. The pH was then measured, and a plot of the increase of optical density at  $600 m\mu$  with time was made. The exact composition of the reaction media is given below, under Results.

*Proteolytic studies.* These studies were carried out separately from the turbidimetric studies. Tubes were incubated in a water bath at  $37^\circ C.$ ; 0.1-ml. aliquots of the reaction mixture were taken at zero time and every 3 min. thereafter until the fifteenth minute, when aliquots were taken every 5 min. until the experiment was concluded after 30 min. The increase of ninhydrin positive material was measured as previously described (9) and compared with the turbidimetric data. Data are reported in Table 1 in terms of bonds split/30,000 mol. wt.

*Distribution of nitrogen and phosphorus.* To determine the distribution of N and P between the supernatant and precipitate, the following experiment was performed. Two reaction tubes containing 75 mg. of  $\beta$ -casein, 0.75 mg. of

TABLE 1  
Extent of proteolysis during the action of snake venom on  $\beta$ -casein

System	Bonds hydrolyzed <sup>a</sup>	
	(30,000 mol. wt.)	
	After 6 min.	At end 30 min.
Control <sup>b</sup>	1.2	3.7
Control (T = 26° C.)	1.0	3.2
-Mg <sup>++</sup>	1.3	2.4
Substrate = 400		
Enzyme = 1	0.7	2.1
+ 3.3 × 10 <sup>-8</sup> M versene (-Mg <sup>++</sup> )	0	0
+ 3.3 × 10 <sup>-2</sup> M versene (-Mg <sup>++</sup> )	0	0
+ 8.2 × 10 <sup>-2</sup> M veronal	0.8	2.5
+ 3.3 × 10 <sup>-1</sup> M veronal	0	0
+ 3.3 × 10 <sup>-1</sup> M NaCl	0.7	2.7
+ 6.6 × 10 <sup>-1</sup> M NaCl	1.2	2.7

<sup>a</sup> All values reported are the averages of at least two independent determinations.

<sup>b</sup> Control system consisted of a substrate-to-enzyme ratio of  $\frac{100}{1}$  in an unbuffered medium of pH 9.5 at 36° C. containing 3.3 × 10<sup>-3</sup> M MgCl<sub>2</sub>. All other systems were the same except as indicated.

enzyme, and 50  $\mu$ moles of Mg<sup>++</sup> in a volume of 10 ml., pH 9.7, were incubated at 36° C. A third control tube contained no enzyme. Aliquots were taken at 0, 15, and 30 min. to determine total N, total P, and inorganic P of the supernatant after the solution had been clarified by centrifugation at room temperature at 2,000 g for 30 min. The extent of proteolysis was also measured, using the whole reaction mixture as described above, and the pH was also determined at the end of 30 min. The precipitate could be further fractionated, as will be described under Results and Discussion.

#### RESULTS

*Turbidimetric studies.* Figure 1 is the plot of a typical experiment in which snake venom was incubated at 38° C. in a final volume of 3.0 ml. with  $\beta$ -casein (0.5%) (substrate to enzyme weight ratio of 100:1). The reaction underwent a pH shift of 1.2 units toward the acid side from an initial pH of 9.5, after correcting for pH changes in the control. Also present in the medium was MgCl<sub>2</sub> (3.3 × 10<sup>-3</sup> M). It is noted that an S-shaped curve was obtained with a lag of about 4 min. and with a slope of  $\frac{0.243 \text{ O.D. units}}{\text{minutes}}$  for the straight line portion of the curve (between 4 and 11 min.). When the enzyme preparation was boiled for 5 min. and then incubated with the casein under the same conditions, there was no reaction. The addition of boiled enzyme to the system described above was neither inhibitory nor stimulatory. When the substrate-to-enzyme ratio was varied over an eightfold range by varying the enzyme concentration, the series of plots illustrated in Figure 2 was obtained. As the  $\beta$ -casein to snake venom ratio increased, the lag period increased, and the slope of the straight-line portion of the curve decreased, as did the pH shift. Also shown in Figure 2 is the plot obtained when the temperature of the reaction was reduced to 25° C. with a substrate-to-enzyme ratio of 100:1. The lag

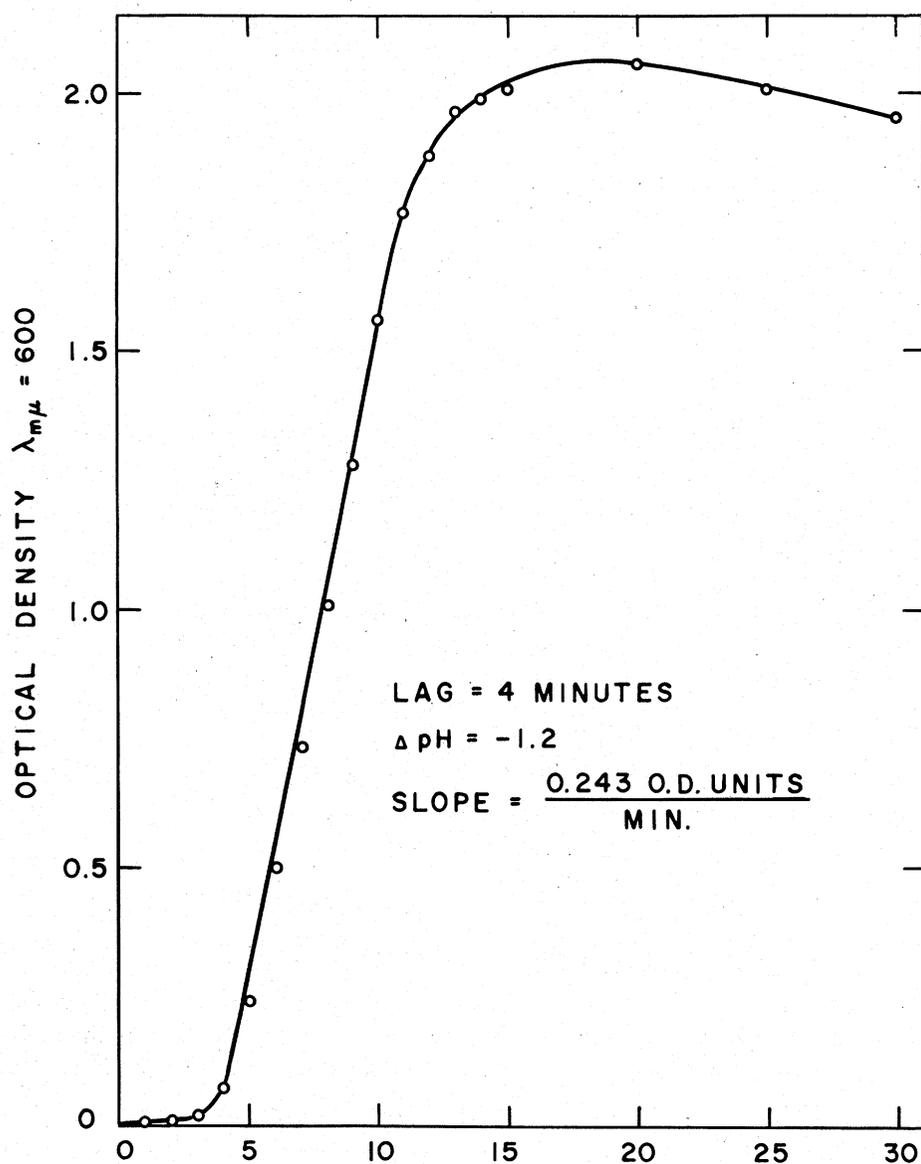


FIG. 1. Incubation of  $\beta$ -casein with snake venom at  $38^\circ \text{C}$ . in the presence of  $3.3 \times 10^{-3} \text{ M}$   $\text{MgCl}_2$  at an initial pH of 9.5.

period was greatly extended, and the slope was reduced by approximately one-half.

It was found that when calcium ions replaced magnesium, the reaction proceeded at the same rate as in the presence of magnesium. However, the omission of divalent metal ions resulted in an increase in the lag and decrease in the slope, as seen in Figure 3. The addition of boiled enzyme to the system

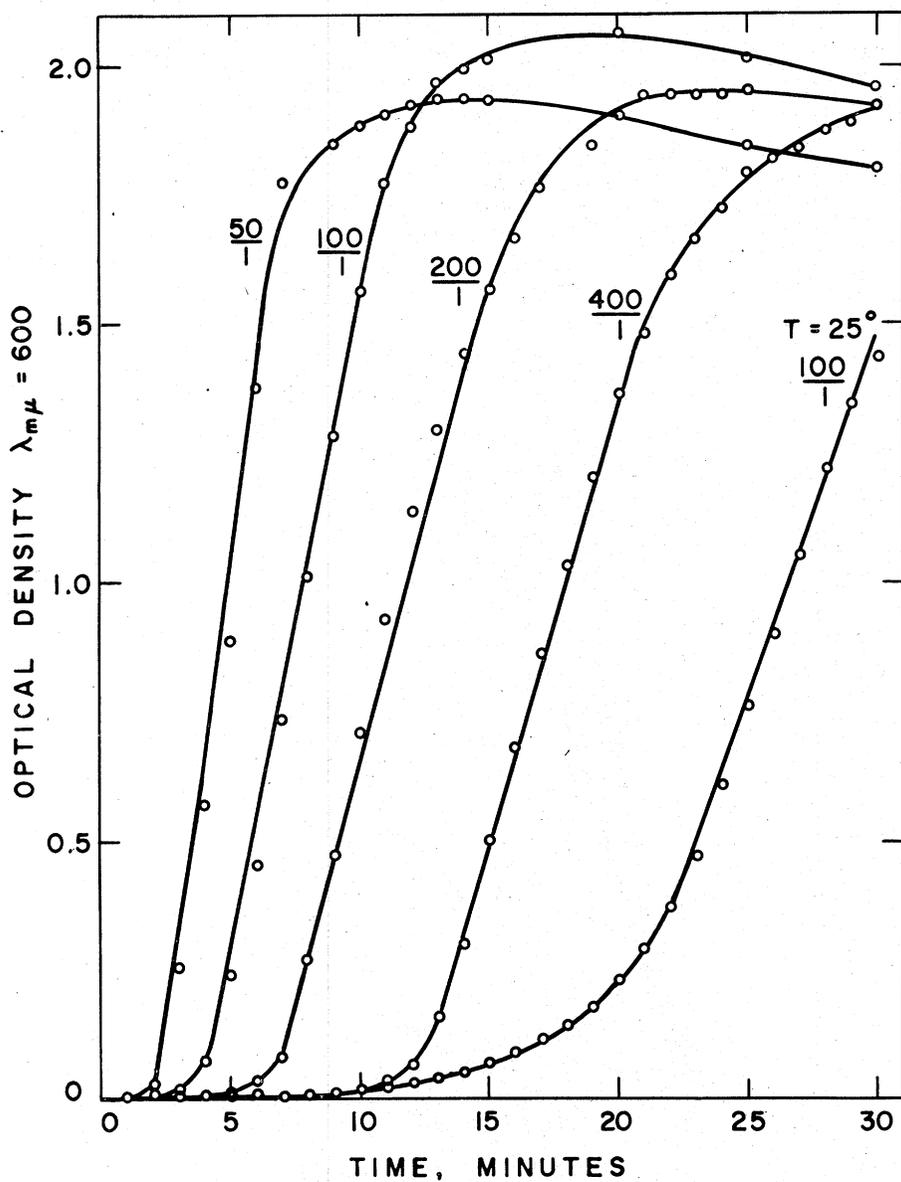


FIG. 2. Incubation of  $\beta$ -casein with snake venom at 38° C.—varying substrate to enzyme concentration in the presence of  $3.3 \times 10^{-3} M$   $MgCl_2$  at an initial pH of 9.5.  $(\frac{50}{1})$  slope =  $\frac{0.348 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.4$ ,  $(\frac{100}{1})$  slope =  $\frac{0.243 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.2$ ,  $(\frac{200}{1})$  slope =  $\frac{0.185 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.0$ ,  $(\frac{400}{1})$  slope =  $\frac{0.166 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -0.6$ .  $T = 25^\circ C$ .  $(\frac{100}{1})$  slope =  $\frac{0.133 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.1$ .

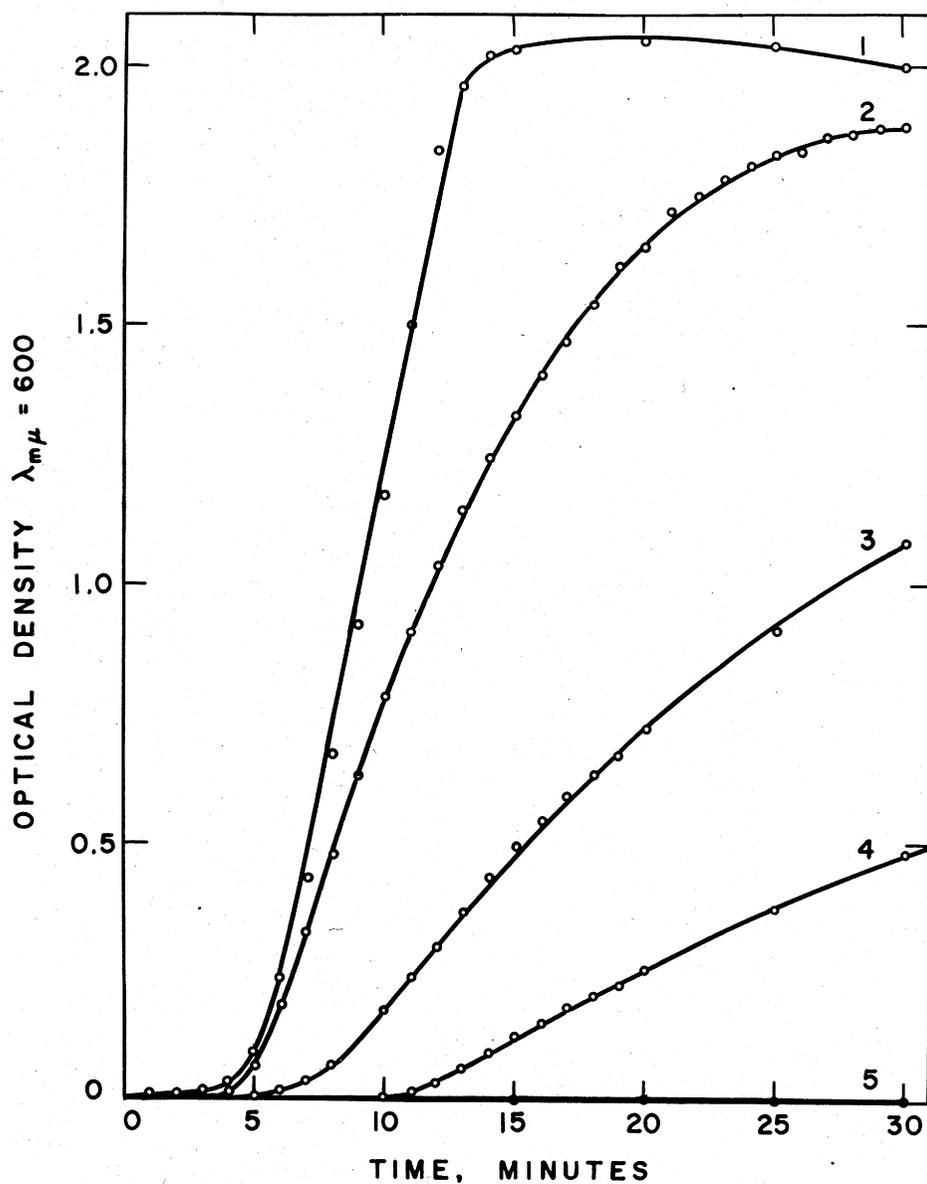


FIG. 3. Effect of magnesium and versene on the reaction of  $\beta$ -casein and snake venom at  $38^{\circ}$  C. and initial pH 9.5. Substrate-to-enzyme ratio  $\frac{100}{1}$  (1) +  $3.3 \times 10^{-3} M$   $MgCl_2$  slope =  $\frac{0.249 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.1$  (2)  $3.3 \times 10^{-3} M$   $MgCl_2$  +  $3.3 \times 10^{-3} M$  versene slope =  $\frac{0.139 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -1.2$  (3)  $-MgCl_2$  slope =  $\frac{0.058 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -0.40$  (4)  $-MgCl_2$  +  $3.3 \times 10^{-3} M$  versene slope =  $\frac{0.025 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta pH = -0.10$  (5)  $-MgCl_2$  +  $3.3 \times 10^{-3} M$  versene slope = 0  $\Delta pH = 0$ .

containing no magnesium is without effect. Versene, on the other hand, inhibited the reaction, and this inhibition could be overcome by the addition of magnesium ions, as is also seen in Figure 3. It was also found in this connection that the  $\beta$ -casein had no major metal contamination, so that the activity in the absence of added divalent metals must be due to trace contaminants in the casein or enzyme preparation.

When the initial pH of the reaction was varied between 6.2 and 9.9 in the unbuffered system, there was no change in the lag period (4-6 min.), but a decreasing shift in pH was observed with decreasing initial pH until pH 7.5, where no pH shift was observed. The value for the slope of the straight line portion of the curve did not vary appreciably over the pH range investigated. When the reaction was carried out in varying concentrations of veronal buffer at pH 9.5, it was noted that the reaction was inhibited, as can be seen in Figure 4. Increasing concentration of NaCl also inhibited the system, but for reasons different from veronal, as will be shown below. It is also seen in Figure 4 that somewhat higher NaCl concentrations were required to achieve inhibitions comparable to those with veronal (see legend of Figure 4).

*Proteolytic studies.* It is well known that snake venoms possess many enzymic activities (18), including proteolytic activities (4). The preparation from *C. adamanteus* was treated with diisopropylfluorophosphate (DFP), which is known to inhibit trypsin and chymotrypsin (4), in a manner previously described (9), and allowed to react with  $\beta$ -casein. The casein was degraded as before without inhibition. When the reaction was followed by measuring the increase in ninhydrin-positive material, it was found that after 30 min., there were about four bonds hydrolyzed, even when the enzyme was treated with DFP. Table 1 gives the results of a series of experiments in which the reaction of snake venom with  $\beta$ -casein was followed by the ninhydrin method. It can be seen that about four bonds are hydrolyzed after 30 min., but hydrolysis can be detected very early, even before turbidity formation can be visually detected. It would appear that the effect of a reduction in temperature is primarily to increase the lag period, since proteolysis was not influenced greatly. In this connection Berridge (2) has shown that the temperature coefficient for aggregation of para-casein with calcium ions is much greater than the temperature coefficient for the rennin-catalyzed proteolysis of casein. This finding was confirmed by Claesson and Nitschmann (3) and extended to other casein systems by Zittle and Pepper (20). The absence of magnesium ions from the medium reduced the over-all hydrolysis, and when versene was added, the proteolytic activity was abolished. This was true even with  $3.3 \times 10^{-3} M$  versene, which did not prevent the appearance of a slight turbidity [see Figure 3, Plot (4)]. It is interesting to note that high concentrations of veronal buffer inhibit proteolysis as well as turbidity formation, whereas the highest NaCl concentrations used inhibited proteolysis by about 25%. These salt concentrations did inhibit the turbidity development, especially at  $6.6 \times 10^{-1} M$  NaCl [See Figure 4, Plot (7, 8)]. It can be speculated that veronal, at high concentrations, inhibits the proteolytic enzyme, whereas NaCl merely inhibits

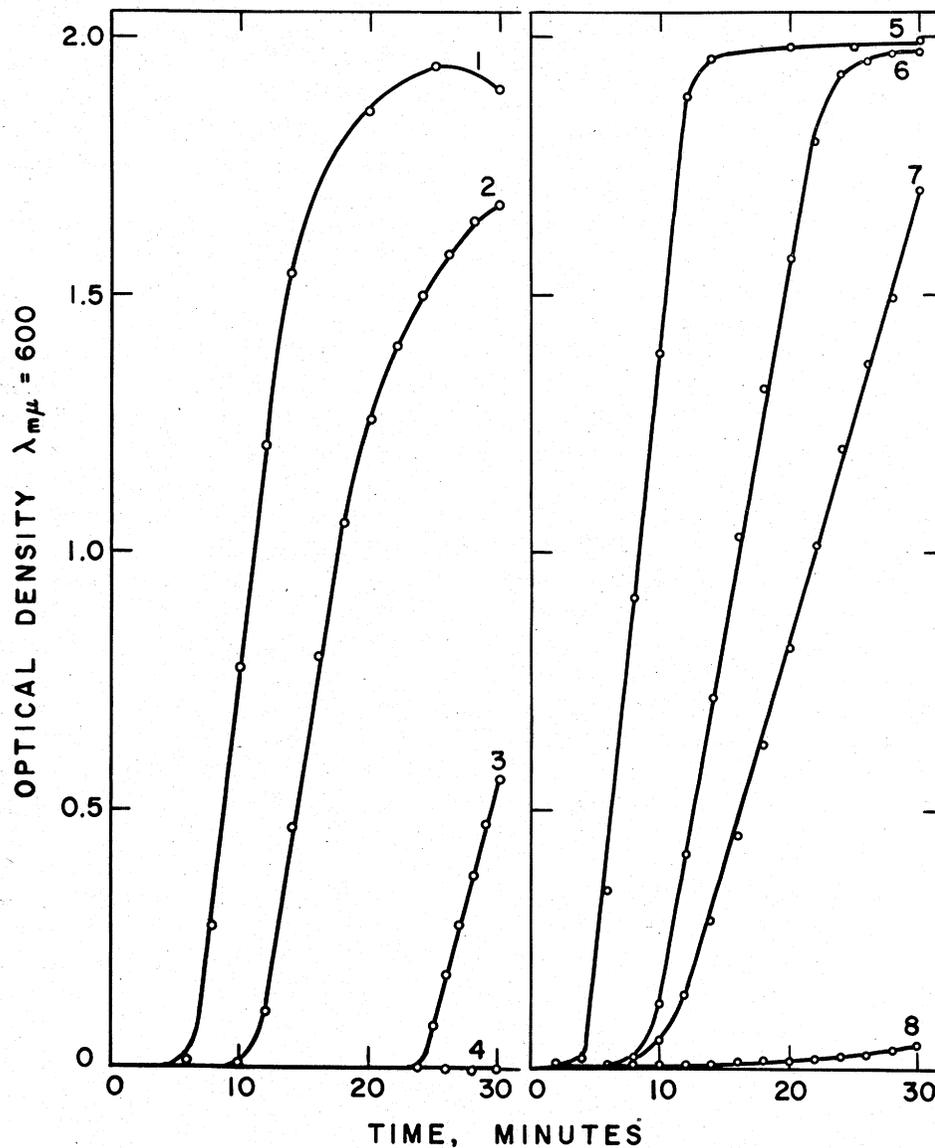


FIG. 4. Effect of varying veronal and NaCl on the reaction of  $\beta$ -casein and snake venom at  $38^{\circ}\text{C}$ . and initial  $\text{pH} = 9.5$ . Substrate-to-enzyme ratio  $\frac{100}{1}$ . (1)  $3.3 \times 10^{-2} M$  veronal slope =  $\frac{0.207 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = -0.2$  (2)  $8.2 \times 10^{-2} M$  veronal slope =  $\frac{0.151 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = 0$  (3)  $1.6 \times 10^{-1} M$  veronal slope =  $\frac{0.094 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = 0$  (4)  $3.3 \times 10^{-1} M$  veronal slope = 0  $\Delta\text{pH} = 0$ . (5)  $3.3 \times 10^{-2} M$  NaCl slope =  $\frac{0.251 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = -0.9$  (6)  $1.6 \times 10^{-1} M$  NaCl slope =  $\frac{0.139 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = -0.60$  (7)  $3.3 \times 10^{-1} M$  NaCl slope =  $\frac{0.088 \text{ O.D. units}}{\text{minutes}}$ ,  $\Delta\text{pH} = -0.7$  (8)  $6.6 \times 10^{-1} M$  NaCl slope  $\cong 0$   $\Delta\text{pH} = 0$ .

the formation of the precipitate. This remains to be investigated further. As further evidence that a proteolytic reaction has taken place, the precipitate was collected by centrifugation, and crudely fractionated into at least three fractions. This was accomplished as follows: The precipitate was dissolved with 0.1 *N* NaOH and immediately dialyzed against water at 4° C. for 48 hr., with one change of the dialysis medium. This procedure resulted in a precipitate which was removed by centrifugation in the cold (ca. 10% of original *N*). The supernatant was allowed to stand at 37° C. under toluene, which resulted in the appearance of a second precipitate which was again separated by centrifugation, this time at room temperature (ca. 2.5% of original *N*). The third fraction was obtained by lyophilization of the supernatant solution (ca. 10-15% of original *N*). It was also found that patterns obtained by moving boundary electrophoresis of the original precipitate indicated a minimum of three fractions.

*Distribution of nitrogen and phosphorus.* Snake venom was allowed to react with  $\beta$ -casein for 30 min., and the precipitate was removed by centrifugation. It was then found that the supernatant contained 70-80% of the original nitrogen of the medium, whereas it contained essentially 100% of the phosphorus. No inorganic phosphorus was formed during the course of the reaction. All the phosphorus of the original  $\beta$ -casein fraction remained organically bound in the soluble fraction. It was also found that upon paper electrophoresis (veronal buffer pH 8.5  $\mu = 0.05$ , 100 V, 15.5 hr.), a component of the supernatant migrated to the positive pole with a greater mobility than  $\beta$ -casein, indicating a greater number of negative charges. Under the same conditions, the precipitated material moved toward the negative electrode.

#### DISCUSSION

The action of various proteolytic enzymes on casein has been extensively studied by Mattenheimer and Nitschmann (11). These workers observed that where casein became clottable by calcium ions, the reaction velocity curves showed two phases, a primary reaction yielding about 2% nonprotein-nitrogen (NPN), very rapidly followed by a general proteolytic breakdown, giving straight curves up to much higher NPN values. The clotting point was always near the end point of the primary reaction. However, when  $\alpha$ -casein and  $\beta$ -casein were compared, it was found that rennin (13) and pepsin (19) act on  $\alpha$ -casein in the specific manner described above, whereas these enzymes hydrolyzed  $\beta$ -casein in a nonspecific manner (straight NPN/time curve). The present study would seem to indicate that the reaction of snake venom on  $\beta$ -casein is generally akin to the action of rennin and pepsin on  $\alpha$ -casein. There is a rapid proteolysis before turbidity can be detected, which is then accompanied by a further increase in proteolysis. The Carlsberg group have advanced a rational two-step theory to explain the clotting of milk by rennin (8). This theory states that the clot is due to precipitation of calcium paracasein after rennin-catalyzed proteolysis of the original casein. In addition, the S-shaped curves obtained are similar to that obtained for the action of rennin on skimmilk (3).

ENZYMIC DEGRADATION OF  $\beta$ -CASEIN

The present reaction is similar in some respects to the action of trypsin on  $\beta$ -casein (15). In the latter case, on short-time incubations, a phosphorus-free fraction was obtained which consisted of 25% of the total nitrogen. Such a fraction was obtained in the present study, under somewhat different conditions, e.g., pH. In addition, the snake venom also showed a definite metal requirement and was inhibited by versene, whereas trypsin is not inhibited by this reagent (4).

REFERENCES

- (1) BALLS, A. K., AND JANSEN, E. F. Stoichiometric Inhibition of Chymotrypsin. *Advances in Enzymol.*, 13: 221. 1952.
- (2) BERRIDGE, N. J. The Second Phase of Rennet Coagulation. *Nature*, 149: 194. 1942.
- (3) CLAESSON, O., AND NITSCHMANN, H. Optical Investigation of the Rennet Clotting of Milk. *Acta Agr. Scand.*, 7: 341. 1957.
- (4) DEUTSCH, H. F., AND DINIZ, C. R. Some Proteolytic Activities of Snake Venom. *J. Biol. Chem.*, 216: 17. 1955.
- (5) FISKE, C. H., AND SUBBAROW, Y. The Colorimetric Determination of Phosphorus. *J. Biol. Chem.*, 66: 375. 1925.
- (6) HIPP, N. J., GROVES, M. L., CUSTER, J. H., AND McMEEKIN, T. L. Separation of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -Casein. *J. Dairy Sci.*, 35: 272. 1952.
- (7) HOFMAN, T. The Action of Phosphatases on Caseins and Low Molecular-Weight Phosphates. *Biochem. J.*, 69: 139. 1958.
- (8) HOLTER, H. Über die Labwirkung. *Biochem. Z.*, 255: 160. 1932.
- (9) KALAN, E. B., AND TELKA, M. The Action of Phosphatases on Casein Fractions. *Arch. Biochem. Biophys.*, 79: 275. 1959.
- (10) MA, T. S., AND ZUAZAGA, G. Micro-Kjeldahl Determination of Nitrogen. *Ind. Eng. Chem., Anal. Ed.*, 14: 280. 1942.
- (11) MATTENHEIMER, H., AND NITSCHMANN, H. Das Lab und Seine Wirkung auf das Casein der Milch VIII. Die Abspaltung von Nicht-Protein-Stickstoff (NPN) aus Casein durch Verschiedene Proteolytische Fermente, Verglichen mit der Abspaltung durch Lab. *Helv. Chim. Acta*, 38: 687. 1955.
- (12) MOORE, S., AND STEIN, W. H. Procedures for the Chromatographic Determination of Amino Acids on Four Per cent Cross-Linked Sulfonated Polystyrene Resins. *J. Biol. Chem.*, 211: 893. 1954.
- (13) NITSCHMANN, H., AND KELLER, W. Das Lab und Seine Wirkung auf das Casein der Milch IX. Über die Abspaltung von Nicht-Protein-Stickstoff (NPN) aus Isoliertem  $\alpha$ - und  $\beta$ -Casein durch Lab. *Helv. Chim. Acta*, 38: 942. 1955.
- (14) PERLMANN, G. E. Phosphodiester Linkages in Proteins. *Biochim. et Biophys. Acta*, 13: 452. 1954.
- (15) PETERSON, R. F., NAUMAN, L. W., AND McMEEKIN, T. L. The Separation and Amino Acid Composition of a Pure Phosphopeptide Prepared from  $\beta$ -Casein by the Action of Trypsin. *J. Am. Chem. Soc.*, 80: 95. 1958.
- (16) SINSHEIMER, R. L., AND KOERNER, J. F. A Purification of Venom Phosphodiesterase. *J. Biol. Chem.*, 198: 293. 1952.
- (17) UMBREIT, W. W., BURRIS, R. H., AND STAUFFER, J. F. p. 165. *Manometric Techniques*. Burgess Publishing Co., Minneapolis, Minn. 1946.
- (18) VENOMS. Ed., Buckley, E. E., and Porges, N. American Association for the Advancement of Science, Washington, D. C. 1956.
- (19) ZITTLE, C. A., AND CERBULIS, J. Clotting of Casein with Pepsin: Amount and Nature of the Soluble Products. *J. Dairy Sci.*, 41: 241. 1958.
- (20) ZITTLE, C. A., AND PEPPER, L. Influence of Hydrogen and Calcium Ion Concentrations, Temperature, and Other Factors on the Rate of Aggregation of Casein. *J. Dairy Sci.*, 41: 1671. 1958.