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ACTIVATION OF PRORENNIN AT LOW pH VALUES

by

NAZAR A. SHUKRI

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INTRODUCTION

Rennin is the main milk-clotting enzyme in the fourth stomach of young calves. This enzyme has been crystallized and studied by several workers such as Hankinson (20), Berridge (1), and Ernstrom (10), and found to be heterogeneous (10). It is a proteolytic enzyme, which exhibits its maximum activity on hemoglobin, bovine serum albumin, and casein in the region of pH 3.4-4.0 (2,6,11,13). It has a specificity similar to, but narrower than, that of pepsin (11).

Proteolytic enzymes of the digestive tract are produced as inactive proteins, presumably to prevent them from attacking surrounding tissues. Rennin is no exception. It is secreted as an inactive precursor or zymogen called prorennin which is transformed into active rennin in the environment of the abomasum.

Conversion of prorennin to rennin is mainly an autocatalytic process at pH 4.4 (4), pH 4.7 (15,28,29), and pH 5.0 (29). Peptide material is split from prorennin during the process of activation (4,15,16). If the activation of prorennin is represented by the following equation:

Prorennin $\xrightarrow{\text{Rennin}}$ Rennin + Peptide material

the assumption might be made that this reaction is proteolytic, since rennin is a proteolytic enzyme (2,6,11,13). If this

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reaction is proteolytic one might expect the pH optimum for activation to coincide with the pH optimum for proteolytic activity. In other words, a maximum rate of activation should occur in the region of pH 3.0-4.0, and on both sides of this range the rate should decrease. There is such agreement above pH 4.0 where the rate of activation decreases as the pH increases to 5.5 and becomes negligible at pH 6.0. However as the pH goes below 4.0 the rate of activation increases in contrast to the proteolytic nature of the enzyme. This contradiction between the proteolytic nature of the enzyme and the rate of activation of prorennin was questioned for the first time by Rand and Ernstrom (29), when they made the following statement: "Rennin exhibits its maximum proteolytic activity in the region of pH 3.0-4.0 (8). Therefore, if the autocatalytic activation of prorennin is proteolytic, it is difficult to explain why the rate of activation increases as the pH is reduced to very low values. Further studies of the activation reaction below pH 3.0 will be necessary to clarify this interesting behavior."

The purpose of this work has been to study the nature of the activation of prorennin at low pH values, and to determine whether or not the reaction is autocatalytic in highly acid solutions.

Rennin is secreted as an inactive precursor or zymogen which is converted to an active protein in the environment of the calf's stomach where it functions. Although this fact has been known since the work of Hammarsten in 1872 (19), Kleiner and Tauber (24) in 1932 were the first to partially isolate this precursor (Prorennin) and study its properties. They demonstrated that it was stable in the region of pH 9.0-10.0 where rennin was rapidly inactivated. Kleiner and Tauber also reported that the rate of conversion of prorennin to rennin increased with acidity to pH 1.0. These workers (25) also found that prorennin had no clotting power, and could be activated completely pH 3.6 within seven minutes. Ege and Lundsteen (9) confirmed the findings that rennin was destroyed in alkaline solutions, and that the rate of activation of prorennin increased as the pH was reduced from 5.0 to 3.0.

Berridge (32) pointed out that there were some indications that the activation of prorennin was a proteolytic process, but that a proper understanding would require activation studies on pure prorennin.

Flotmann (12,14,15) devised a method for purifying prorennin and found that the zymogen was heterogeneous, and could be fractionated into at least two components. He called the

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main fraction prorennin-B, and the minor fraction prorennin-A. After activation, each of the above fractions gave rise to different fractions of rennin. He reported that activation at pH 4.7 was partially autocatalytic, while that at pH 2.0 was similar to a first order reaction. Also, his results showed that activation of prorennin was brought about by hydrolysis of peptide material from the N-terminal end of the prorennin molecule. Foltmann, by applying Sanger's method (31) for determining N-terminal residues, found that alanine was the N-terminal amino acid on prorennin. He also analyzed rennin fractions after activation by the same method, but his results were inconclusive. However, Jirgensons et al. (27) reported that the N-terminal and the C-terminal groups on rennin were glycine and leucine (or isoleucine) respectively. The C-terminal amino acid on prorennin, has not yet been reported.

Bundy et al. (4), described a new method for purifying prorennin and claimed that their zymogen was homogeneous in contrast to the findings of Foltmann (12). These workers based their conclusion on sedimentation analysis and chromatography. Their findings also suggested the autocatalytic nature of the activation of prorennin at pH 4.4, and the release of peptide material during the process. It is noteworthy that the prorennin used in their work was extracted from frozen fresh calf abomasum tissue by acetone at -5°C.

Rand and Ernstrom (28,29) studied the activation of prorennin in the region of pH 2.0-5.5, and demonstrated that activation at pH 5.0 and 4.7 was predominantly autocatalytic. At pH values below 4.7 it was too rapid, even at low temperature, to follow accurately.

Recently, Foltmann (16) reported the amino acid composition of prorennin, rennin, and the peptides released during the process of activation. He found that the amino acid composition of prorennin-B was similar to that of total prorennin. He also found that chromatographically purified B-rennin, resulting from activation of prorennin-B, had almost the same amino acid composition as crystallized rennin. Although prorennin-B was chromatographically homogeneous, the enzyme derived from it was heterogeneous. Foltmann's explanation for this phenomenon was that the activation process probably takes different pathways leading to different enzyme fractions. He was able to account for all the amino acids in prorennin from the amino acid composition of rennin and the peptides released during activation. Table 1 summarizes some of Foltmann's amino acid studies.

TABLE 1. Amino acid composition of prorennin-B, total prorennin, B-rennin, and total rennin (Moles/10 grams of protein) (16).

Amino acid	Prorennin-B	Total prorennin	B-rennin	Total rennin
Lys.....	35.8	35.3	26.5	24.0
His.....	13.8	13.9	13.4	14.1
NH3.....		114.8		113.3
Arg.....	19.4	18.5	15.8	16.7
Asp.....	92.2	92.7	97.7	103.3
Thr.....	56.4	57.2	61.0	61.8
Ser.....	84.4	80.6	86.9	80.1
Glu.....	100.3	101.5	94.3	93.3
Pro.....	38.6	41.0	39.2	43.7
Gly.....	78.7	77.7	79.8	75.4
Ala.....	41.5	41.7	41.8	41.1
Cys.....	14.0	13.3	15.9	12.3
Val.....	63.0	57.5	68.1	66.3
Met.....	18.7	18.9	21.7	21.4
Ileu....	52.6	50.5	49.4	50.6
Leu.....	70.6	70.5	60.4	64.0
Tyr.....	50.7	52.2	50.1	50.8
Phe.....	45.5	45.2	44.9	45.0
Try.....	11.2	11.2	13.6	12.4
Amide...	94	94	100	100

Djurtoft et al. (7) determined the molecular weight of prorennin and rennin by gel filtration on a column of sephadex G-100, and by sedimentation-diffusion. They reported that the molecular weight of prorennin and rennin were approximately 36,000 and 31,000 respectively. The most interesting point in this study that these workers did not observe heterogeneity by either of the two methods (gel filtration and sedimentation).

EXPERIMENTAL METHODS

Purification of prorennin: Partially purified prorennin
was prepared by a modification of Foltmann's method (12).
The procedure was as follows:

1 - Five unsalted dry-blown calf stomachs that had been
    stored at -23C. were ground and macerated in two
    liters of 0.1M sodium borate containing 10% sodium
    chloride. The mixture was agitated over night at
    3C. with a slow-speed mechanical stirrer. The un-
    dissolved materials were separated by centrifuging
    at 15 C. in an International centrifuge at 5,000 g
    for 30 minutes.

2 - The pH of the extract was adjusted at 7.0 with 3N
    HCl, and potassium aluminum sulfate $(Al_2(SO_4)_3K_2$
    $SO_4.24H_2O)$ was added at the rate of 10 grams per
    liter of solution on an anhydrous basis. The acidic
    salt was neutralized immediately to pH 6.3 with
    saturated disodium phosphate (Na_2HPO_4), and the gel
    which formed was removed by centrifugation.

3 - The supernatant from step 2 was adjusted to pH 8.0
    with 3N NaOH, saturated with sodium chloride, and
    left over night at 3C. The pH was adjusted to 7.0
    with 3N HCl and the enzyme was precipitated by
    centrifuging at 15C. for two hours at 5,000 g. The
    precipitate was redissolved in a minimum of 0.1M
    borate buffer at pH 8.0.

4 - After the solution was filtered, step 3 was repeated.

5 - To the supernatant from the last step, potassium
    aluminum sulfate $(Al_2(SO_4)_3 K_2SO_4.24H_2O)$ was added
    at the rate of 5 grams per liter, on an anhydrous
    basis, and neutralized immediately to pH 7.0 with
    saturated disodium phosphate. The gel was removed
    by centrifuging for 30 minutes at the same tempera-
    ture and speed as in previous steps.

6 - The supernatant resulting from step 5 was dialyzed
    against 0.04M borate buffer at pH 8.0 for 96 hours.
    During this period the buffer was changed three times.

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The dialyzed supernatant was freeze dried, and the prorennin powder stored in the refrigerator at 1C.

The final preparation which was used during this study had a specific activity of 190 prorennin units (PU) per mg. N. and less than 0.4% of its potential activity was active rennin.

Determination of milk-clotting activity: Activity was measured on a substrate suggested by Berridge (2,3), using a method described by Ernstrom (10) in which solutions of unknown activity were compared with a standard rennet. The reference was maintained at a constant strength throughout this study. The substrate mentioned above was made by dissolving 120 grams low-heat nonfat dry milk in one liter 0.01M $CaCl_2$. During the course of this study the same lot of nonfat dry milk, stored at -23C., was used for preparation of the substrate.

Nitrogen determination: The nitrogen content of pro-rennin solutions was measured by a semimicro Kjeldahl method (22).

Buffer: Phosphate buffer at a concentration of 0.025M (pH 6.3) was used for diluting activation mixtures for the milk-clotting test. This buffer was prepared by dissolving 0.85 gram Na_2HPO_4 and 2.62 grams $NaH_2PO_4.H_2O$ in one liter distilled water.

Activation mixtures: Acetate buffer at a concentration of 0.02M was used as an enzyme-activating medium for experi-

ments carried out at pH 5.0, 4.0, and 3.5. Activation at pH 2.0 was carried out in 0.51N lactic acid. When glycerol was required, the desired glycerol-water solution was prepared and used as a solvent for preparing the activation mixture.

Throughout this work and for each individual experiment, approximately 70 mg. partially purified prorennin were dissolved in 15 ml. of the proper activation solution. From the activation mixture, one ml. aliquot samples were withdrawn and diluted for the milk-clotting test. The activity, expressed as rennin units per ml., was plotted against time.

RESULTS

Preliminary experiments were concerned with reducing
the rate of activation of prorennin at pH 4.0 and lower in
order to study the kinetics of activation more conveniently.
Activation at low temperature was first attempted as a mean
of slowing the reaction. A series of experiments at pH 2.0
were conducted at 1 C in a controlled-temperature water bath.
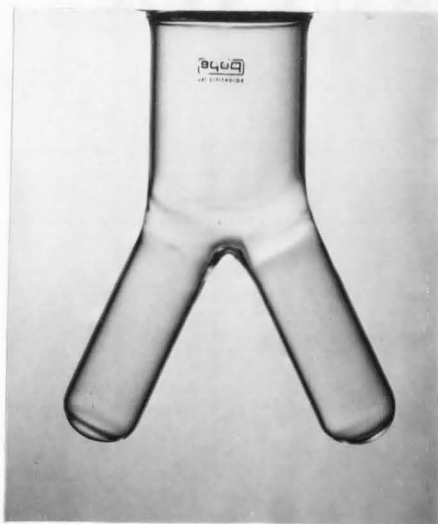Special glassware, as shown in Figure 1, was used in which

Figure 1. The glassware used for instant mixing of pro-
rennin and activation solution at low temperature.

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the activation solution and prorennin powder could be kept separate and instantly mixed at the proper time. The activation solution in these experiments consisted of a lactic acid solution of a concentration such that when mixed with the enzyme the pH was 2.0. After the enzyme and the activation solution were brought to the required temperature, they were mixed by tipping the apparatus 90 degrees without taking it out of the water bath. Samples were withdrawn at proper intervals for activity measurement. It was found that activation at 1 C was much too rapid to follow accurately.

An attempt was then made to carry out the activation below 0 C. An anti-freeze was sought that would reduce the freezing point of the activation mixture without interfering in any way with the activation reaction. Ethylene glycol was used at the rate of 2 ml per 9 ml distilled water. These experiments were carried out as previously described. An anti-freeze was also used in the water bath to protect it from freezing.

At that temperature a significant reduction in the rate of prorennin activation was noted, and development of maximum activity required three hours. Actually, it may have been possible to carry out the proposed experiments under these conditions. Nevertheless, it seemed desirable to find a more convenient procedure, particularly in view of difficulties encountered in sampling and maintaining the temperature of

samples during transfer and dilution. Another attempt was
made to run a series of experiments in the ice cream hardening
room at -23 C. In this room it was possible to temper every
piece of glassware to the proper temperature without fear of
temperature change. An activation solution was prepared by
mixing equal amounts of water and ethylene glycol and ad-
justing it to pH 2.0 with lactic acid. A control solution
at the same pH in the absence of ethylene glycol reached
maximum activity within 30 minutes at room temperature. How-
ever, at -23 C there was no significant activity even after
75 minutes. After 20 hours the activity was only 25% of the
potential activity of the sample. The activation mixture
was removed from the hardening room and left at room tempera-
ture for 30 minutes. No further increase in activity was
noted. It appeared that ethylene glycol may have had a
destructive effect on the enzyme, although this was not
verified.

The necessity of working below the freezing point of
water required a soluble compound that would be an effective
anti-freeze, but would not interfere with the activation of
prorennin or be destructive to the activated enzyme. It was
found that glycerol was not only an effective anti-freeze,
but that it was able to significantly reduce the rate of
activation at 25 C. Therefore, the presence of glycerol in
activation mixtures would make it possible to carry out low

pH activation studies at room temperature, provided it could be shown that glycerol did not affect the activation reaction in any way other than to reduce its rate.

Effect of glycerol on activation of prorennin: Desired amounts of glycerol were mixed with acetate buffers or distilled water on a volume basis, and the mixture adjusted to the required pH. These activation solutions along with enzyme samples were tempered to 25 C in a water bath. Activation and activity tests were carried out as previously described. Activity was calculated as prorennin units (PU) per milliliter when compared to a standard rennet having a value of 100 RU/ml (10). Prorennin units (PU) and rennin units (RU) were considered equivalent.

Activation of prorennin at pH 5.0 was known to be autocatalytic (29). Therefore the effect of glycerol on activation was first studied at that pH. Figure 2 shows activation curves for prorennin at pH 5.0 in the presence of 0 and 20% glycerol v/v. An S shaped activation curve, characteristic of autocatalytic activation, was obtained in each case. Maximum activities recovered from the two samples were very close. However, a remarkable difference in the rate of activation was clearly evident. Maximum activities were recovered after 60 and 78 hour for samples containing 0 and 20% glycerol v/v respectively.

Applying the equation proposed by Herriott (21) for

Figure 2. Activation of prorennin, in 0 and 20% glycerol

v/v, at pH 5.0 and 25 C.

2.1
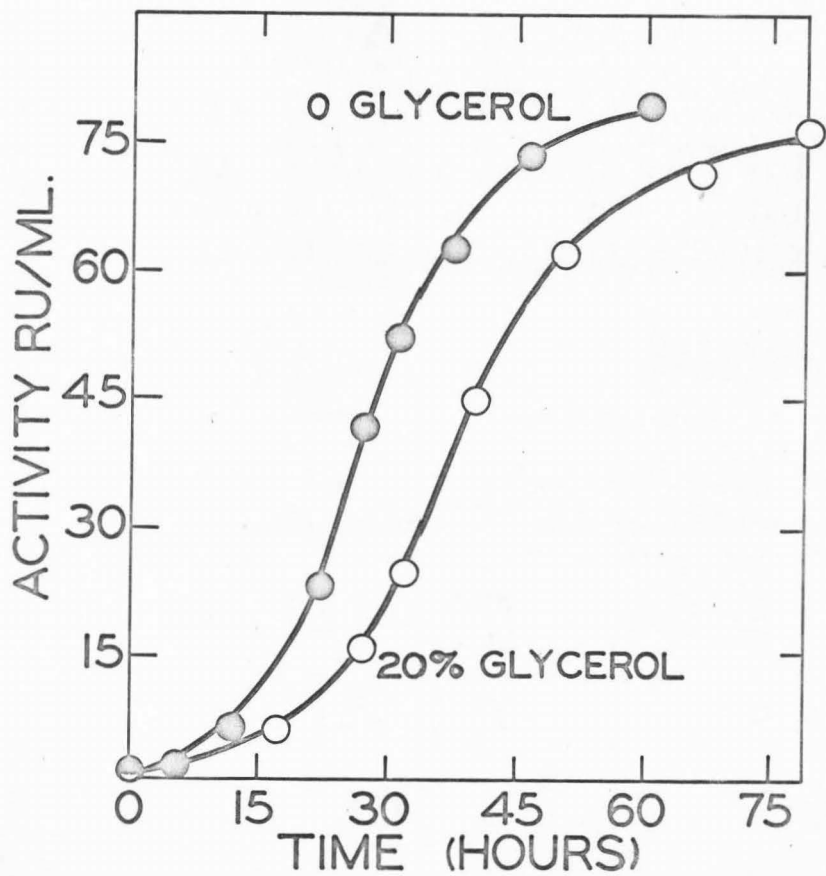
testing the autocatalysis theory of pepsinogen, straight
lines resulted for both samples as given in Figure 3. Auto-
catalytic activation occurred in the presence or absence of
glycerol at pH 5.0, and glycerol appeared to have no effect
other than to reduce the rate of activation.

Curves showing the activation of prorennin at pH 2.0
in 45, 50, and 55% glycerol v/v are found in Figure 4. The
effect of glycerol on reducing the rate of activation was
proportional to its concentration in the activation mixture.
Maximum activity was achieved in four hours in 45% glycerol,
about nine hours in 50% glycerol, and about 16 hours in 55%
glycerol. Again, glycerol seemed to have no noticeable
affect on the nature of the activation or on the yield of
active rennin.

Low pH activation in the presence of glycerol: Acetate
buffers at concentration of 0.02M were used as activating
solutions in the region of pH 3.5-5.0, and 0.51N lactic acid
was used at pH 2.0.

At pH 4.0 in the presence of 45% glycerol the activation
curve had only a faint S shape near its beginning where the
process tended to go rather fast (Figure 6). Evidence of
some autocatalysis was still present, and a straight line,
with some deviation at the beginning, was obtained by applying
Herriott's equation (Figure 7). Activation of prorennin at
pH 4.0 seemed to be border-line between the predominantly

Figure 3. A plot of $\ln \dfrac{A}{Ae - A}$ * vs time for the activation

of prorennin at pH 5.0 and 25 C.

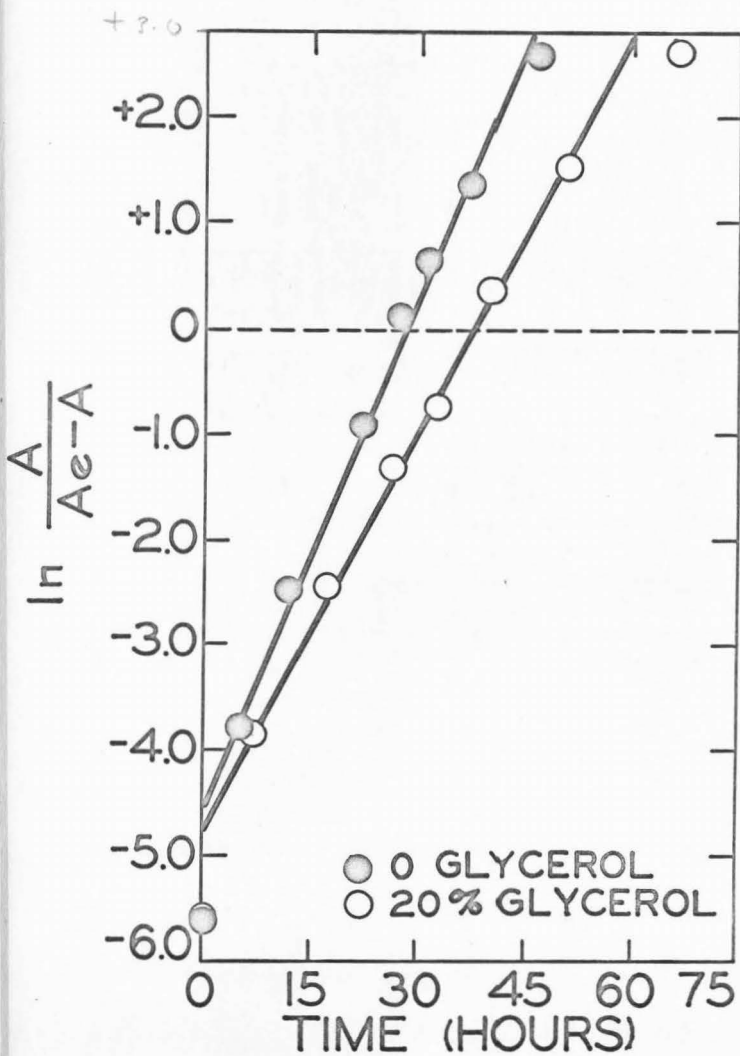*A = activity at time t
Ae = final or equilibrium activity

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Figure 4. Activation of prorennin at pH 2.0 and 25 C, in
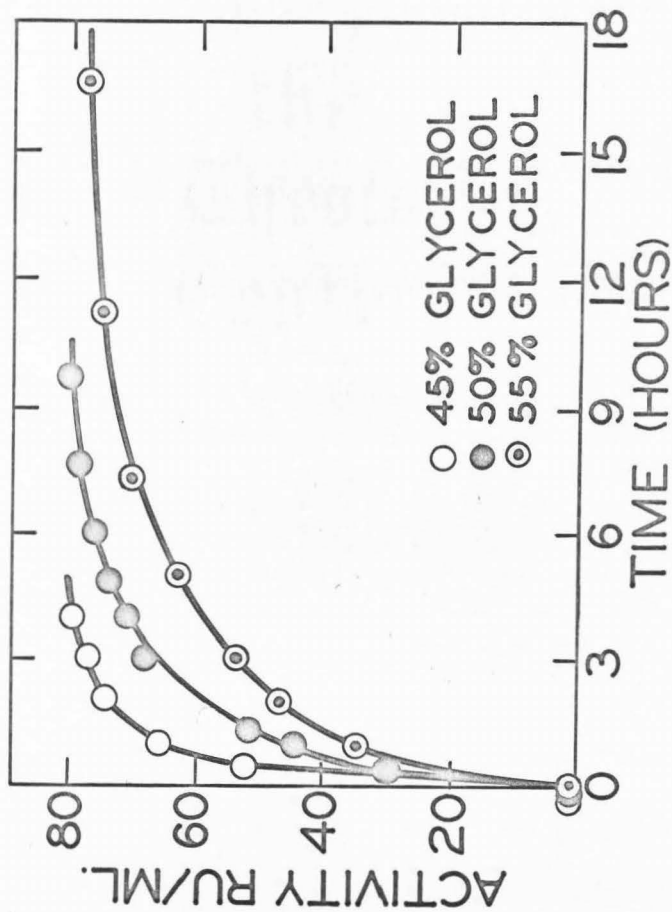the presence of different amounts of glycerol.

Figure 5. A plot of log substrate (prorennin concentration) vs time for the activation of prorennin at pH 2.0 and 25 C.
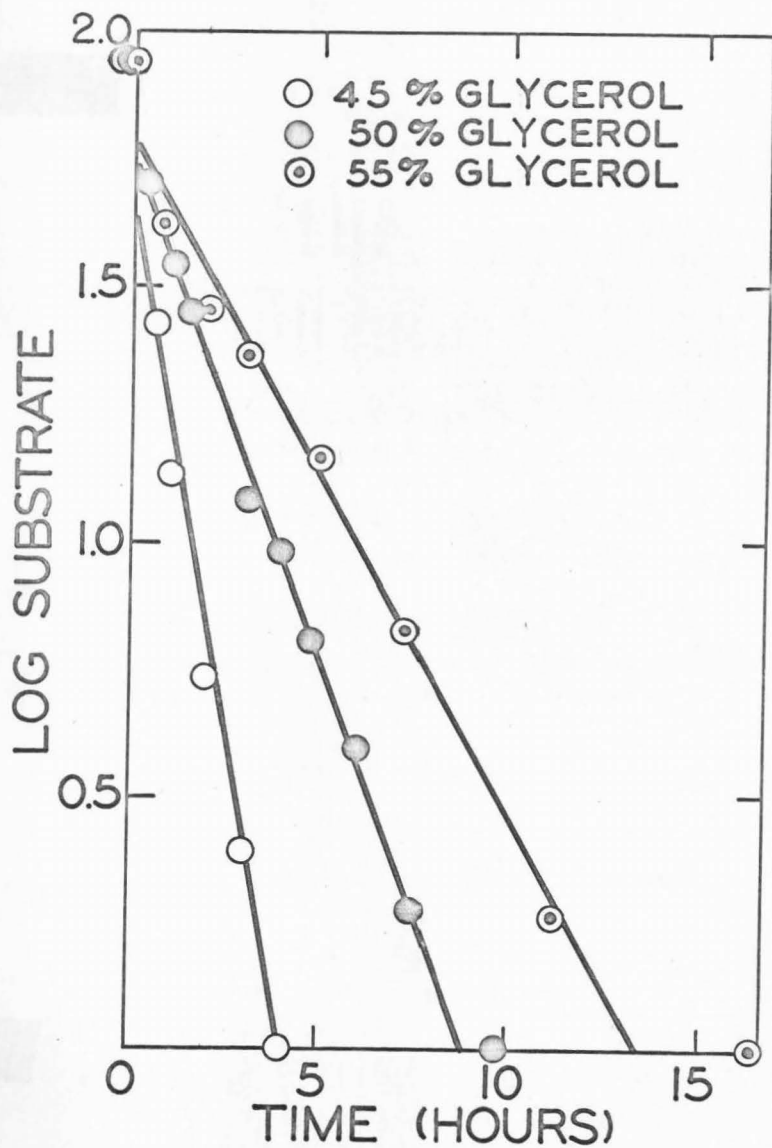
Figure 6. Activation of prorennin in 45% glycerol v/v, at pH 4.0 and 25 C.
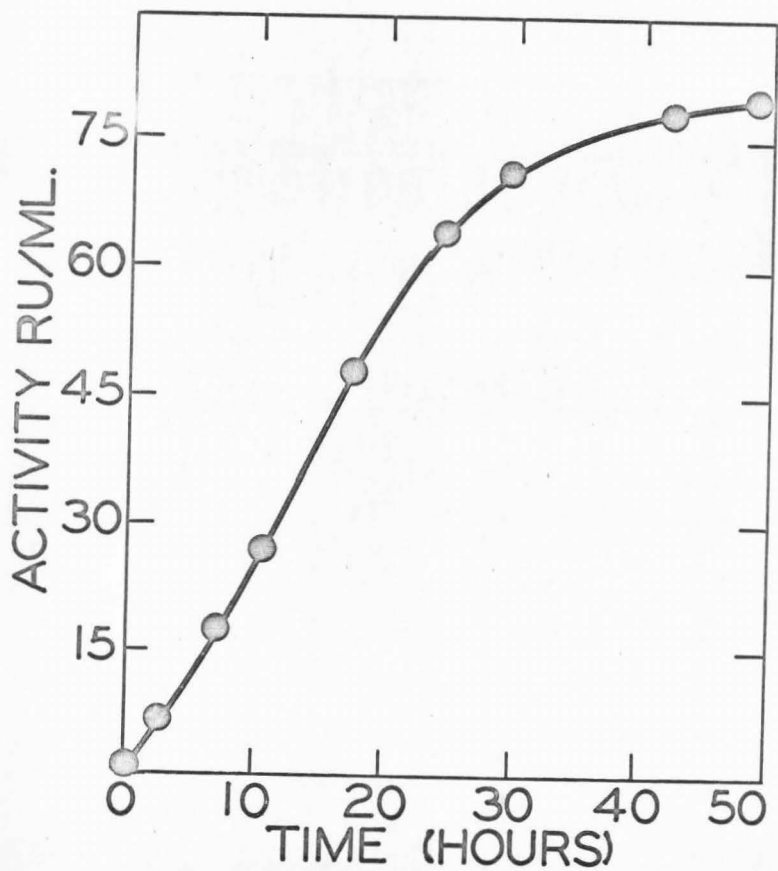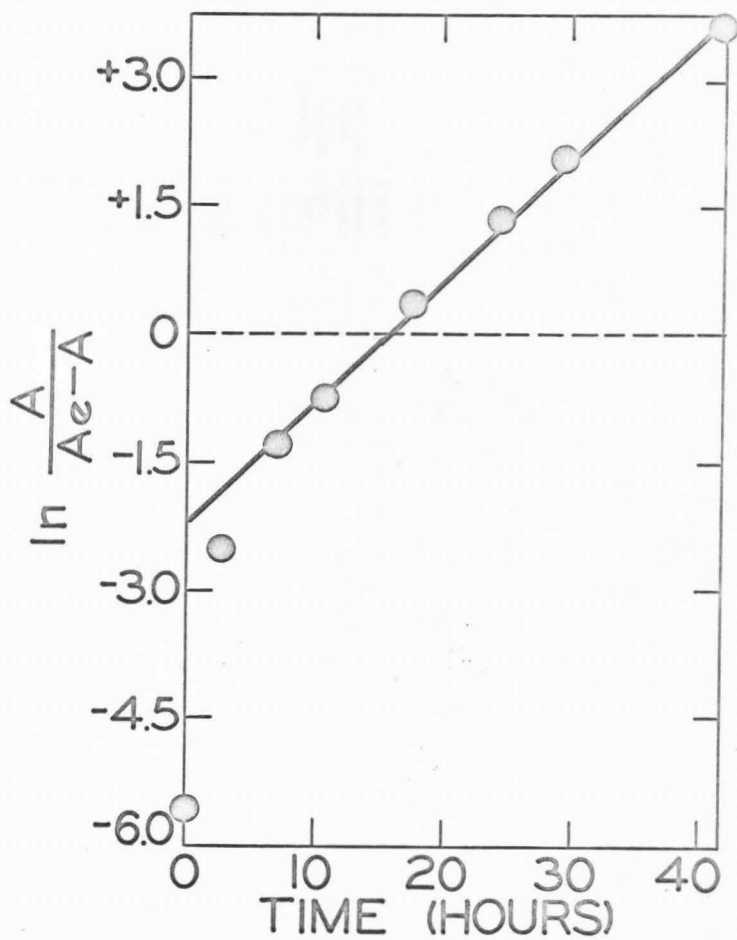
3.1

Figure 7. A plot of $\ln \dfrac{A}{Ae - A}$ * vs time for the activation
of prorennin in 45% glycerol, at pH 4.0 and 25 C.

*A = activity at time t
Ae = final or equilibrium activity

[3 1]

autocatalytic reaction that occurred at higher pH values
and that which approached a first order reaction at lower
pH values.

Figure 8 represents the activation of prorennin at pH
3.5 in the presence of 45% glycerol v/v. At that pH there
was no more evidence of autocatalysis, and the activation
appeared to follow first order kinetics (5). Plotting the
logarithm of substrate concentration (prorennin) against
time, a straight line was obtained, as given in Figure 9.
At pH 2.0 three different levels of glycerol were used: 45%,
50%, and 55%. Again the reaction appeared to follow the
first order kinetics, and its reaction rate decreased with
increasing glycerol concentration as shown in Figures 4
and 5.

Figure 8.   Activation of prorennin in 45% glycerol v/v, at
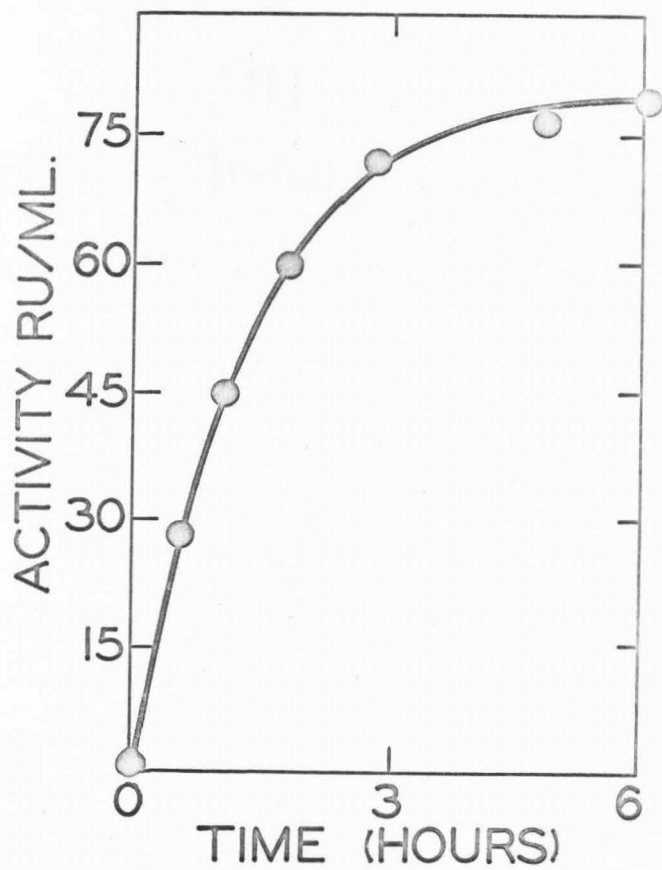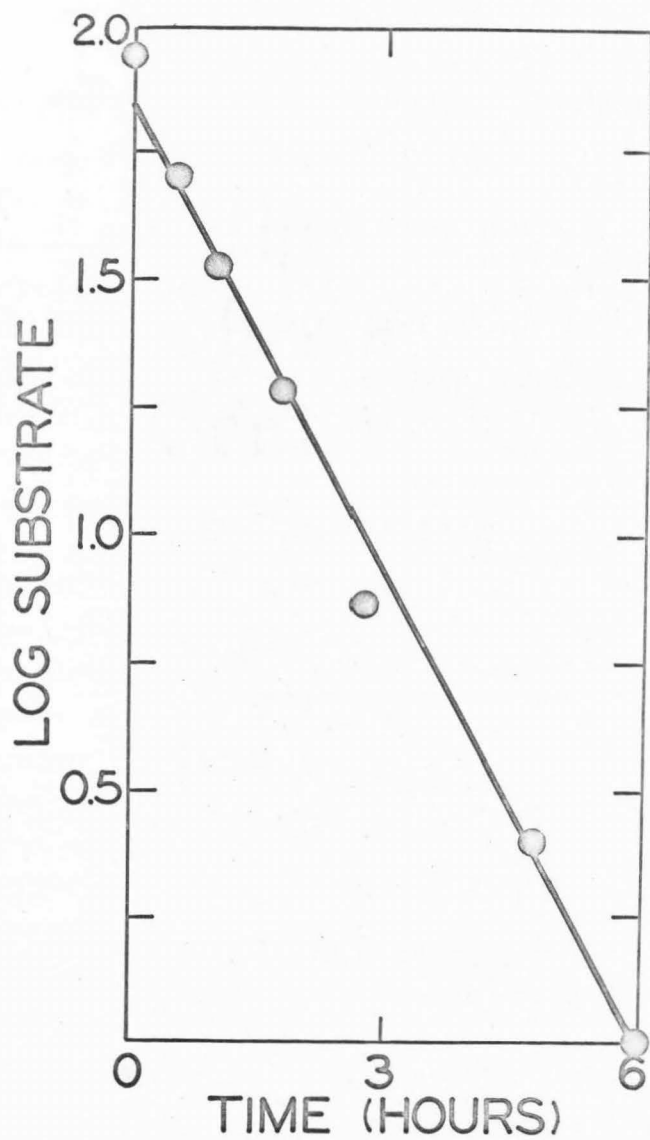pH 3.5 and 25 C.

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Figure 9. A plot of log substrate (prorennin concentration)
vs time for the activation of prorennin in 45%
glycerol, at pH 3.5 and 25 C.

DISCUSSION

Activation of prorennin at low pH values is very rapid, and the reaction is difficult to follow accurately. The discovery of the glycerol effect on the rate of activation made it possible to accomplish this work with a high degree of accuracy. Activation of prorennin in the presence of glycerol revealed the possibility that two different mechanisms may be involved. Autocatalytic activation at pH 5.0 was verified. It was also demonstrated that the activation followed first order kinetics at pH 3.5 and below.

The effect of glycerol on the rate of activation was remarkable. It was thought that this effect may have been due to an increase in viscosity of the activation mixture introduced by glycerol. This was ruled out when sucrose solutions, having the same viscosity, did not produce the same effect. It has long been known that some substances such as $(NH_4)_2SO_4$ and sucrose have the ability to stabilize proteins against denaturation (26,33). These substances can modify the structure of solvents, which in turn interfere with interaction between a solvent and macromolecules, and indirectly change the conformation and behavior of large molecules. It could be that the effect of glycerol on pro-rennin activation is due to a similar stabilizing effect.

Rice et al. (30), in a comparative study of the papain-digestion of native human serum albumin, and of the same protein after denaturation by heat and urea, observed some interesting effects of sodium caprylate and some other sodium salts. It is well established that proteins become more susceptible to enzyme hydrolysis after denaturation. The presence of these salts stabilize the protein during treatment with heat and urea such that the rate of subsequent hydrolysis is not appreciably different from that of native protein. This also could be the case with activation of prorennin in the presence of glycerol. Glycerol has been used as a rennin preservative, and as a solvent for rennin extraction (8).

Two possible mechanisms for the activation of prorennin open interesting possibility for further investigation. This should be directed toward a determination and identification of the peptide materials released during activation at different pH values. If different activation mechanisms are involved, there may be differences between peptides set free during autocatalytic activation and those liberated during first order activation.

Recently, Foltmann (16) reported the amino acid composition of peptide material liberated during activation of prorennin at pH 2.0, but he did not identify the peptide bonds involved. He stated that his results sustained his

assumption that transformation of prorennin into rennin took place through limited proteolysis. However, this assumption was based only on peptide liberation experiments at pH 2.0. Since the activation reaction at pH 2.0 appears to be different from that at pH 5.0, it may be that his conclusions should be restricted to activation at pH 2.0 and not extended to the overall process. This does not rule out the possibility that there are no differences in the structural changes of prorennin during autocatalytic activation and first order activation, but one should oppose any conclusion based on experimental work done at one pH value.

The assumption was made in the introduction of this paper that activation of prorennin is proteolytic. This was based on the fact that rennin is a proteolytic enzyme (2,6,11,13). Nevertheless, such an assumption resulted in a contradiction between the known proteolytic pH optimum of the enzyme and its rate of activation at low pH values. This contradiction could be accepted as fact if rennin acted optimally only in the region of pH 3.0-4.0. However, the literature tells of several enzymes that act optimally at more than one pH value (18). Pepsin acts optimally on protein substrates at pH 2.0 and on synthetic substrates at pH 4.0 (17). Papain (23) is similar to pepsin in that it shows different pH optima depending on the nature of the substrate. From this it is possible to speculate that rennin may have pH optima that

are substrate dependent. On the other hand the possibility exists that under some circumstances, activation of prorennin may not be proteolytic, but may result from the splitting of an ester-type linkage.

CONCLUSIONS

1. Glycerol has the ability to reduce the rate of activation of prorennin.

2. Glycerol exhibited its effect from pH 5.0 to pH 2.0.

3. The effect of glycerol increased with increasing concentration in the activation mixture.

4. Glycerol did not appear to have any effect other than to reduce the rate of activation.

5. Activation of prorennin in glycerol is autocatalytic at pH 5.0, but follows first order kinetics at pH 3.5 and below.

6. The borderline between the two reactions seemed to occur near pH 4.0.

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