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ENZYME DEGRADATION DURING SHEARING AND FOAM FORMATION

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemical Engineering

1983

154-5450

ABSTRACT

ENZYME DEGRADATION DURING SHEARING AND FOAM FORMATION

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In order to examine the mechanism of enzyme degradation in a shear field, rennet solutions were subjected to turbulent shear in a stirred tank. The results suggest that shear caused little or no loss of activity and that the formation of foam in the stirring process was the more likely cause of activity loss. Addition of an antifoaming agent (polypropylene glycol) to the tank during stirring resulted in no detectable loss of activity. Conversely, foaming caused by sparging (without stirring) with air or nitrogen resulted in as much as 95% loss. It is suggested that activity loss occurred during the process of bubble formation.

ACKNOWLEDGMENTS

I would like to thank Dr. Donald Anderson for all his support and guidance during my research.

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TABLE OF NOMENCLATURE

<u>Symbol</u>	<u>Definition</u>
c	Concentration
c_o	Initial concentration
K_1	Constant
M	Molar concentration
t	Exposure time in stirred tank
t_c	Coagulation time
t_o	Delay time

INTRODUCTION

In 1970, Charm and Wong³ showed that the enzymes catalase, carboxypeptidase, and rennet lost activity when sheared in a coaxial cylinder viscometer. They attributed the loss of activity to the shearing alone. They also found that when shearing was stopped, rennet regained much of its activity over a period of 100 minutes. Tirrell and Middleman⁸ found that urease lost activity when sheared during the hydrolysis of urea and that some of the damage was reversible. However, in 1979, Thomas and Dunhill⁷ studied catalase and urease in a sealed couette viscometer using the same shear rates as Charm and Wong and found no significant loss of activity of the enzymes over extended periods of time. When they repeated the same experiments in an open viscometer, the enzymes lost activity not only when sheared, but also under zero shear. In neither case was the activity loss as great as reported by Charm and Wong. Thomas and Dunhill suggested that enzyme degradation thought to be caused by shearing may in fact, be a result of other factors such as:⁷ 1) pressure, 2) heat denaturation, 3) gas-liquid surface denaturation caused by oxidation, 4) cavitation, and 5) metal contamination. In an open system, denaturation by oxidation and foaming are especially important to take into consideration.

EXPERIMENTAL METHODS

The coagulation of milk by rennet is a two step process. Milk contains micelles which are stabilized by k-casein. In the first step the k-casein is hydrolysed, leaving the micelles unstable. The casein micelles aggregate in the second step to form a coagulum.⁶ Several studies have shown that coagulation time varies with pH, temperature, and the type of milk used.^{2,4}

Kopelman and Cogan⁵ used the sudden increase in viscosity at the onset of coagulation as an indicator of coagulation time. This time is correlated with enzyme concentration and used as an assay. Viscosity was followed continuously, using a Brookfield viscometer, by Kopelman and Cogan and in this work. A small sample adaptor was used here.

Rennet solutions* contained 4% of a 1 M acetate buffer (pH 5.7) to keep coagulation times in a reasonable range and were maintained at 14°C. Reconstituted milk was made with 16% skim milk powder in 0.01 M CaCl₂ solution. The milk was gently shaken for 15 minutes and heated to 40°C in a water bath for 10 minutes prior to each assay. The reaction was started by adding 0.3 ml of enzyme solution to 16 ml of heated milk in a test tube. The milk-enzyme solution was gently mixed and quickly added to the viscometer which was maintained at 40°C in a water bath. The output from the viscometer was continuously recorded on a strip

* Rennet was obtained from the Sigma Chemical Company.

chart recorder. At the onset of coagulation, a sharp increase in viscosity is observed. Coagulation time, t_c , was found by extending the tangent to the viscosity curve back to the base line (see Figure 1). The intersection of the base line and the tangent is defined as t_c .

Kopelman and Cogan⁵ found that coagulation time decreased with increasing rennet concentration and coagulation times were correlated by the relationship:

$$t_c = t_o + K_1/c$$

t_c : coagulation time

t_o : delay time characteristic of the system

K_1 : constant

c : percent enzyme concentration in enzyme solution

In this work, concentrations from 0.05% to 0.7% were assayed and a calibration plot of data for known concentrations is shown in Figure 2.

APPARATUS

Stirred Tank

The stirred tank used to shear rennet solutions is shown in Figure 3. The tank was designed following Standard Tank Configurations¹ often used in industry: a) fluid depth equal to the tank diameter, b) impeller diameter equal to one third of the tank diameter, c) impeller distance from the bottom equal to one third of the tank diameter, d) impeller blade width equal to one fifth of the impeller diameter. The impeller, as shown in Figure 3 has six blades, each 0.56 centimeters in length. The temperature of the enzyme solution was maintained by circulating water at 14°C through the tank jacket. A cover was used to prevent spillage from the tank.

METHODS AND RESULTS

Shearing in Stirred Tank

The stirred tank was filled with 450 ml of distilled water containing 4% of 1 M acetate buffer. Two enzyme concentrations were used for this test: a) 0.55% and b) 0.67%. The enzyme solution was cooled to 14°C before stirring. For each concentration separate tests were run at impeller speeds of 2500, 2750, and 3000 RPM. Samples were taken intermittently over a 100 minute period. For each sample, Figure 2 was used to find the apparent concentration from the recorded coagulation time. This concentration was compared with the initial concentration to find the percent loss of activity for the solution. The results for the two concentrations are shown in Figures 4 and 5. In all cases coagulation time increased with the length of time in the stirred tank. As the impeller speed was increased, more activity was lost at corresponding times in the tank. For example, from Figure 4 it can be seen that the solution had 67% of the initial activity left after 50 minutes in the tank at 2500 RPM, while at 3000 RPM only 32% of the activity was left. It is interesting to note that the lower concentration lost more activity at corresponding times in the tank and at all impeller speeds, than the larger concentration. In all experiments a stable foam developed almost immediately after shearing began. There was noticeably more foam at higher speeds and with the larger enzyme concentration.

Shearing in Stirred Tank with Antifoam

Polypropylene glycol, an antifoaming agent, was added to a 0.55% rennet solution and sheared at 3000 RPM in the stirred tank. Four concentrations of antifoam were tested, taking samples over a 100 minute period. Figure 6 shows that with an antifoam concentration of $5.56 \times 10^{-5}\%$, no significant activity was lost after 70 minutes of shearing. Very little foam developed at this antifoam concentration and the foam collapsed within a few seconds when the impeller was stopped to take samples. As the polypropylene glycol concentration was decreased, more foam developed while shearing and more activity was lost.

Foaming of Rennet Solutions Without Shear

A sparger was used to foam rennet solutions to find the effect of foaming alone on the activity. A 50 ml solution with 0.55% rennet was sparged in a graduated cylinder until 21 ml of the liquid was foamed. While sparging, samples were taken of the foam and the liquid. After sparging was stopped, another sample was taken of the liquid including the collapsed foam. These samples were assayed and compared to an initial sample taken before sparging. The results, given in Table 1, show that 50% of the initial activity was gone from the sample taken after sparging. The samples taken during the foaming process indicate that the foam had a significantly higher activity than the liquid. A small amount of brown precipitate was observed in the foam.

A second test was performed in which an enzyme solution ($c=0.55\%$) was sparged for a period of 15.8 minutes. Three

foam samples were taken while sparging, as well as a liquid sample after sparging was stopped. As Table 2 shows, the final solution had only 5.1% of the original activity.

The sparging experiments were repeated using nitrogen rather than air. In the first experiment 50 ml of a 0.55% rennet solution was sparged until 26 ml of the liquid was foamed. Samples were taken of the liquid before sparging, the foam and liquid while sparging, and the liquid after sparging. Table 3 shows that, as before, the rennet was more concentrated in the foam. The liquid after foaming lost 40% of the initial activity. Table 4 shows the results when a rennet solution was sparged for 5 minutes. The liquid after sparging lost 70% of the initial activity.

DISCUSSION

Activity loss was found in the shearing and foaming experiments. In both cases, the loss was related to the amount of foam formed as well as the duration of the foaming process. As found by Thomas and Dunhill⁷, the enzyme proved to be more shear resistant at the higher enzyme concentration. When antifoam was added to the stirred tank, the enzyme damage was reduced or eliminated.

That activity was lost in the sparging experiments without shear, suggests that factors other than shear may be responsible for the enzyme damage in these experiments and in previous work. The experimental results in Table 1 indicate that the rennet concentration in the foam was much higher than in the liquid being sparged. This was also shown in the stirred tank by stirring a 0.387% rennet solution at 2500 RPM for 50 minutes. Samples were taken of the foam and liquid. The rennet concentration in the foam was 0.20% while the concentration in the liquid was 0.097%. The brown precipitate found in the foam in both the stirred tank and the sparger experiments is further evidence that the rennet concentrated at the surface of the liquid where foaming occurred.

When an antifoaming agent, polypropylene glycol was added to the stirred tank before shearing, there was a noticeable decrease in foam development. There was a correlation between activity loss and the amount of foam that developed from stirring. Whether the antifoam protected the

enzyme by preventing the formation of foam or by some other means is not known.

In order to check whether enzyme damage occurred at the time of foam development or with time in the foam phase, a 0.55% rennet solution was sheared in the stirred tank at 3000 RPM for 30 minutes. After stirring, two samples were taken of the foam. One sample was collapsed immediately with polypropylene glycol and assayed. The other sample was allowed to sit for two hours in an open container before it was collapsed and assayed. The apparent rennet concentrations for the two samples were essentially the same suggesting that enzyme damage occurred during the formation of the foam. This is consistent with previous studies of denaturation of plasma-lipoproteins in bubble or film oxygenation of dog blood plasma.⁹

The sparging experiments with nitrogen were done to determine if enzyme denaturation was caused by oxidation or the foaming process itself. As shown in Table 4, a rennet solution lost 70% of the initial activity when sparged with nitrogen for 5 minutes suggesting that the foaming process is responsible for enzyme damage.

In conclusion, this study suggests that previous reports of damage to enzymes when subjected to shear may be incorrect and that much, if not all, of the damage likely occurred at forming gas-liquid interfaces independent of the presence of shear.

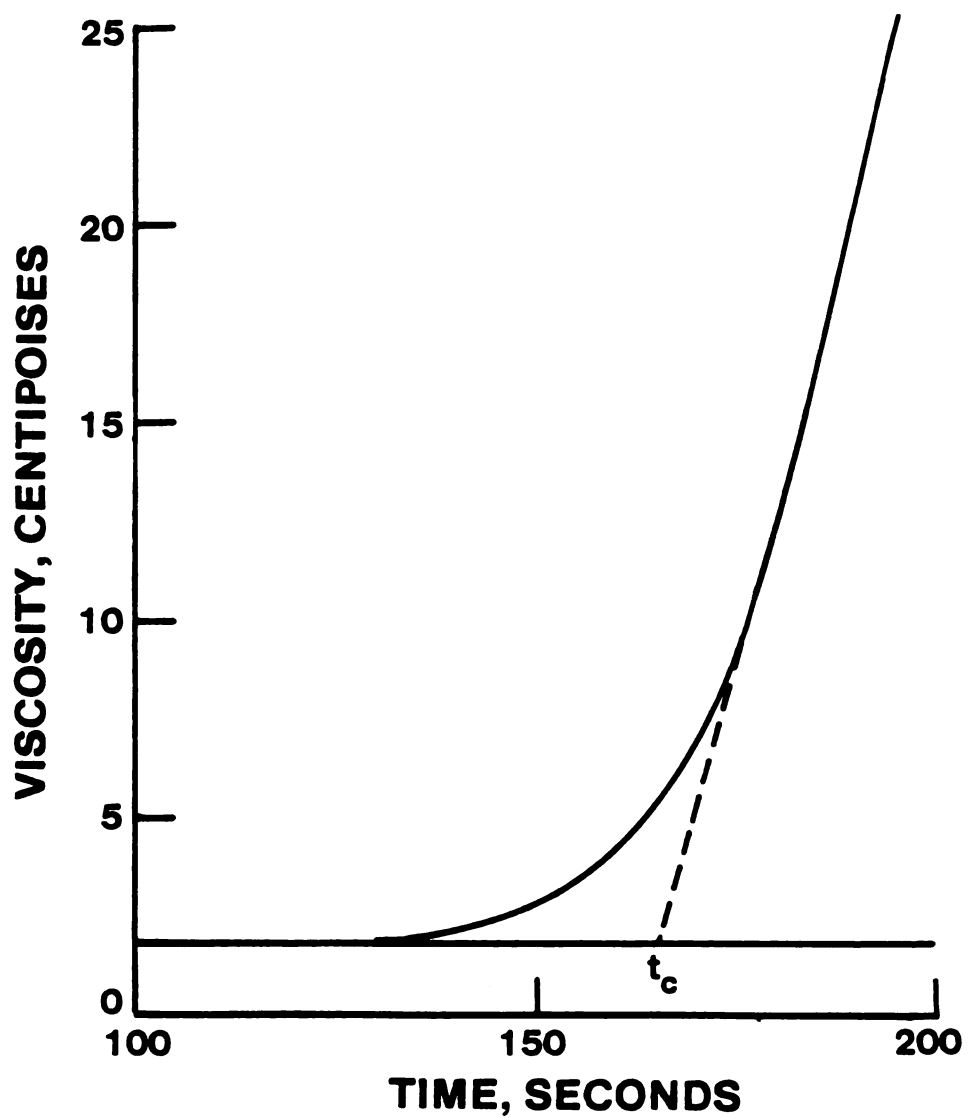


Figure 1. Viscosity of Milk-Rennet Solution as a Function of Time During Assay Procedure

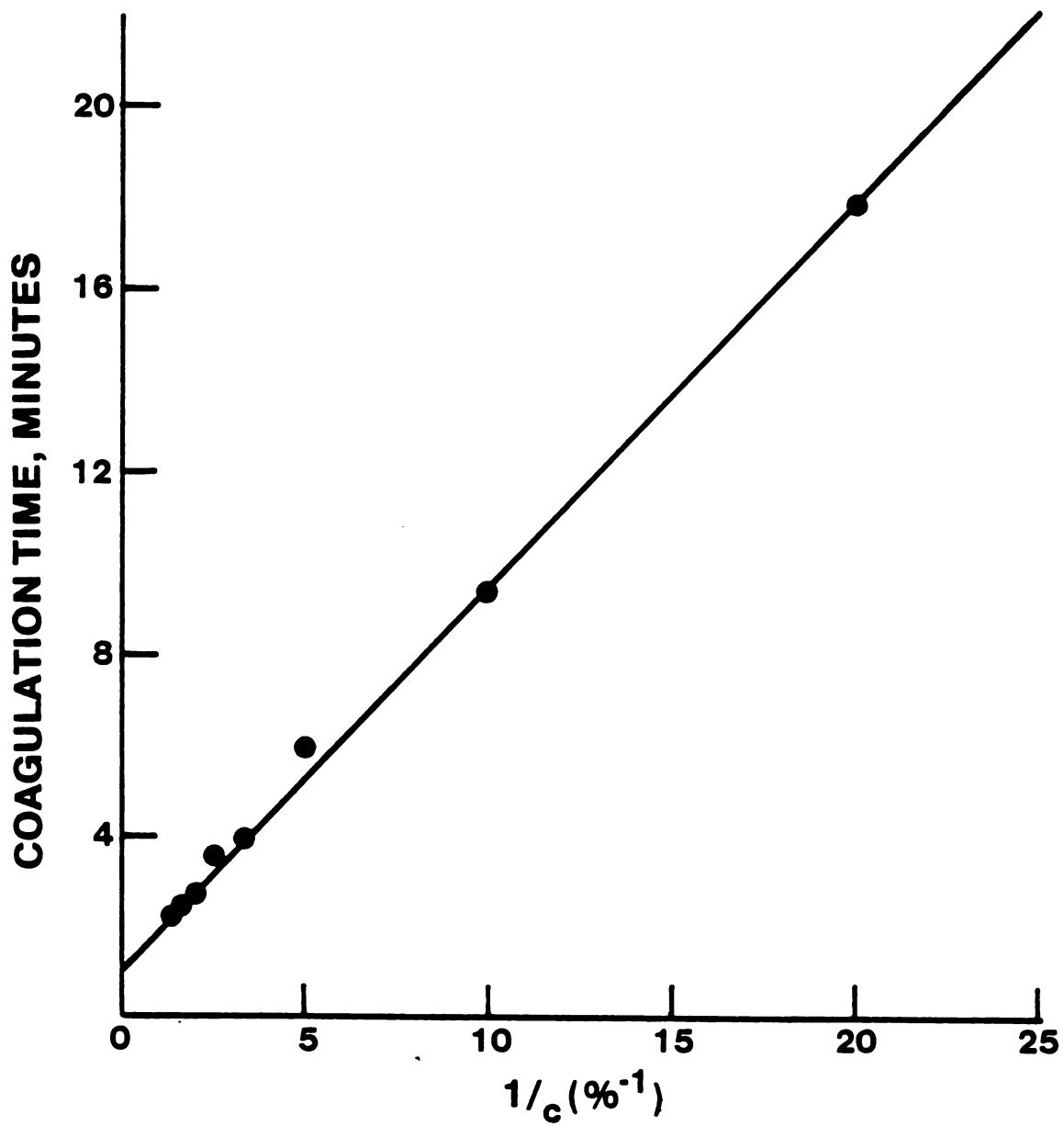


Figure 2. Coagulation Time of Milk as a Function of Reciprocal Concentration of Rennet

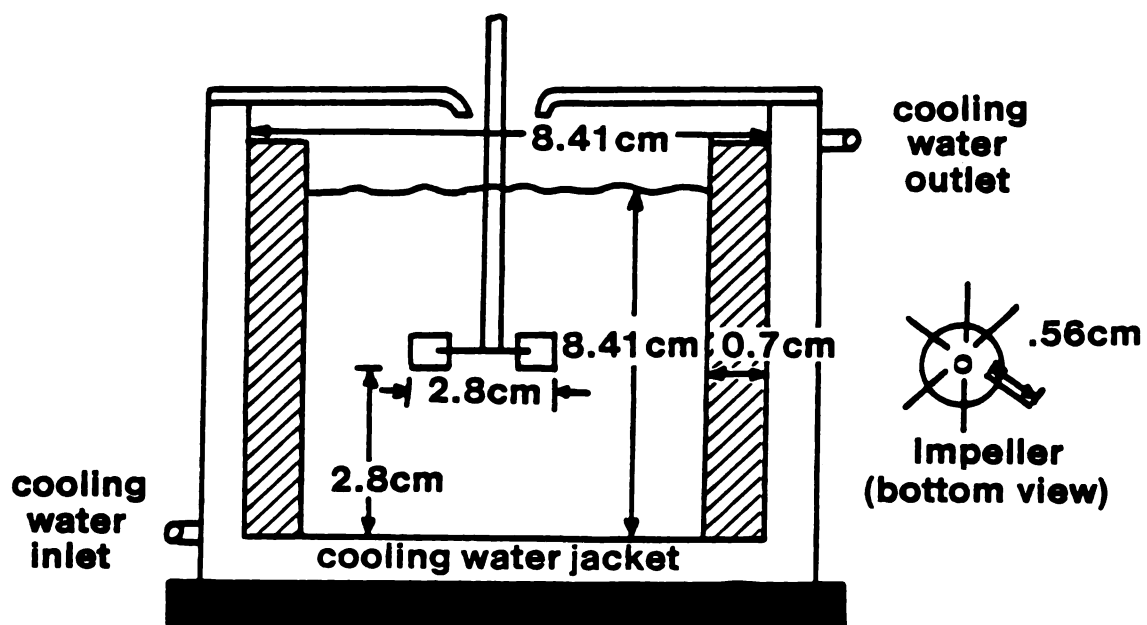


Figure 3. Schematic of Stirred Tank Used for Shear Damage Studies

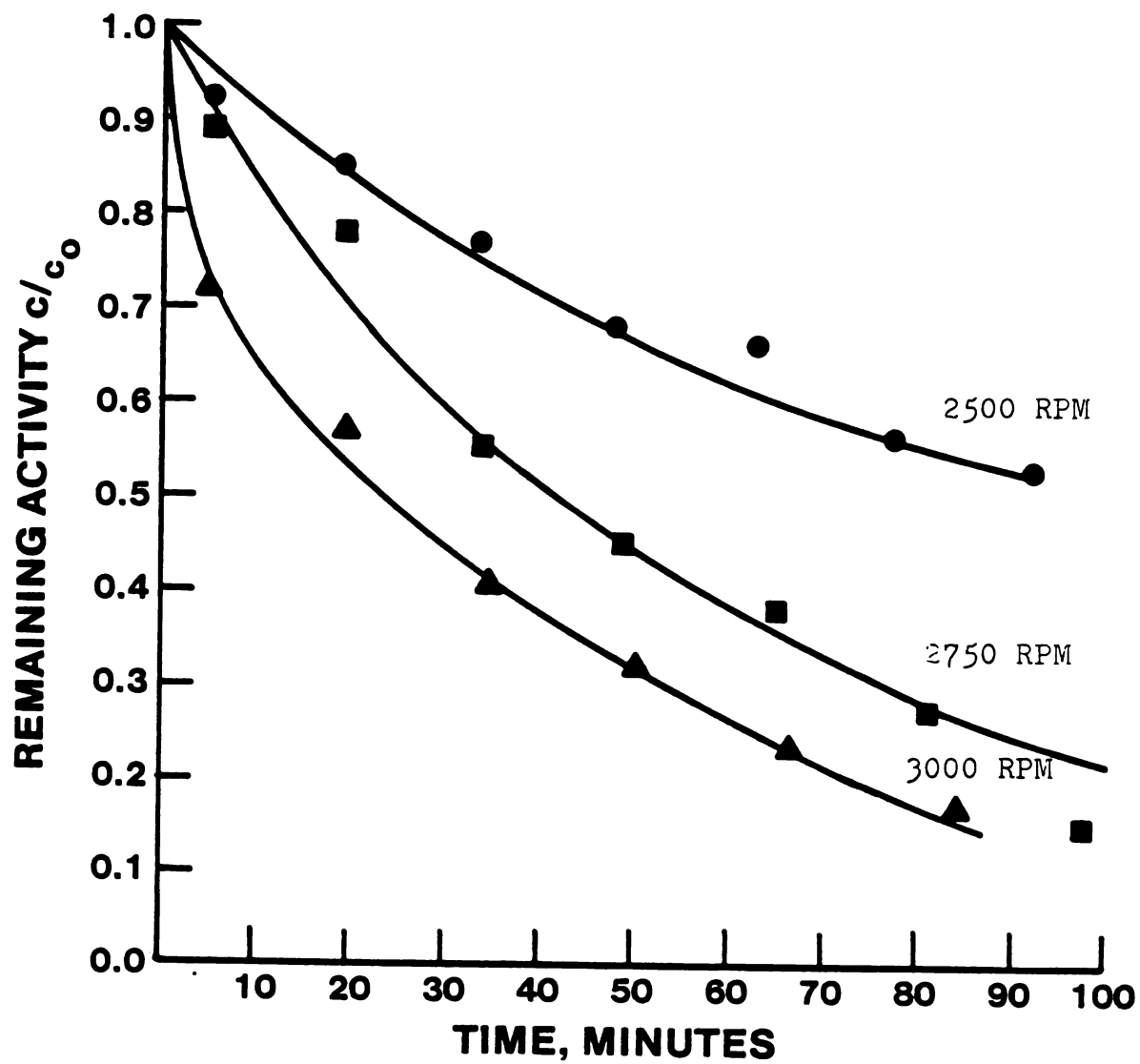


Figure 4. Rennet Activity Loss During Shear in a Stirred Tank ($c=0.66\%$)

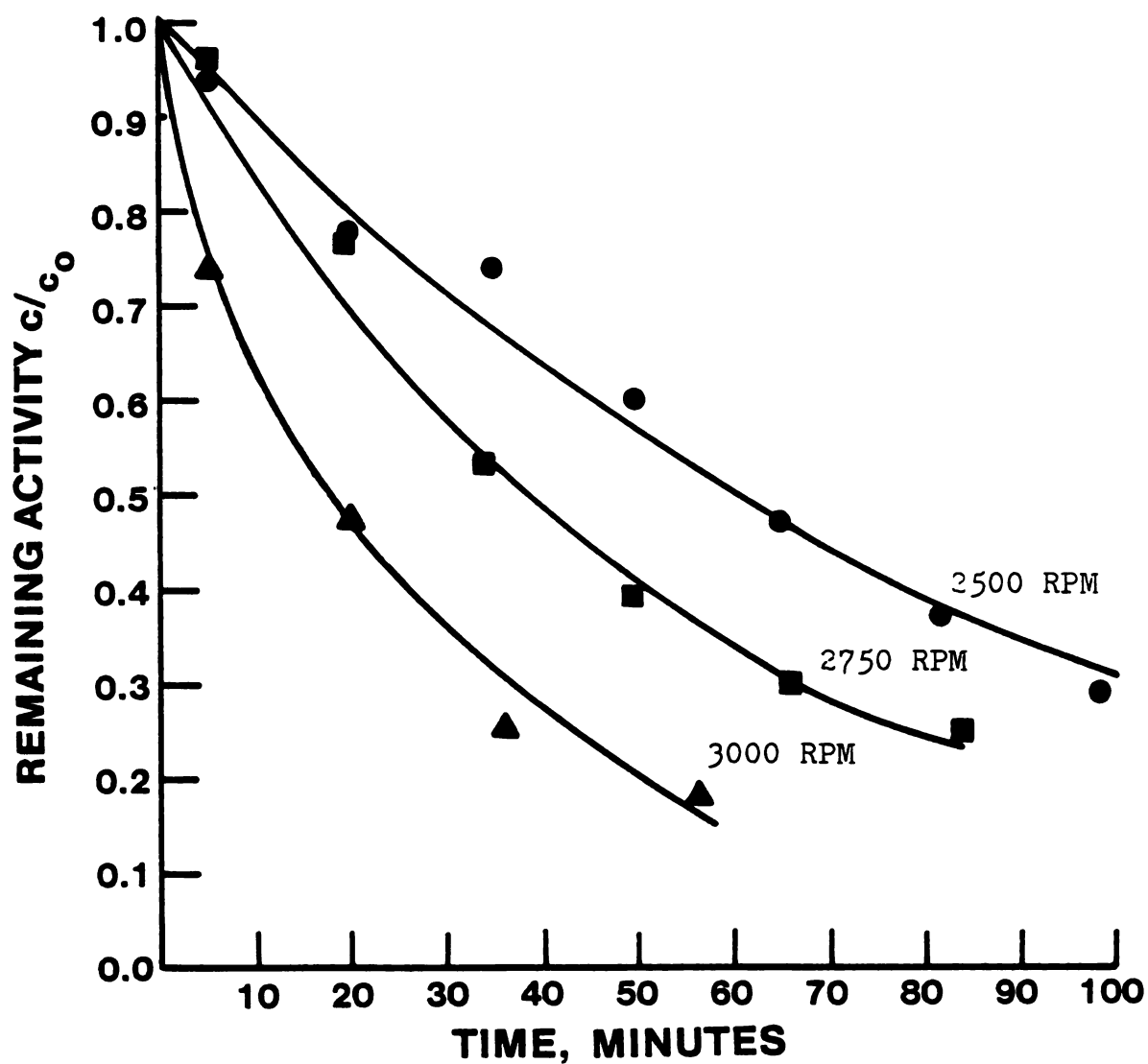


Figure 5. Rennet Activity Loss During Shear in a Stirred Tank ($c=0.55\%$)

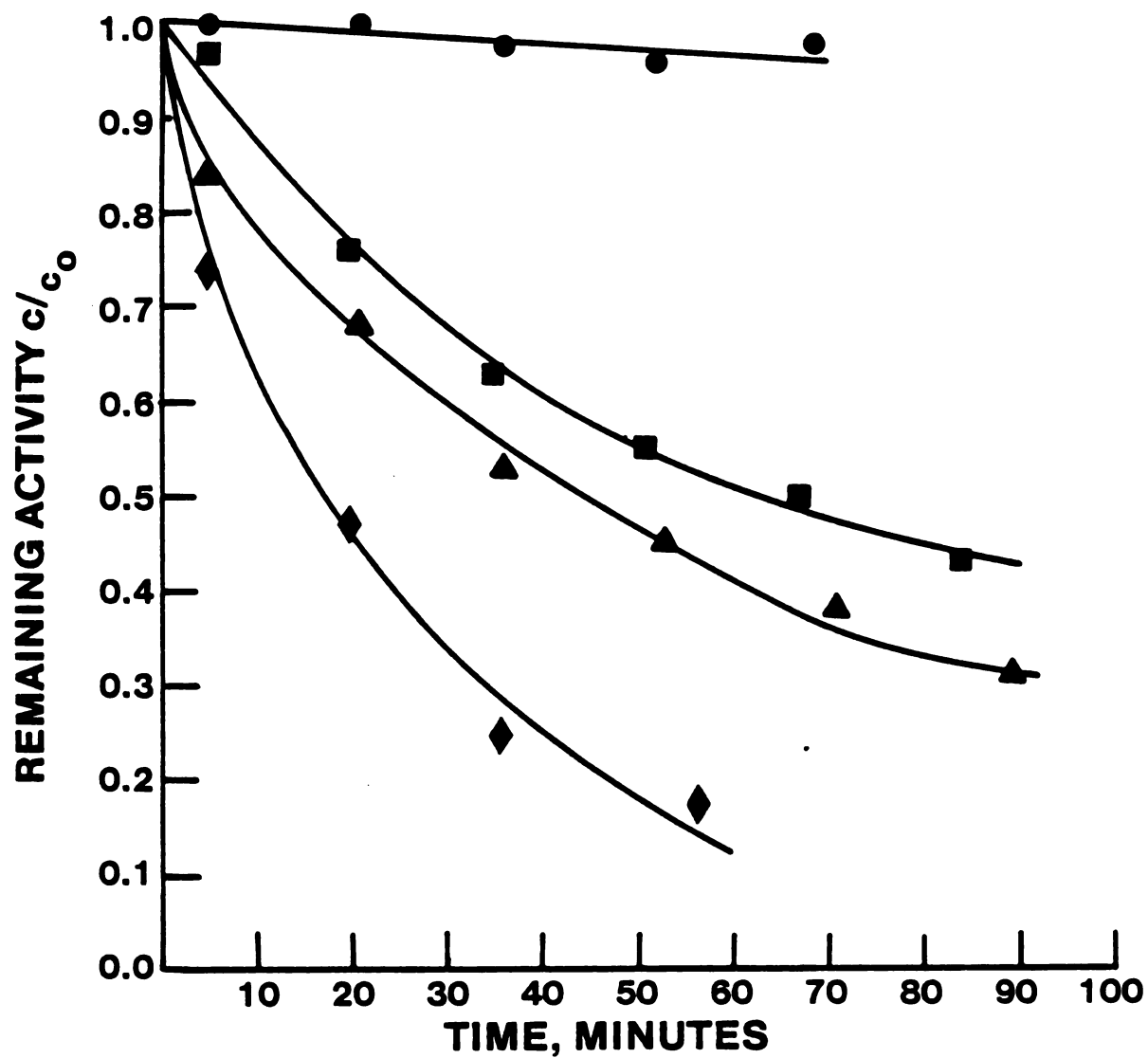


Figure 6. Rennet Activity Loss During Shear in a Stirred Tank with Polypropylene Glycol (●5.56x10⁻⁵% - Polypropylene Glycol, ■1.39x10⁻⁵% - Polypropylene Glycol, ▲1.11x10⁻⁵% - Polypropylene Glycol, ◆No Polypropylene Glycol)

Table 1 Sparging of rennet solution with air*	
<u>Sample</u>	<u>% Activity</u>
Initial liquid (c=0.55%)	100.0
Foam - while sparging	66.6
Liquid - while sparging	41.2
Liquid - after sparging	50.0

*Initial 50 ml sample was sparged until 21 ml became foam

Table 2 Sparging of rennett solution with air	
<u>Sample</u>	<u>% Activity</u>
Initial liquid	100.0
Foam - 3 minutes	81.4
Foam - 8 minutes	18.0
Foam - 15.8 minutes	5.8
Liquid - after sparging	5.1

Table 3 Sparging of rennet solution with nitrogen*	
<u>Sample</u>	<u>% Activity</u>
Initial Liquid (c = 0.55%)	100.0
Foam - while sparging	146.0
Liquid - while sparging	65.0
Liquid - after sparging	60.0

*Initial 50 ml sample was sparged until 26 ml became foam

Table 4 Sparging of rennet solution with nitrogen for 5 minutes	
<u>Sample</u>	<u>% Activity</u>
Initial Liquid	100.0
Liquid - after sparging 5 minutes	31.4

RECOMMENDATIONS

Further study is recommended in the following areas. The stirred tank experiments should be repeated using several rennet concentrations to verify the pattern found in earlier experiments in which lower enzyme concentrations lost more activity at corresponding times and impeller speeds. The second recommendation is to repeat all experiments using other enzymes such as urease, catalase, and carboxypeptidase. Charm and Wong³ and Tirrel⁸ reported that these enzymes lost activity in a shear field. It is important to discover if the reported activity loss was due to bubble formation as shown with rennet.

APPENDICES

APPENDIX A

DATA FOR MILK-RENNET SYSTEM

TABLE A1
Coagulation Time of Milk as a Function of
Reciprocal Concentration of Rennet

<u>c (g/10 ml)</u>	<u>1/c (%⁻¹)</u>	<u>t_c,minutes</u>
.07	1.44	2.3
.06	1.68	2.4
.05	2.01	2.8
.04	2.51	3.6
.03	3.34	3.9
.02	5.01	5.9
.01	10.01	9.4
.005	20.01	17.8

TABLE A2
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.66%, 2500 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.2	.87	100
5.00	2.3	.80	92
19.15	2.4	.74	85
33.40	2.6	.67	77
47.95	2.7	.59	68
62.70	2.8	.57	66
77.33	3.0	.49	56
92.20	3.1	.47	53

TABLE A3
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.66%, 2750 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.3	.80	100
5.00	2.5	.71	89
19.62	2.6	.63	78
34.03	3.1	.44	55
49.15	3.6	.36	45
64.80	4.0	.30	38
80.92	5.1	.22	27
98.20	8.1	.12	15

TABLE A4
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.66%, 3000 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.3	.87	100
5.00	2.6	.63	72
19.50	3.0	.50	57
34.50	3.6	.36	41
50.20	4.2	.28	32
66.62	5.3	.20	23
84.30	6.9	.15	17

TABLE A5
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.55%, 2500 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.6	.63	100
5.00	2.7	.59	94
19.58	3.0	.49	78
34.50	3.1	.47	74
49.60	3.5	.38	60
65.00	4.1	.29	47
81.57	4.8	.23	37
98.30	5.7	.18	29

TABLE A6
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.55%, 2750 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.4	.74	100
5.00	2.5	.71	96
19.32	2.7	.57	77
33.92	3.4	.39	53
49.40	4.2	.29	39
66.00	5.0	.22	30
83.85	6.0	.18	24

TABLE A7
Rennet Activity Loss During Shear in a
Stirred Tank (c=0.55%, 3000 RPM)

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.7	.59	100
5.00	3.2	.44	74
19.98	4.3	.27	47
36.20	6.9	.15	25
56.70	8.9	.11	18

TABLE A8
Rennet Activity Loss During Shear in a Stirred
Tank with $1.11 \times 10^{-5}\%$ Polypropylene Glycol

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	3.0	.48	100
5.00	3.3	.40	84
20.80	3.9	.32	68
36.20	4.5	.25	53
53.00	5.1	.22	45
70.50	5.9	.18	38
88.92	6.9	.15	31

TABLE A9
Rennet Activity Loss During Shear in a Stirred
Tank with $1.39 \times 10^{-5}\%$ Polypropylene Glycol

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	2.7	.57	100
5.00	2.8	.56	97
19.88	3.2	.44	76
35.12	3.6	.36	63
50.80	3.9	.31	55
67.10	4.2	.29	50
83.60	4.7	.24	43

TABLE A10
 Rennet Activity Loss During Shear in a Stirred
 Tank with $5.56 \times 10^{-5}\%$ Polypropylene Glycol

<u>Time,mins</u>	<u>t_c,mins</u>	<u>c (%)</u>	<u>% Activity</u>
0.00	3.38	.35	100
5.00	3.38	.35	100
20.80	3.28	.36	101
36.30	3.45	.34	98
52.00	3.48	.32	96
68.30	3.45	.34	98

APPENDIX B

APPENDIX B

DISCUSSION ON COUETTE VISCOMETER

In a separate set of experiments a couette viscometer was used to shear rennet solutions. Under laminar conditions, shear stress in a couette viscometer can be defined as follows:¹

$$s = u/b$$

s= shear stress (sec^{-1})
u= linear velocity of fluid
b= gap width of viscometer

Where:

$$u = RN$$

R= outer radius of viscometer
N= revolutions per second

By shearing rennet solutions at different speeds in the couette viscometer and measuring the activity of the rennet over time it was hoped to find a correlation between loss of activity in the viscometer and in the stirred tank. If such a correlation was found, the shear field in the stirred tank could be modeled.

A rennet concentration of 0.55% was used in the couette experiments. The solutions were sheared at 233 RPM and 417 RPM. In both cases the activity loss was minimal. No correlation could be made between the shear field in the couette viscometer and the stirred tank. When bubble formation rather than shear was found to be the cause of activity loss it was decided to exclude the couette viscometer data from the main body of this report.

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LIST OF REFERENCES

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