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Heat Denaturation of Serum Proteins in Skimmilk and Whey as Measured by the Harland-Ashworth Test

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HEAT DENATURATION OF SERUM PROTEINS IN SKIMMILK AND

WHEY AS MEASURED BY THE HARLAND-ASHWORTH TEST

by

Lynn Verl Ogden

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Dairy Science

UTAH STATE UNIVERSITY
Logan, Utah

1967

ACKNOWLEDGMENTS

This work was made possible by the efforts of many people who advised and gave moral support to me during the course of this thesis preparation.

I thank Dr. C. A. Ernstrom for the hours he has given in consultation on reasoning, methods, and conclusions. I extend appreciation to the other members of the committee, Paul B. Larson, George E. Stoddard, and Harris Van Orden, for their help; to my roommates for their understanding; to Nazar Shukri for his suggestions and encouragement; to my fiancée, Laurel Croff, for many hours spent typing; and to the professors and students in the Department. Everyone was willing to help with equipment and instruction whenever possible.

Lynn Verl Ogden

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ABSTRACT

Heat Denaturation of Serum Proteins in Skimmilk and

Whey as Measured by the Harland-Ashworth Test

by

Lynn Verl Ogden, Master of Science

Utah State University, 1967

Major Professor: C. A. Ernstrom
Department: Dairy Science

Milk serum proteins have been shown by other workers to be partially protected from heat denaturation by the presence of milk solids (8, 15, 16) and whole caseinate (14). This study revealed that some serum proteins (β-lactoglobulin), as shown by the Harland-Ashworth test (14, 17), were more readily denatured in skimmilk than in rennet whey. A comparison of the heat denaturation rate of serum proteins in whey as opposed to those in whey containing 2.5 percent isoelectric casein was also studied. A striking protection of serum proteins seemed apparent in the presence of isoelectric casein. However, it was learned that isoelectric casein interferes with results of the Harland-Ashworth test, and by taking this interference into account, that isoelectric casein has no affect on the heat denaturation rate of serum proteins. Some proteins are more sensitive to heat in the presence of miceller casein than in the presence of isoelectric casein or no casein at all.

The addition of sulfhydryl-group blocking agents (P-chloromercuriben-zoic acid and N-ethylmaleimide) inhibited serum protein denaturation in both skimmilk and rennet whey. Reactions of sulfhydryl groups following their liberation by heat resulted in much of the physical instability

measured by the Harland-Ashworth test. The complex between β-lactoglobulin and κ-casein, if it had any affect at all, was not one of the significant sulfhydryl-group reactions involved in the heat denaturation of the serum proteins.

INTRODUCTION

A test developed by Harland and Ashworth (11) for the determination of undenatured serum proteins in milk is used extensively as a control procedure in the manufacture of nonfat dry milk (NDM). Since denaturation of serum proteins in milk progresses with the application of heat, the Harland-Ashworth test serves as a practical method of classifying NDM according to the amount or degree of heat applied during processing (2). Bakeries require high-heat NDM because of its ability to absorb water and yield a better loaf volume (18). Low-heat NDM is required for fortification of Cottage-cheese milk (3, 8, 21) and for beverage purposes (9).

While the Harland-Ashworth test has proved to be of great practical value to the dairy industry, additional information is needed about its response to certain heat-induced changes involving the nonserum-protein constituents in milk. A heat-induced complex between β-lactoglobulin (the major serum protein) and κ-casein has been proposed and supported by evidence which suggests that the interaction involves sulfhydryl groups (29, 34). In performing the Harland-Ashworth test, casein and heat-denatured serum proteins are precipitated by saturating milk with sodium chloride. Heating serum proteins in the presence of casein or caseinate micelles could result in complexing such that the β-lactoglobulin would be precipitated by the saturated salt solution to a different extent that would occur if casein were not present.

The purpose of this work was to use the Harland-Ashworth test to compare the rate of heat denaturation of serum proteins in skimmilk,

rennet whey, and solutions of isoelectric casein in rennet whey, and
to determine how the test is affected when sulfhydryl-group blocking
agents are added to these systems prior to heating.

REVIEW OF LITERATURE

The Harland-Ashworth test

The original Harland-Ashworth test (11) was developed to determine
the suitability of NDM for use by the baking industry. Actually, the
test measured the amount of undenatured serum protein nitrogen (WPN)
in milk powder. Since serum proteins are denatured by heat, the
percentage of the original undenatured serum protein remaining after
processing reflects the amount of heat applied to the milk prior to
drying (1). Standards adopted by the American Dry Milk Institute (ADMI)
require that high-heat powder contain not more than 1.5 mg. of un-
denatured WPN per gram of powder. Low-heat powder cannot contain less
than 6.0 mg WPN per gram, medium-heat powder must contain from 1.51 to
5.99 mg WPN per gram.

Whey proteins comprise all the proteins in milk except the caseins
and include β-lactoglobulin, α-lactalbumin, milk serum albumin, the
euglobulins and pseudoglobulins (32). Swanson et al. (27) and Overman
(22) demonstrated that the WPN content of milk fluctuates throughout the
year from about 6.5 to 8.0 mg per gram of solids-not-fat (SNF). The
highest values were observed from October to December and the lowest
from May to June. Therefore, the amount of undenatured WPN in a product
depends not only upon the heat treatment used during processing, but
also upon the original amount of WPN in the sample. This was taken
into consideration when the ADMI standards were established, but even
so, it is possible for milk subjected to different heat treatments at
different seasons of the year to show the same amount of undenatured WPN.

The Harland-Ashworth test makes use of the fact that casein and heat-denatured whey proteins are insoluble in saturated solutions of sodium chloride in the pH range of normal milk. These insoluble substances are separated by filtration, and the filtrate, which contains the undenatured whey proteins, is acidified with HCl to produce a turbid solution. The turbidity can be measured in a colorimeter and related directly to the WPN content of the sample (17). Leighton (19) improved the reproducibility of the test by standardizing the procedure for reconstituting the NDM, and by controlling the acidification technique to give a constant pH for development of turbidity in the saturated salt solution.

Factors affecting heat denaturation of milk serum proteins

The effect of casein and milk solids. Krueger et al. (15) and Emmons et al. (8) concluded that in general a high concentration of milk solids resulted in a low rate of denaturation of serum proteins during heating. Krueger et al. (15) used low-heat NDM at concentrations of 1 to 30 g per 100 ml of distilled water. Heat treatments of 70, 75, 77, 80, and 85 C for 30 minutes were applied and the extent of denaturation determined by the Harland-Ashworth test. The percent denaturation decreased with increasing concentration when samples were heated to 70, 75, and 77 C, but at 80 and 85 C it increased with increasing concentration up to 15 g per 100 ml, then remained constant up to 30 g per 100 ml. Later Krueger et al. (16) isolated milk serum proteins by the method of Harland and Ashworth (17) and redissolved them in phosphate buffer at pH 6.6. Serum-protein concentrations were made equal to those described in the previous experiment, and the samples were subjected to

identical heat treatments. The serum proteins alone were more readily denatured than in previous experiment where a complete milk system was used. Results of this experiment might have been more conclusive if the original milk from which the whey protein was isolated had been available for direct comparison and if all the normal serum solids other than casein had been present in the serum protein solutions. Emmons (8) used concentrated skimmilk containing 40 to 50 percent solids and applied heat treatments up to 71 C for 15 minutes. He observed no serum protein denaturation under these conditions. Normally, the serum protein would have been 15 to 20 percent denatured by such a treatment. Kenkare et al. (14) determined that addition of caseinate to neutralized acid whey or ultracentrafuged serum prior to heating resulted in greater heat stability of the serum proteins. Addition of 2.5 percent whole caseinate raised the stability of serum proteins in neutralized acid whey from 30 percent to 95 percent and in ultracentrafuged whey from 55 percent to 100 percent after heating to 91 C for 10 minutes. Stability was determined by the ability of serum proteins to remain suspended following centrifugation at 1000 G.

Effect of pH during heating. Guy et al. (10) observed that the denaturation rate of serum proteins in Cottage-cheese whey (decreased) with decreasing pH at the time of heating. The extent of denaturation ranged from zero at pH 3.4 to 82 percent at pH 7 when whey was heated to 82 C for 30 minutes. It was also determined that adjusting the pH of Cottage-cheese whey after the heat treatment and before analysis also influenced the results. Maximum WPN values were observed when the heated whey was adjusted between pH 5.4 and 6.8 before analysis. Sharp declines were noted outside this range.

Interaction of κ-casein with β-lactoglobulin

Formation of a heat-induced complex between α-casein and β-
lactoglobulin was suggested by McGugan et al. (20). They heated mixtures
of the isolated proteins in 0.1 ionic strength phosphate buffer (pH 6.86)
to 85 C for 30 minutes. Electrophoresis in the same buffer yielded a
single peak of slightly slower mobility than that of the α-casein
peak. An unheated mixture of the two proteins gave two electrophoretic
peaks. Electrophoresis in glycine-HCl buffer at 0.1 ionic strength
and pH 2.45 led to the same conclusion. The possibility of a β-
lactoglobulin-κ-casein complex was later supported by Zittle et al.
(34). They determined that if β-lactoglobulin was heated it could
combine with κ-casein and form a complex which had an electrophoretric
mobility faster than either leased or unleased κ-casein and slower
than either heated or unheated β-lactoglobulin. Sedimentation experi-
ments by the same authors gave S_{20} (Svedberg units) values of 2.7 and
15.1 for β-lactoglobulin and κ-casein respectively. The values for
heated κ-casein were 15.1 and 32.0, and for heated β-lactoglobulin A,
5.3. In unheated mixtures S_{20} values of 2.7 and 17.4 were obtained,
but in heated mixtures a single S_{20} value of 45 was obtained. Zittle
et al. (34) found that the ability of κ-casein to stabilize α_s-casein
was greatly reduced after it had been heated with β-lactoglobulin.
They also determined that heating solutions of κ-casein decreased rennin
coagulation time, but heating κ-casein in the presence of β-lactoglobulin
produced the opposite effect. Zittle et al. (34), supported by this
evidence, concluded that a complex between β-lactoglobulin and
κ-casein did take place during heating.

Trautman and Swanson (28) presented electrophoretic evidence to show that sulfhydryl blocking agents could prevent heat-induced complexing between β-lactoglobulin and κ-casein. They also found that P-chloromercuribenzoic acid (PCMB) nullified the stabilizing effect of the forewarming treatment in the manufacture of evaporated milk (29). Sawyer et al. (24) showed by electrophoresis that complexing of β-lactoglobulin and κ-casein could be prevented when these proteins were heated in the presence of N-ethylmaleimide (NEM). It was concluded that the complex involved sulfhydryl groups, and it was postulated that the interaction might result from an intermolacular disulfide bond between β-lactoglobulin and κ-casein. The reaction of PCMB with sulfhydryl groups is illustrated as follows (7).

$$RSH + Cl.Hg \bigcirc COOH \longrightarrow RSHg \bigcirc COOH + HCl$$

The chemical reaction involved in sulfhydryl group blocking by NEM is as follows (26).

$$RSH + \begin{array}{c} CH = CH \\ | \quad\quad | \\ O^{=}C \quad C^{=}O \\ \diagdown N \diagup \\ C_2H_5 \end{array} \longrightarrow \begin{array}{c} RSCH - CH_2 \\ | \quad\quad | \\ O^{=}C \quad C^{=}O \\ \diagdown N \diagup \\ C_2H_5 \end{array}$$
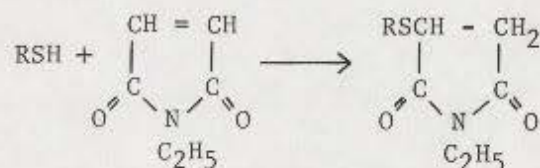
A preliminary report by the Commonwealth Scientific and Industrial Research Organization (4) suggests that disulfide bonds are not involved in heat-induced complexing between β-lactoglobulin and κ-casein. However, no data were presented.

Interaction between β-lactoglobulin and α-lactalbumin

Yamauchi (33), after heating serum protein to 70 C for 10 minutes in dilute NaCl, found a new electrophoretic peak with a mobility between

those of α-lactalbumin and β-lactoglobulin. Hostettler and Stein (12) reported that protein in sediment from stored evaporated milk contained an α-lactalbumin-β-lactoglobulin complex whose electrophoretic mobility was intermediate between those of the individual proteins. Hunziker and Tarassuk (13) showed chromatographically that when α-lactalbumin was heated alone to 75 C for 30 minutes in a 0.2 M phosphate buffer at pH 6.7, the area under its chromatographic peak was reduced by 14 percent; when milk serum proteins were heated together, the area under the α-lactalbumin peak was reduced by 36 percent; and when α-lactalbumin and β-lactoglobulin were heated together, the area under the α-lactalbumin peak was reduced by 84 percent. They also suggested that the inter-action involved sulfhydryl groups similar to the reaction that Sawyer et al. (24) suggested for β-lactoglobulin and κ-casein. However, no evidence was presented to support this suggestion.

Comparison of heat denaturation rates

Fresh raw skimmilk was obtained from the Utah State University
Dairy Products Laboratory and divided into two lots. From one lot whey
was obtained by treating two liters of skimmilk at 37 C with 1 ml of
rennet for 20 minutes. After coagulation, the flask was shaken to break
the curd, and the mixture agitated periodically for several minutes to
allow separation of the whey. The whey was decanted and centrifuged
at about 1000 G to remove fine casein particles. The whey and the milk
from which it was obtained had identical pH values. The whey was divided
into seven 50-ml samples and each one placed in a 30 x 200 mm test tube.
Samples were heated in a water bath for 30 minutes at temperatures of
60, 65, 70, 77, 85, and 93 C ± 1 C. The raw skimmilk was divided into
similar-sized samples and simultaneously subjected to the same heat
treatments as the whey. After heating, the samples were quickly cooled
to 10 C in a water bath. Control samples of both skimmilk and whey
received no heat treatment. The Harland-Ashworth test modified by
Kuramoto et al. (17) was used to determine the extent of denaturation
of serum proteins in each sample. Turbidity was developed in a 250 ml
test tube, but the samples were transferred to cuvettes for turbidity
determinations at 420 mu in a Coleman Universal Spectrophotometer.
Turbidity in filtrates from unheated control samples was arbitrarily
assigned a value of zero percent denaturation and the turbidity in
unacidified filtrates was assigned values of 100 percent denaturation.

The percent serum protein denaturation of heated samples was calculated proportionally from the resulting turbidities.

→ STOP

The possibility that rennet coagulation of milk might reduce the calcium content of the whey that is produced, and that this in turn can influence the rate of denaturation of the serum proteins was investigated. Rennet whey obtained from raw skimmilk was dialyzed back against the skimmilk for 2 days at 4 C to equalize the soluble salts. Samples were then subjected to the heat treatments previously described, and the rate of heat denaturation compared with raw skimmilk by the Harland-Ashworth test.

P-chloromercuribenzoic acid (a sulfhydryl blocking agent) was added to samples of raw skimmilk and to rennet whey obtained from raw skimmilk at a concentration of 2×10^{-4} M. The denaturation rate of the serum proteins in these samples was compared with that in normal raw skimmilk and normal rennet whey obtained from raw skimmilk. This experiment was also repeated using NEM as the sulfhydryl-group blocking agent. In another experiment the rates of heat denaturation of serum proteins in whey containing PCMB, whey containing 2.5 percent added isoelectric casein prepared by the method of Van Slyke and Baker (30), and whey containing both PCMB and isoelectric casein were compared with rennet whey. Rennet in all samples was destroyed by a heat treatment of 60 C for 30 minutes so it would not produce artifacts in the Harland-Ashworth filtrates of those samples containing dissolved isoelectric casein, and to insure that the dissolved casein would not coagulate. The pH of all samples was adjusted to 6.7 before heating to 60, 65, 70, 77, 85, and 93 C ± 1 C for 30 minutes.

The Harland-Ashworth test was run on heated (93 C for 30 minutes) and unheated solutions (2.5 percent) of isoelectric casein at pH 6.7 to determine whether any of the isoelectric casein could be found in the filtrate.

Freeze-dried Harland-Ashworth filtrates

Raw skimmilk was obtained from the Utah State University Dairy Products Laboratory and divided into two two-liter lots. One lot was treated with 1 ml of rennet at 35 C for 20 minutes. After coagulation, the curd was broken by agitation, then shaken periodically to aid separation of the curd from whey. The whey was decanted and centrifuged at about 1000 G for 30 minutes to remove fine casein particles. Samples of whey and milk (350 ml) were given simultaneous heat treatments of 65, 77, 85, and 93 C for 30 minutes in 500 ml Erlenmeyer flasks immersed in a hot water bath. Five minutes were allowed for the samples to reach treatment temperatures. These plus the unheated control samples of milk and whey were adjusted to 37 C and saturated with NaCl. They were then incubated for 30 minutes at 37 C and filtered through S and S #602 filter paper. Exactly 100 ml of clear filtrate was collected from each sample and placed in one-inch diameter dialysis casings and dialyzed at 4 C for 3 days against three changes of distilled water. Material remaining in the dialysis bags was shell frozen in 1000 ml flasks in a dry ice-alcohol mixture and dried on a _Virtis_ mobile freeze drying unit. In the same manner, freeze dried Harland-Ashworth filtrates were obtained from samples of milk and whey containing 2×10^{-4} M PCMB, whey containing 2.5 percent dissolved isoelectric casein, and whey containing both isoelectric casein and PCMB. These samples had been heated to 93 C for 30 minutes before subjection to the Harland-Ashworth test.

Identification of serum proteins in
Harland-Ashworth filtrates

Starch gel electrophoresis of whey proteins was carried out by a modification of the procedure described by El-Negomy (7). Forty-four g of hydrolyzed starch[1] were suspended in a mixture of 190 ml of distilled water and 50 ml of tris-citrate buffer (23). The suspension was heated over a direct flame in a 1 liter Erlenmeyer flask. As heating proceeded, the mixture became thick and clear, then became a little less viscous, whereupon 150 g of urea were added. Heating was continued until the mixture boiled lightly. At that time 1.4 g of mercaptoethanol were added, and the flask was removed from the flame and allowed to cool for 10 minutes. Vacuum from a water aspirator was applied to the flask to remove all air bubbles. An excess of the solution was poured into a 12 x 21 x .6 cm frame made by clamping four plexiglass strips onto a piece of glass. A glass plate covered tightly with Saran wrap was placed on top of the gel beginning at one end. The other end was slowly lowered so the excess gel ran out carrying the bubbles with it. A weight was placed on top of the glass cover to prevent bubbles from forming in the gel. The gel was allowed to age at room temperature for 12 to 24 hours.

Sodium borate conductivity buffer was made by mixing 1.424 g of boric acid with .3679 g of NaOH and 1000 ml of water. Vesselinovitch (31) stated that such a buffer would be pH 8.6 but the actual pH was measured at 8.85.

Inserts (5 mm x 6 mm) made from Whatman #3 filter paper were saturated with protein solutions, and the excess moisture blotted off. The inserts were placed in a slit in the gel made by a spatula. The

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[1] Obtained from Connaught Medical Laboratories, Toronto, Canada.

inserts completely penetrated the 6 mm gel and were spaced about 1.5 cm apart across the gel. A drop of amido black solution (25) was placed on one insert. Since the migration rate of the amido black was more rapid than that of the proteins, it was used as an indicator to estimate the extent of migration.

The electrophoresis unit was powered by a Heath kit regulated power supply, model 1p-32 with variable voltage and milliampere gauges. The electrodes consisted of two 10-inch platinum wires immersed in conductance buffer in 9 x 4.5 x 3-inch glass pans. Wicks connecting the conductance buffer with ends of the gel were made from eight thicknesses of Whatman #3 filter paper. The apparatus is shown in Figure 1. Electrophoresis was carried out between 10 C and 15 C. A voltage of 250 V was applied, and the current varied from 50 to 75 ma during the run. The length of runs was 4½ to 5 hours.

After each experiment was completed, the gel was cut with a fine fishing leader into two layers 3 mm thick. The layers were separated by placing Saran wrap on top of the top layer and separating the layers with the fingers while holding the top layer to the Saran wrap with both hands. The Saran adhered to the gel and cleanly removed the top layer of the gel without injury to either layer. The thin layers were stained with a saturated solution of amido black (25) in a solvent of methanol, distilled water, and acetic acid (50: 50: 10 v/v) for 10 minutes and destained for 3 days in three changes of solvent. Protein zones remained black and the gels became white upon destaining.
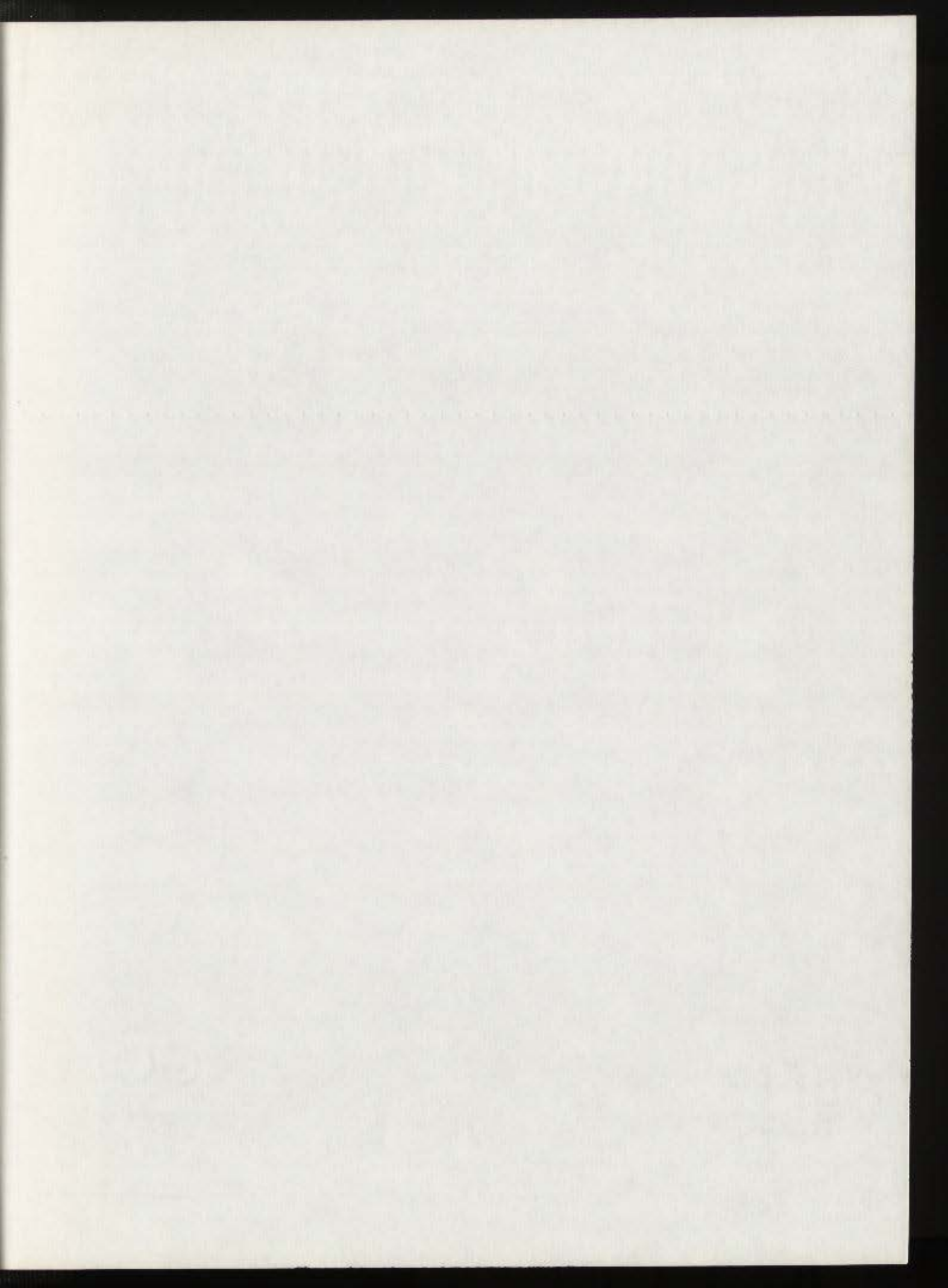
Figure 1. Equipment used for starch-gel electrophoresis.

Heat denaturation of serum proteins in skimmilk and whey

The Harland-Ashworth test was used to measure serum protein denaturation in samples of skimmilk and rennet whey that had been heated to temperatures of 65, 70, 77, 85, and 93 C for 30 minutes. ¶The results of this experiment are presented in Figure 2 and indicate that the serum proteins were more readily heat denatured in skimmilk than in whey. The largest difference in denaturation was noted when both samples were heated to 77 C for 30 minutes. Under these conditions the serum proteins in the whey and milk were 45 and 77 percent denatured respectively. Following the most severe treatment, 93 C for 30 minutes, the serum proteins in the whey and milk were 81 and 93 percent denatured respectively.

During the coagulation of milk by rennet, it is known that additional calcium ions are bound by para-casein. Therefore, it was probable that the soluble calcium concentration in the rennet whey was lower than in the corresponding milk serum. The possibility that differences in heat stability of serum proteins in skimmilk and whey might be due to differences in salt concentration was investigated. Rennet whey from raw skimmilk was dialyzed for 2 days at 4 C against raw skimmilk to equalize the dialyzable constituents. The dialyzed whey and skimmilk were then subjected to the same heat treatments indicated in Figure 2 and tested by the Harland-Ashworth test. The results were practically identical to those shown in Figure 2. At 77 C for 30 minutes, the whey proteins in the whey and skimmilk were 47 and 69 percent denatured respectively. At 93 C for 30 minutes, the serum proteins in whey and
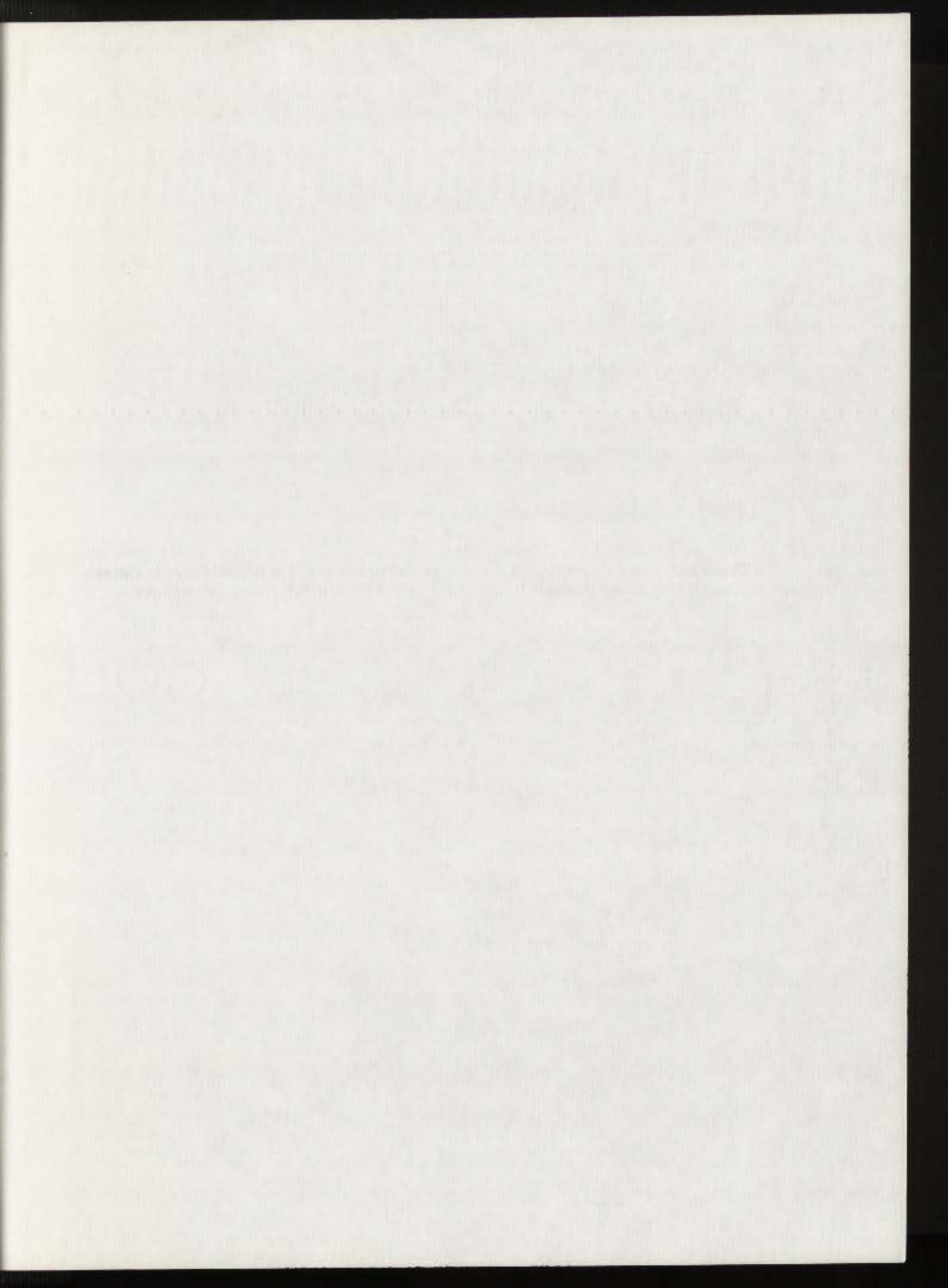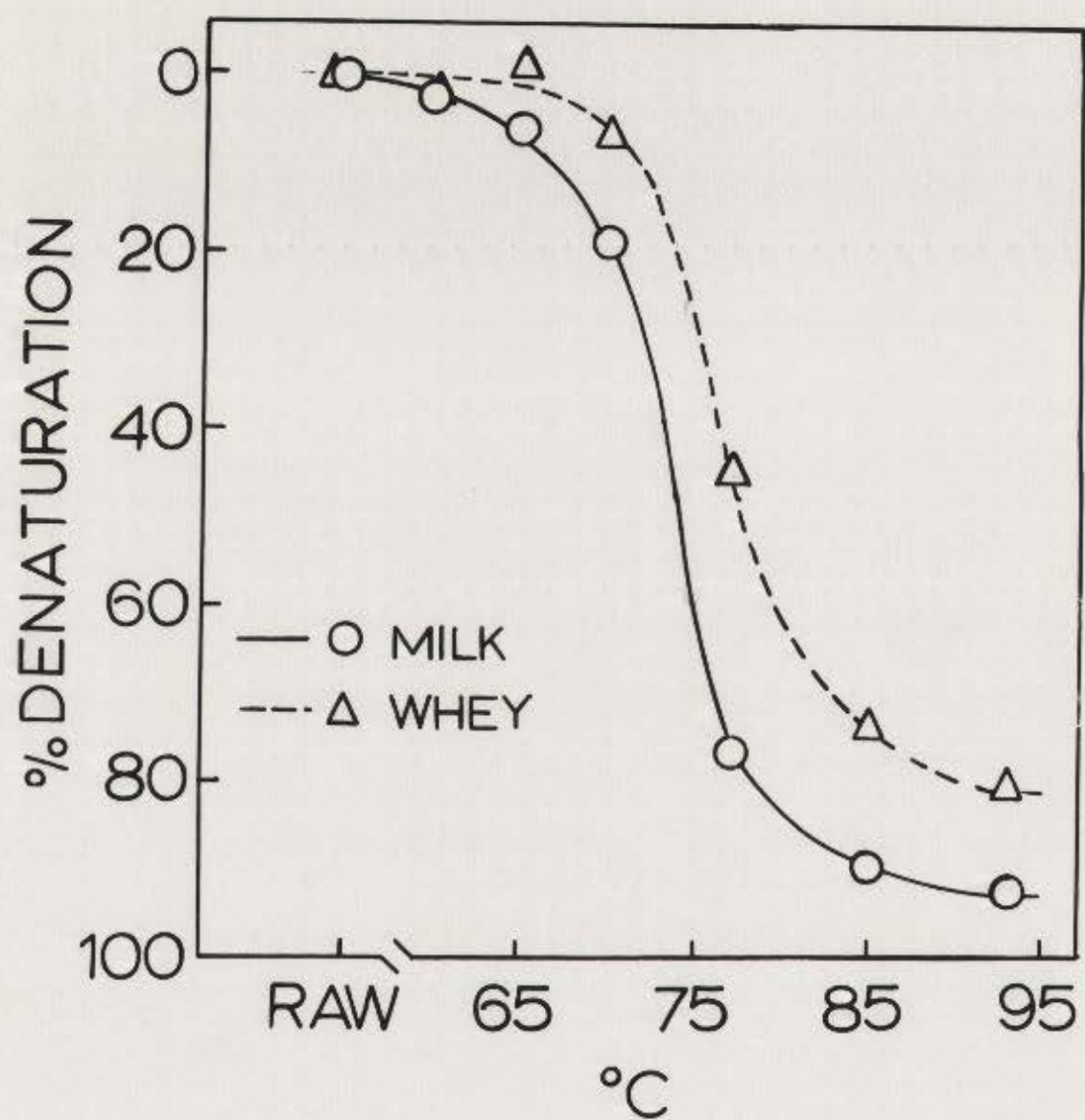
Figure 2. Heat denaturation of serum proteins in skimmilk and rennet whey heated to 65, 70, 77, 85, and 93 C for 30 minutes.

skimmilk were 77 and 87 percent denatured respectively. It was, therefore, concluded that the differences noted in Figure 2 were not due to a discrepancy in dialyzable solids between the milk and whey, and that it was justifiable to use non-dialyzed rennet whey for further experiments.

P-chloromercuribenzoic acid was next added as a sulfhydryl blocking agent to samples of raw skimmilk and rennet whey. These samples were heated as previously described along with skimmilk and whey containing no blocking agents. The results of the Harland-Ashworth analysis of these samples after heating are presented in Figure 3. Addition of PCMB inhibited denaturation of serum proteins in both raw skimmilk and rennet whey. Heating skimmilk to 93 C for 30 minutes resulted in 77 and 89 percent denatured serum proteins respectively in samples with and without PCMB. The same heat treatment applied to rennet whey resulted in 65 and 84 percent denaturation of serum proteins respectively in blocked and unblocked samples. The sulfhydryl blocking experiment was repeated with N-ethylmaleimide (NEM) substituted for PCMB as a blocking agent. Figure 4 shows the effect of NEM on the heat denaturation of serum proteins in skimmilk and rennet whey. The results were similar to those illustrated in Figure 3. However, the serum proteins in skim-milk containing NEM denatured a little more rapidly than those in rennet whey without a blocking agent. When PCMB was used as a blocking agent, the opposite was true; however, in both experiments addition of either blocking agent retarded the heat denaturation rate of serum proteins in both milk and whey.

A report by Kenkare et al. (14) suggested that isolated caseins exhibited a protective effect on serum proteins during heating of acid and ultracentrafuged wheys. However, results shown in Figure 2 indicate
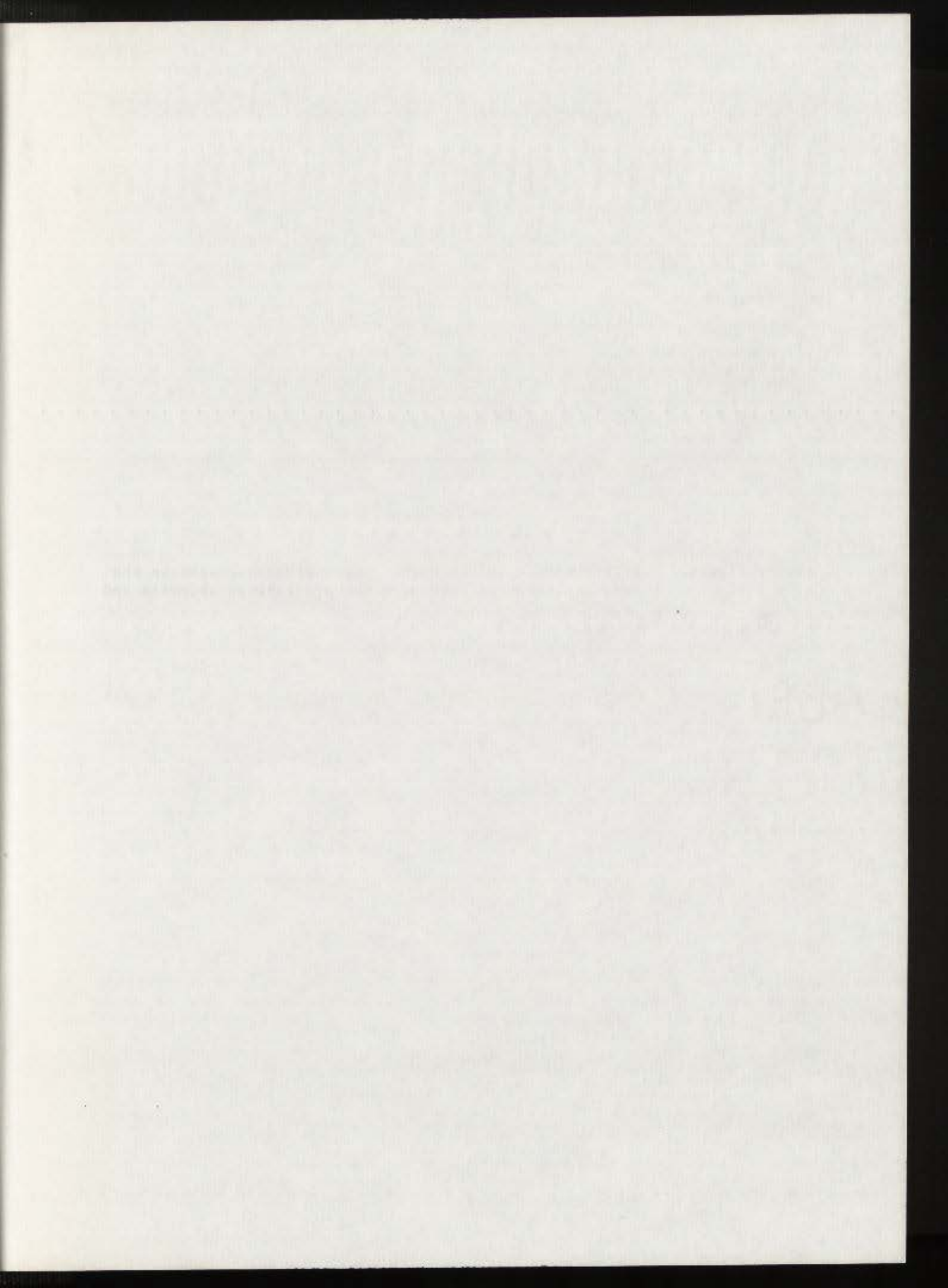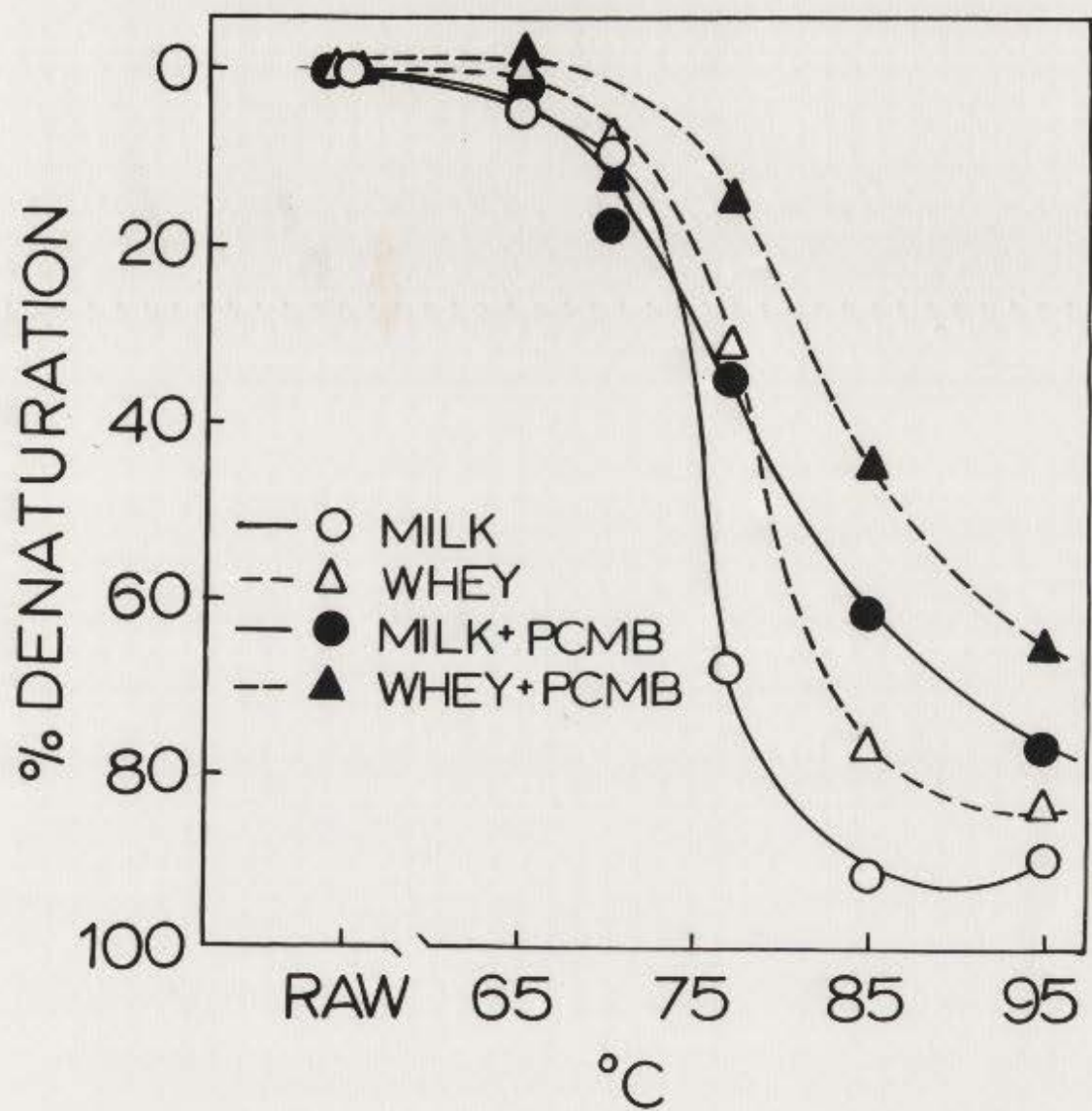
18

Figure 3.   Effect of 2×10^{-4} M P-chloromercuribenzoic acid on the
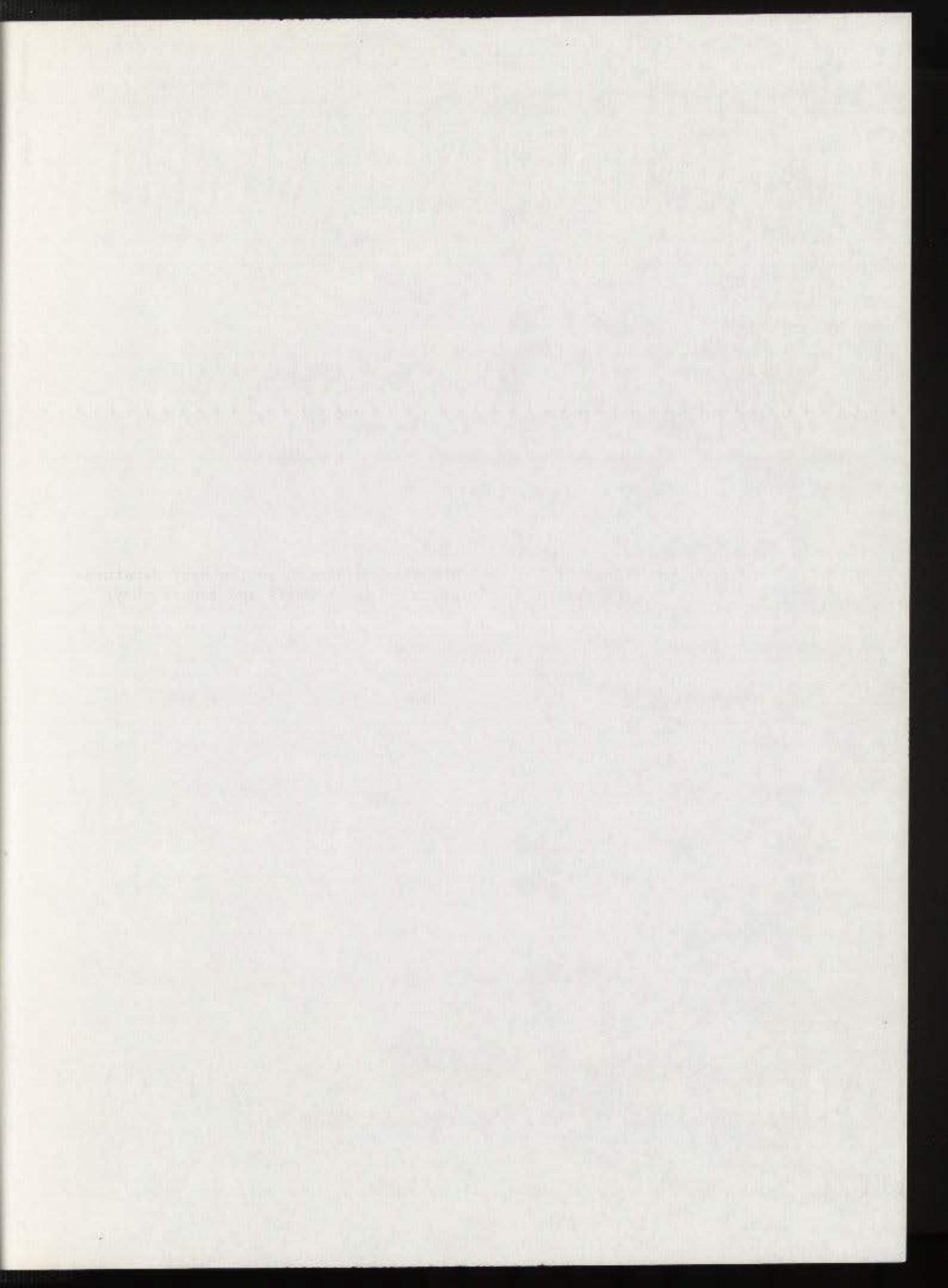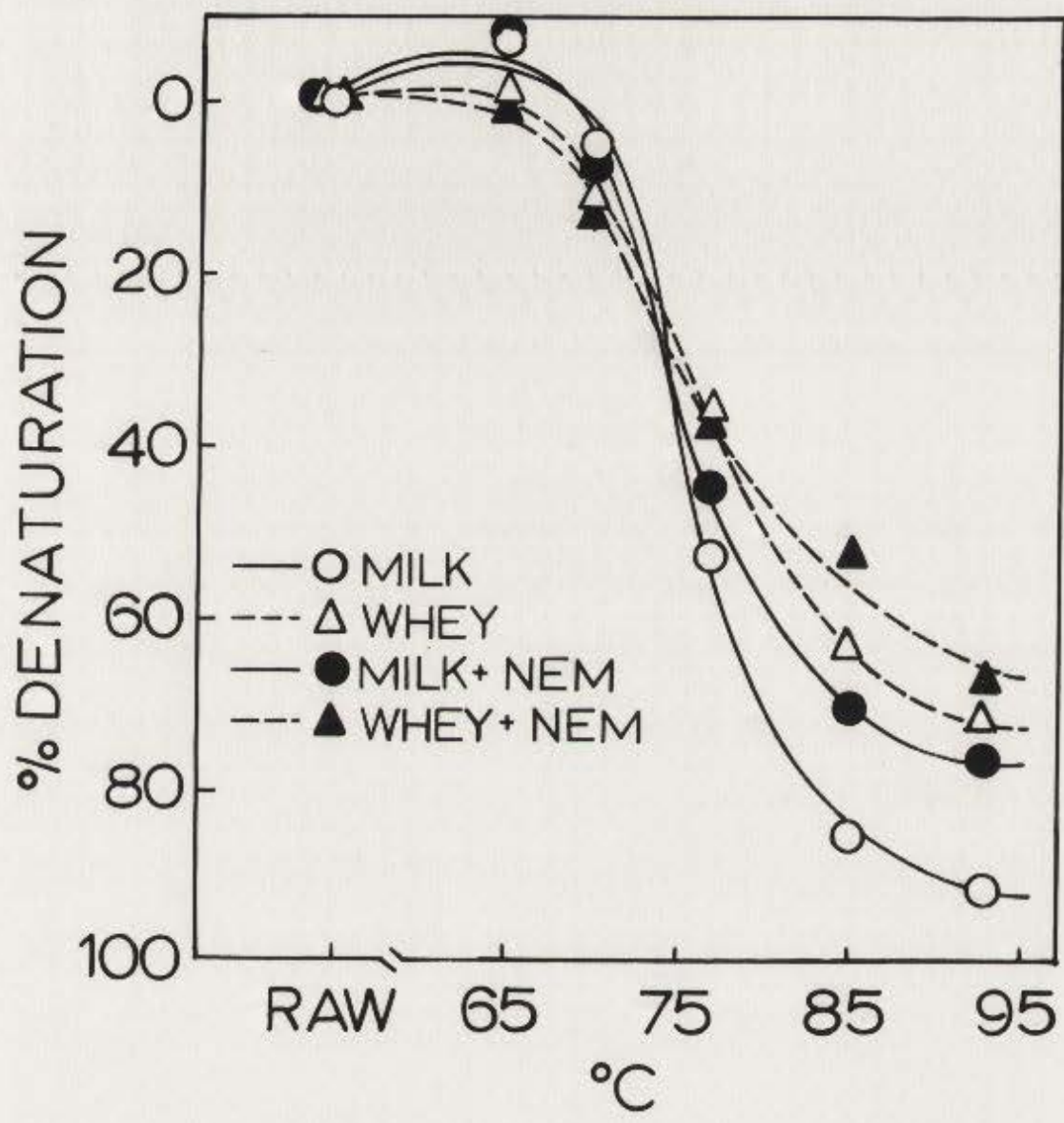heat denaturation rate of serum proteins in skimmilk and
rennet whey.

Figure 4. Effect of 2×10^{-4} M N-ethylmaleimide on the heat denatura-
tion rate of serum proteins in skimmilk and rennet whey.

just the opposite in skimmilk and whey. The casein in skimmilk exists
in a miceller form while that used in the experiments of Kenkare et al.
would have been nonmiceller. An experiment was run to determine whether
nonmiceller casein had the same effect on heat denaturation of serum
proteins as in-tact miceller casein. Isoelectric casein prepared by the
method of Van Slyke and Baker (30) was dissolved in fresh rennet whey
that had been heated to 60 C for 30 minutes to destroy residual rennet.
The final solution contained 2.5 percent casein. This material was
heated in the presence and absence of 2×10^{-4} M PCMB as were control
samples of blocked and unblocked whey. Results of Harland-Ashworth
analyses following heat treatments of these materials are given in
Figure 5 and tabulated in Table 1. The isoelectric casein appeared to
give about the same degree of protection to serum proteins as the PCMB
when samples were heated to 93 C for 30 minutes. Casein and PCMB
together seemed to give substantially more protection to serum proteins
against heat denaturation than the PCMB alone. In the samples heated to
93 C for 30 minutes, the serum proteins were 73.6 percent denatured in
whey, 49.5 percent denatured in whey containing isoelectric casein,
53.5 percent denatured in whey containing PCMB, and only 28.5 percent
denatured in the whey containing both PCMB and isoelectric casein.

On the basis of the Harland-Ashworth test, it appeared that
isoelectric casein dissolved in whey protected serum proteins against
denaturation, and this seemed to be in agreement with the suggestion of
other workers (14). The initial high absorbancy in Harland-Ashworth
filtrates from samples containing casein, and the observed protection
of serum proteins against heat denaturation in the presence of casein
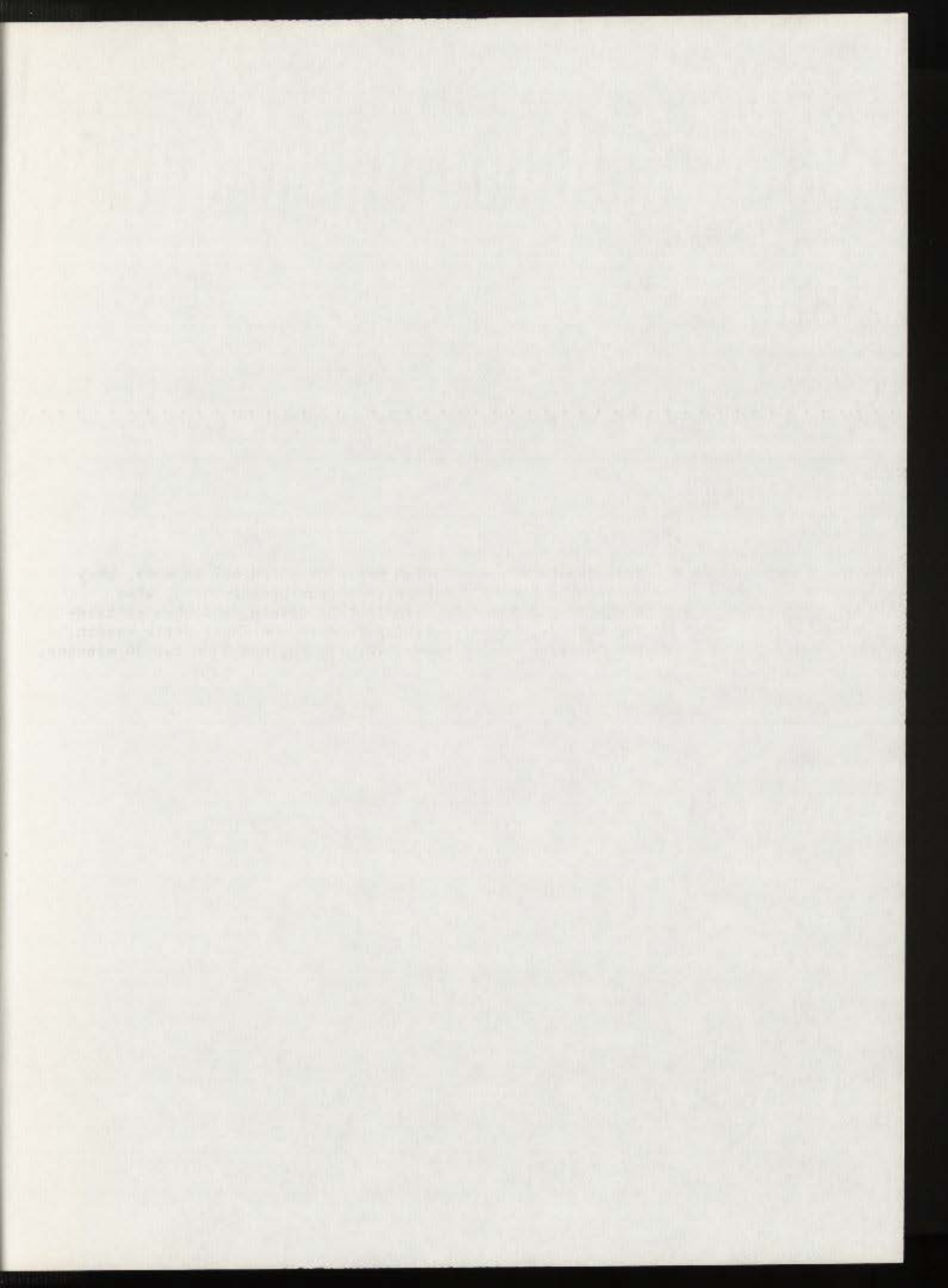was more thoroughly explained by running the Harland-Ashworth test on

Figure 5. Heat denaturation of serum proteins at pH 6.7 in whey, whey
containing 2×10^{-4} M P-chloromercuribenzoic acid, whey
containing 2.5 percent isoelectric casein, and whey contain-
ing both P-chloromercuribenzoic acid and isoelectric casein.
Samples were heated to 65, 70, 77, 85, and 93 C for 30 minutes.
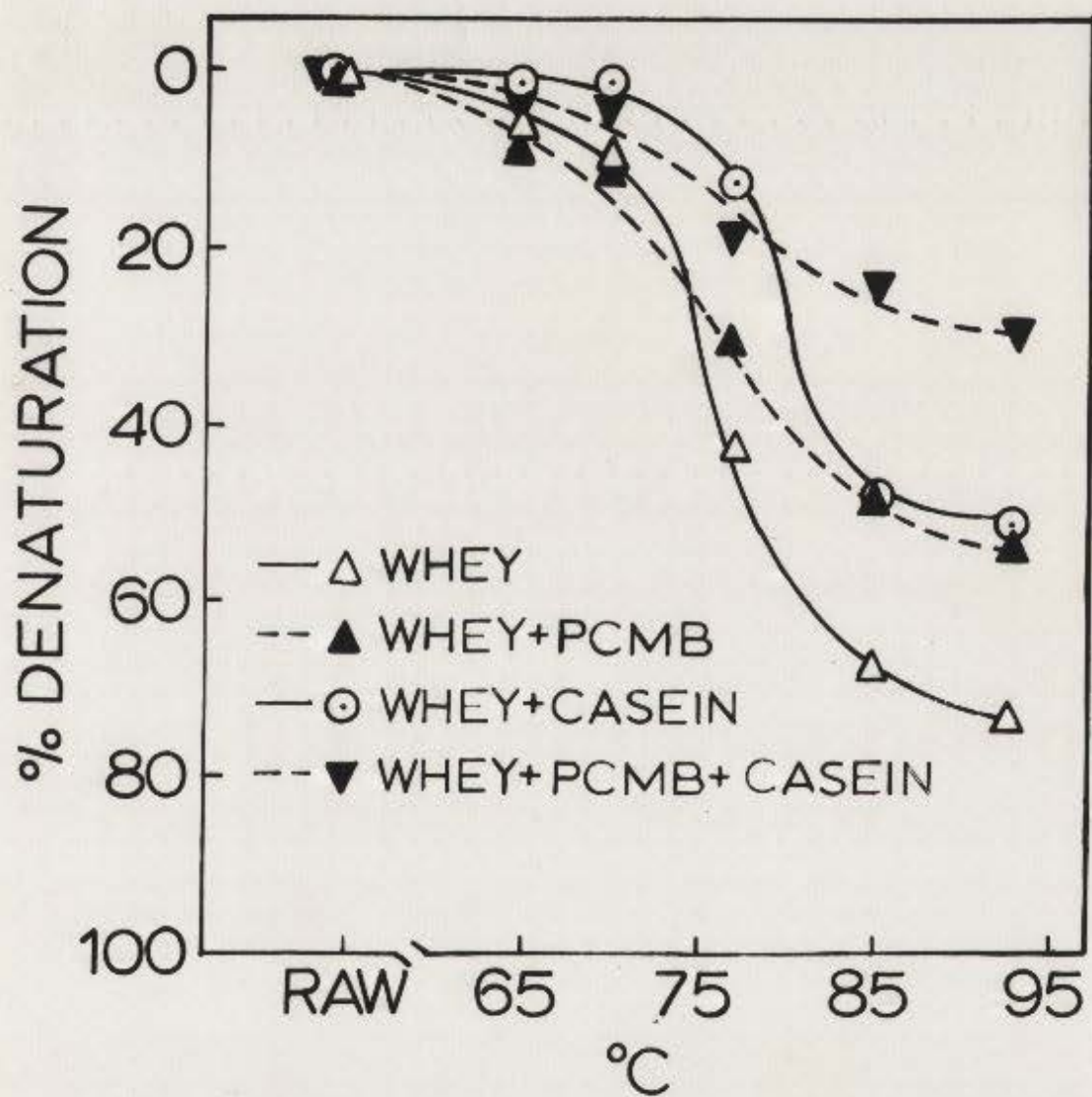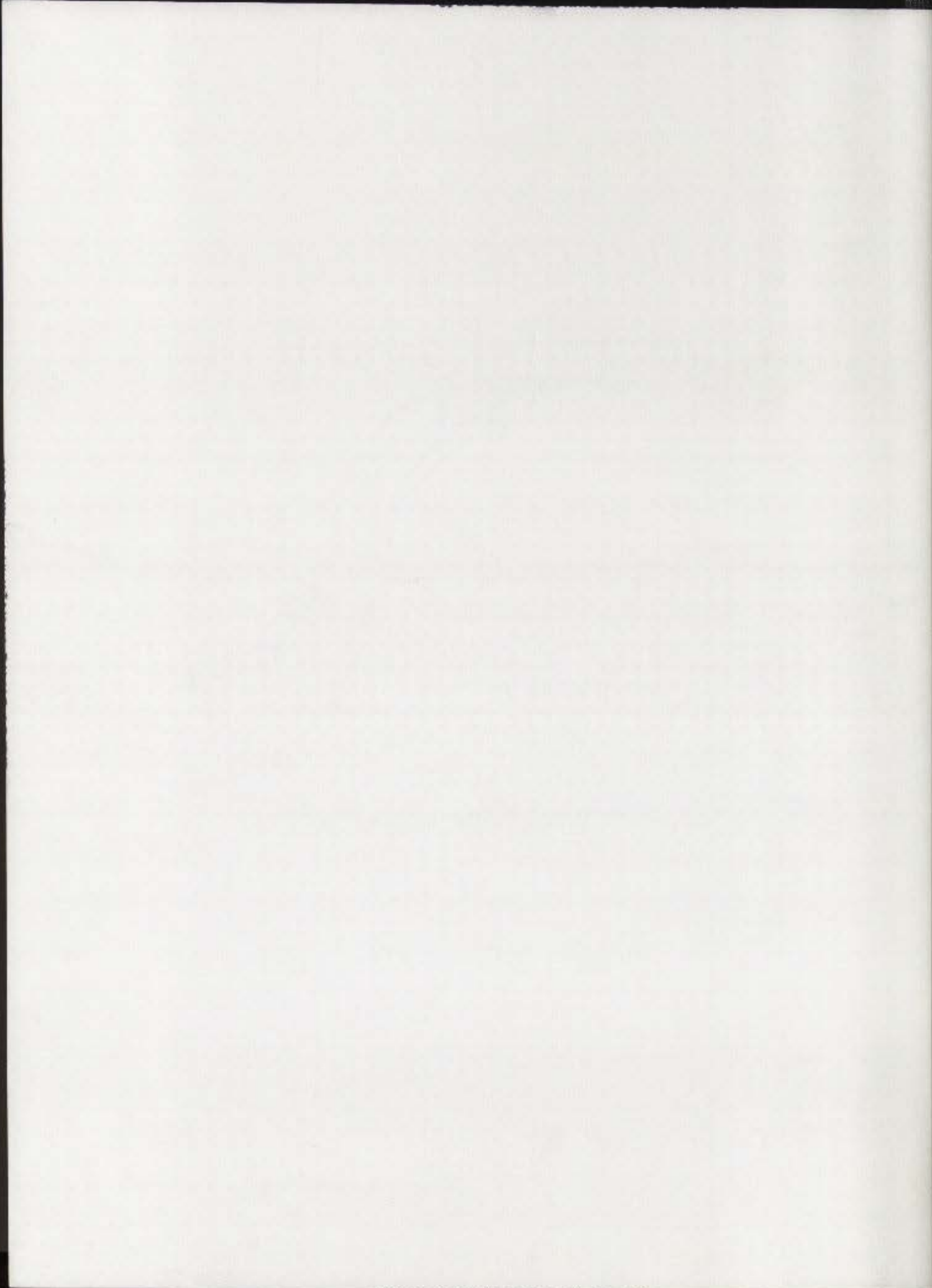
Table 1. Effect of adding 2×10^{-4} M PCMB and isoelectric casein to whey prior to heating on the absorbancy (420 mu) of Harland-Ashworth filtrates and apparent heat denaturation of serum proteins

Heat treatment	Whey		Whey + PCMB		Whey + casein		Whey + PCMB + casein	
	Absorbancy	Denaturation	Absorbancy	Denaturation	Absorbancy	Denaturation	Absorbancy	Denaturation
(°C)	(A_s)	(%)	(A_s)	(%)	(A_s)	(%)	(A_s)	(%)
Control	.447		.447		.547		.536	
65	.425	9.1	.401	9.8	.541	1.1	.521	3.0
70	.402	18.6	.395	11.1	.541	1.1	.506	5.4
77	.258	42.5	.313	30.0	.454	17.3	.440	19.5
85	.147	67.1	.230	48.4	.282	48.5	.402	24.6
93	.118	74.6	.205	53.5	.277	49.5	.376	28.5

heated and unheated solutions of 2.5 percent isoelectric casein. The

results are shown in Table 2. The unheated sample gave an average

absorbancy value of 0.088. Heating the sample increased the absorbancy

to 0.159. This showed that some component in isoelectric casein was

slightly soluble in a saturated salt solution at the pH of normal milk,

and that heating increased the amount of salt-soluble casein enough to

account for the apparent protection observed in the samples of whey

containing isoelectric casein.

Table 2. Absorbancy at 420 mu of Harland-Ashworth filtrates from heated
and unheated solutions (pH 6.7) of 2.5 percent isoelectric
casein

	Absorbancy	
Control	Heated to 93 C for 30 minutes	
(A_s)	(A_s)	
.090	.156	
.086	.161	

Identification of protein components in Harland-Ashworth filtrates

Bovine α-lactalbumin and crystalline β-lactoglobulin obtained from

Nutritional Biochemicals Corporation and bovine serum albumin obtained

from Pentex Incorporated were compared in starch gel electrophoresis

with the solids in Harland-Ashworth filtrates obtained from raw skimmilk

and rennet whey. The results are presented in Figure 6. All the Harland-

Ashworth filtrates were resolved into three bands which corresponded with

the bands produced by the purified proteins. The band of slowest

mobility was identified as bovine serum albumin, the one with medium

Figure 6. Starch-gel electrophoresis of proteins from Harland-Ashworth
filtrates obtained from 1. skimmilk, and 2. rennet whey
compared with one percent solutions of 3. bovine serum
albumin, 4. β-lactoglobulin, 5. α-lactalbumin. Samples were
dissolved in 7 m urea. Electrophoresis was run for 4½ hours
at 12 C, 250 V, and a current of 57-75 ma. A is α-lactalbumin,
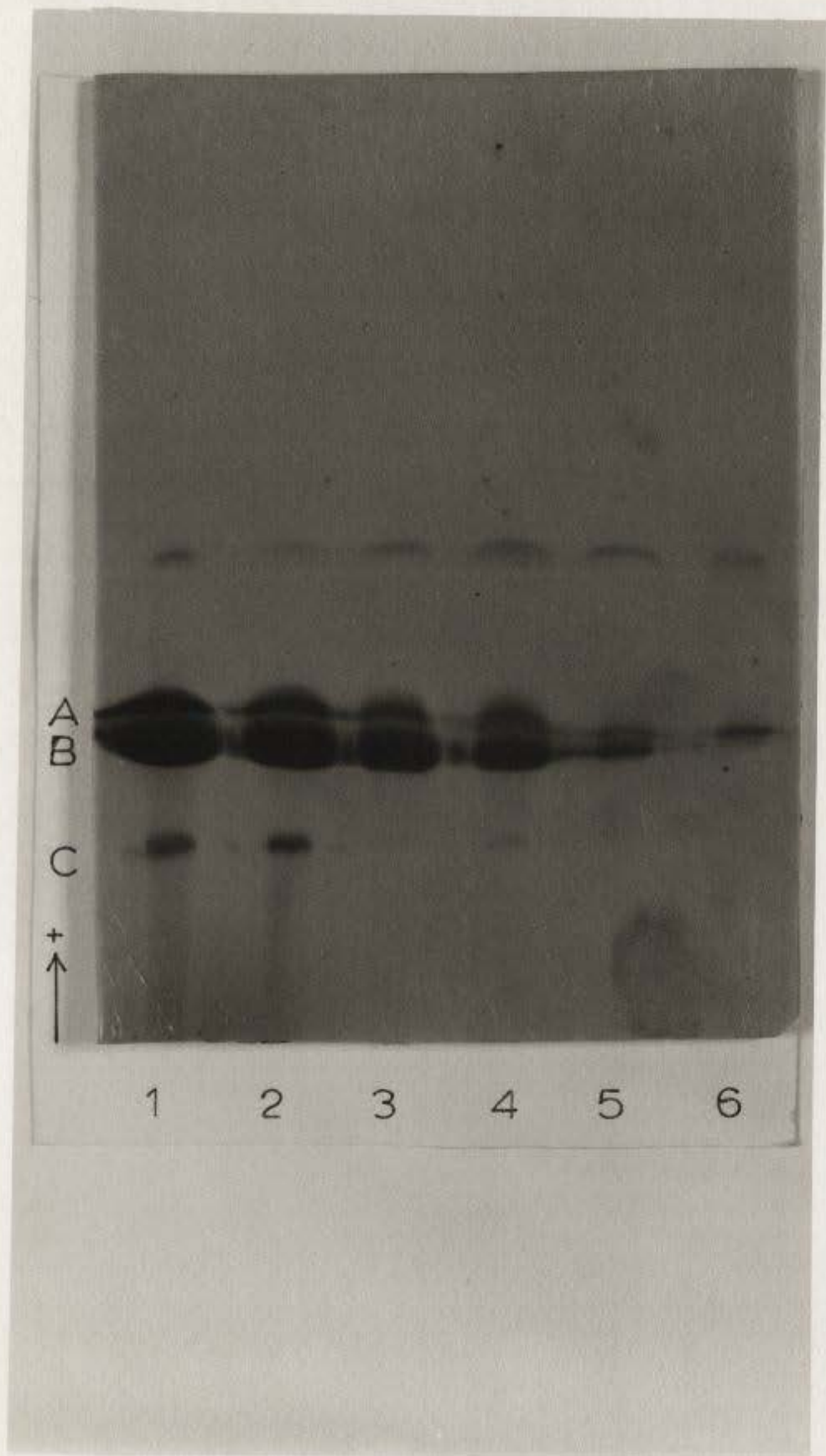B is β-lactoglobulin, and C is bovine serum albumin.
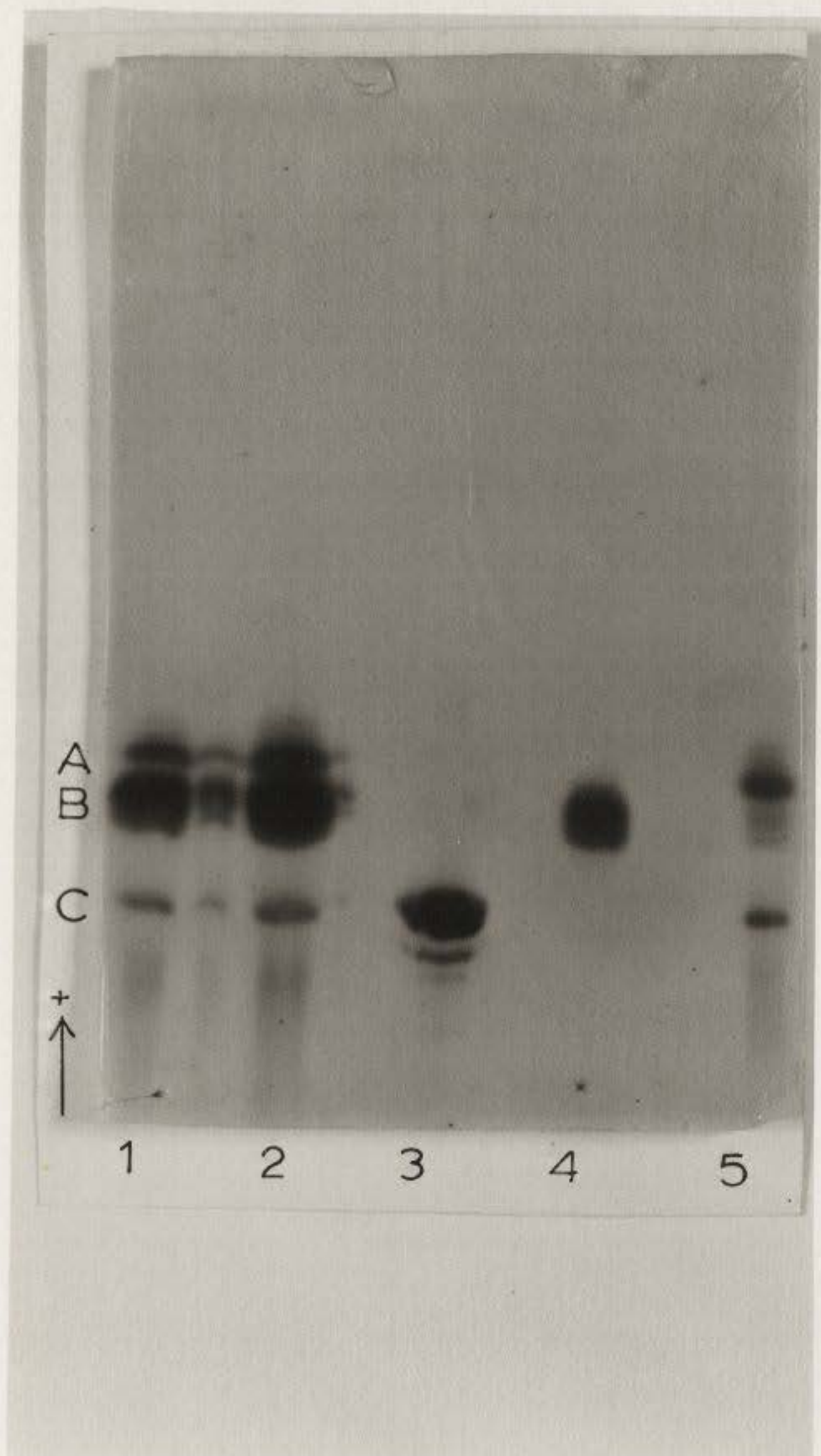
mobility was β-lactoglobulin, and the protein with the highest mobility was α-lactalbumin. It was evident that the purified α-lactalbumin sample was contaminated with bovine serum albumin.

Solids in Harland-Ashworth filtrates from milk and whey treated at 77 C and 93 C for 30 minutes and from unheated control samples of milk and whey were isolated and dried. Solids equivalent to the amount in 10 ml of each filtrate was dissolved in one-half ml of 7 M urea solution which in turn was used to saturate a filter paper insert. Electrophoresis of the proteins in these filtrates was carried out, and the results presented in Figure 7. The serum albumin in both the milk and whey samples was denatured and removed by a heat treatment of 77 C for 30 minutes. Following a heat treatment of 93 C for 30 minutes, the β-lactoglobulin was completely denatured and none remained in the Harland-Ashworth filtrate from the skimmilk; however, some still remained in the Harland-Ashworth filtrate from whey heated to the same temperature. The β-lactoglobulin in the whey filtrate accounted for the comparatively high turbidity in Harland-Ashworth filtrates of whey over those of milk.

Electrophoresis was run on freeze-dried Harland-Ashworth filtrates from whey, skimmilk, whey containing 2×10^{-4} M PCMB, whey containing 2.5 percent isoelectric casein, and whey containing both PCMB and iso-electric casein. These results revealed that the proteins protected from denaturation by blocking agents during the heating of whey and skim-milk included both β-lactoglobulin and α-lactalbumin. All samples were heated to 93 C for 30 minutes before the Harland-Ashworth filtrates were collected. The results are presented in Figure 8. The lack of serum proteins in the heated Harland-Ashworth filtrate from whey containing isoelectric casein supported the theory that isoelectric

Figure 7.   Starch-gel electrophoresis of proteins from Harland-Ashworth filtrates obtained from 1. raw whey, 2. raw milk, 3. whey treated at 77 C for 30 minutes, 4. skimmilk treated at 77 C for 30 minutes, 5. whey treated at 93 C for 30 minutes, 6. milk treated at 93 C for 30 minutes. All samples were dissolved in 7 m urea. Electrophoresis was run for 5 hours at 12 C, 250 V, and a current of 60-75 ma. A is α-lactalbumin, B is β-lactoglobulin.

Figure 8.   Starch-gel electrophoresis of proteins from Harland-Ashworth filtrates obtained from 1. whey containing 2×10^{-4} P-chloromercuribenzoic acid and 2.5 percent isoelectric casein, 2. whey containing 2.5 percent isoelectric casein, 3. whey containing 2×10^{-4} P-chloromercuribenzoic acid, 4. skimmilk containing 2×10^{-4} M P-chloromercuribenzoic acid, 5. whey, 6. skimmilk. All samples were dissolved in .25 ml of 7 m urea. Electrophoresis was carried out for 5 hours at 10 C, 250 V, and a current of 50-60 ma. A is α-lactalbumin and B is β-lactoglobulin.

casein, not serum proteins, were responsible for the high turbidities in samples containing isoelectric casein. Such turbidities in the high-heat Harland-Ashworth filtrates would indicate that isoelectric casein protected serum proteins from denaturation only if we continued to assume that all casein was precipitated by saturation with NaCl and filtered out in the Harland-Ashworth test. However, our results have shown that this is not the case and that heating systems containing isoelectric casein apparently produces anomolous results in the Harland-Ashworth test.

DISCUSSION AND CONCLUSIONS

The ability of whole casein to protect milk-serum proteins against loss of physical stability during heating and the ability of high milk-solids concentration to protect serum proteins against heat denaturation has been proposed by several workers (8, 14, 15, 16).

Work reported in this thesis has revealed that some serum proteins (β-lactoglobulin) are more succeptable to heat denaturation in skimmilk than in rennet whey. Isoelectric casein, on the other hand, appeared to increase the heat stability of serum proteins in whey. Further examination showed that the apparent heat stability of serum proteins in the presence of isoelectric casein was actually due to the heat degradation of the casein which produced erroneous results in the Harland-Ashworth test. Therefore, contrary to earlier suggestions, isoelectric casein actually had no apparent effect on the heat stability of serum proteins when the effect of heat on casein was taken into account. It appeared that β-lactoglobulin was more sensitive to heat in the presence of miceller casein than in the presence of non-miceller casein or no casein at all. The condition of the casein was assumed to be the only variable.

It was originally thought that a heat-induced interaction between β-lactoglobulin and κ-casein involving sulfhydryl groups (28) might be responsible for some serum proteins being precipitated with the casein when running the Harland-Ashworth test. Had this been true, heating skimmilk in the presence of sulfhydryl group blocking agents (28) might have blocked the reaction. As a result, more undenatured serum proteins

would be present in skimmilk after heating and the difference between the denaturation rate of serum proteins in heated skimmilk and whey would have been eliminated.

When PCMB and NEM were added to skimmilk and whey prior to heating, the Harland-Ashworth test indicated much less serum protein denatured in both whey and skimmilk. This led to the conclusion that sulfhydryl groups are involved in heat-induced changes which cause serum proteins to precipitate in saturated NaCl solutions. Sulfhydryl blocking agents produced about as much improvement in the heat stability of serum proteins in whey as they did in skimmilk; therefore, if these agents prevented the formation of a β-lactoglobulin-κ-casein complex during the heating of skimmilk, it appeared that this particular complex had little or no effect on the results of the Harland-Ashworth test. It is possible that the blocking agents prevent heat-induced complexing of β-lactoglobulin and α-lactalbumin (13) causing the observed heat stability in blocked samples.

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