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EFFECT OF VARIOUS MILK CLOTTING ENZYMES ON THE DETERMINATION OF CASEIN BY DYE BINDING PROCEDURES

by

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INTRODUCTION

Various laboratory procedures have been used for determining the percentage of protein in milk. Nearly all of these procedures were sufficiently accurate; however, none was simple, rapid and inexpensive enough to be suitable for routine analysis.

Generally, milk is purchased according to its butterfat content and its weight. There is a considerable anomaly in valuing milk solely by these two criteria, particularly when milk is converted into products such as cottage cheese, which essentially is derived from the protein content of milk, namely casein. When the casein factor is considered in milk purchases, the cheese yeild can be predicted more accurately. At the same time knowledge of casein content makes it possible to eliminate milk that might decrease cheese yield due to low casein content.

The main reasons why the fat content of milk is an important criterion for milk pricing are the relatively high value of butter, and the easy procedures by which milkfat percentage may be determined. On the other hand, the reasons why milk caseins are not considered directly in milk purchasing are the tedious and time consuming procedures used for determining the percentage casein in milk. Moreover, nonfat dry milk, of which proteins constitute a high ratio, was an inexpensive product until recent years. However, usage of nonfat dry milk has expanded; it is used in many food items such as baked goods, confectionary products, sausages and baby food. As a result, its price has increased steadily and at a rate that encouraged the dairy industry to continue to meet the increasing market demands. Compared with the prices of butter, the Dairy Situation records

show that nonfat dry milk increased three folds in the last eight years. On a wholesale price basis, during the period from March 1966 to September 1973, butter price increased from 63.4¢/lb to a peak of 70¢/lb in 1972, but dropped to 63.4¢/lb again in September 1973. During this same period, nonfat dry milk wholesale price increased from 15.6¢/lb to 45.8¢/lb. In the winter and spring of 1974, the price of nonfat dry milk rose to 70¢/lb.

The objective of the research reported in this thesis was to study the effect of various milk clotting enzymes on the accuracy of measuring casein by the dye binding procedure which could be used to measure the casein content of milk as a factor in milk pricing in addition to the conventional milkfat and weight basis.

The investigation reported herein is an extension of the work done at this University by Dr. Ross Mickelsen and Dr. Nazar A. Shukri on measuring casein by dye binding.

LITERATURE REVIEW

Principles of Protein Determination by Acid Dyes.

The precipitation of proteins by acid dyes was first reported by Chapman et al (6) in 1927, when they observed that in titrating a protein, organic acid dyes reacted similarly to simple acids. Also in 1929, Stearn and Stearn (21) observed that a chemical reaction took place between dye ions and the oppositely charged ions of protein. The above observations were further investigated by Fraenkel-Conrat and Cooper (9) in proteins from various sources. They indicated that the polar groups in protein bind dyes of the opposite charges resulting in a protein-dye complex in the form of a precipitate.

Quantitative determination of proteins by acid dyes has been agreed upon by many workers. Certain acid dyes form insoluble precipitates with certain proteins. The removal of protein by the formation of insoluble precipitates with the dye will carry the reaction further to completion than if the end-products were soluble. The fact that different proteins were titrated to the same equivalence regardless of the dye used in the titration, indicates that the reaction takes place in stoichiometric proportions (6). Ashworth and Chaudry (2) reported that it is commonly assumed that each unit weight of protein binds and precipitates a constant amount of dye due to a stoichiometric reaction between the basic amino groups of the protein and the acid groups of the dye. The ratio of dye bound per unit weight of protein is called the Dye Binding Capacity (DBC), which under certain conditions appears to be constant. This consistancy is the basis for the quantitative methods for measuring proteins by dye

binding techniques . Stearn and Stearn (21) showed that in a buffer at pH 2.2, the acid orange G dye combined quantitatively with basic groups of proteins. Also they showed that casein and beta-lactoglobulin bound, 0.34 and 0.58 MM of dye per gram of protein respectively. Schobler et al (17) indicated that the fractions of casein have binding capacities in the ratio of 1.0:0.63:0.26 for alpha, beta and gama casein, respectively. Also, it was reported (3) that whey proteins gave higher binding values than casein, while dialized nitrogen fractions bound no dye, thus demonstrating that no reaction occurs between nonprotein nitrogen and acid dyes.

Microanalytical methods developed by Fraenkel-Conrat and Cooper (9) for estimating the number of acid and basic groups on proteins were based on the tendency of the polar groups to bind the opposite charge, which resulted in precipitation of the protein-dye complex. The acid orange G dye combined quantitatively with the basic groups on protein in a buffer of pH 2.2. The basic dye, safranine O, reacted with acid groups on the protein at pH 11.50. However, the extent of combination in this latter case was affected by other factors such as protein concentration and therefore was not linear.

A variety of proteins was shown by Perman (13) to combine with metaphosphoric acid to form an insoluble precipitate in which the phosphorus content was equivalent to the number of positively charged groups on the protein. Also, egg albumen was found (14) to react with metaphosphoric acid forming a precipitate containing a constant proportion of unbound metaphosphate.

Dyes Used for Milk Protein Estimation.

Milk has a very accessable protein system that rapidly interacts with azo dyes. It is most suitable in adopting automated protein

determination by dye binding. Traditionally, four dyes have been used for quantitative measurement of milk proteins: amido black 10B, Buffalo black, orange G and amino black 10B. Dolby (8) investigated amido black 10B and orange G methods for protein estimation in milk. He observed that dye samples of various degrees of purity all gave a linear relationship between dye and protein precipitation as determined by optical density. However, with the less puredye samples, slightly less dye was bound per unit weight of protein. Also, he noted that amido black, though reacting with protein in the same molar ratio as orange G, gave much more sensitive optical indication of protein content. Shiga et al. (19), also noted the superior sensitivity of amido black 10B compared with orange G. Ashworth and Chaudry (2) observed that orange G dye showed more stoichiometry in its reaction with milk proteins than did amido black 10B, although the latter gave a higher DBC. However, the DBC for amido black 10B decreased more rapidly with increased dye: protein ratios than did corresponding values for orange G dye.

Schobler and Hetzel (17) described a method employing amino black dye which forms an insoluble dye-protein complexes. The precipitate may be removed by either filtration or centrifugation and the supernatant read in a spectrophotometer to determine the concentration of the residual dye.

Vanderzant and Tennison (30) determined milk proteins by using buffalo black dye in a phosphate buffer at pH 2.3. They observed a linear relation between optical density values of the supernatant fluid and the protein values as determined by the Kjeldahl method.

Effect of Variation in Experimental Conditions on the Amount of Dye Bound by Proteins.

Fraenkel-Conrat and Cooper (9) indicated that for the purpose of

convenience and practicality, the protein-dye combination was carried out in a buffered solution, thus circumventing the need for pH measuring and adjusting. Buffers of 2.2 and 11.5 were selected because Chapman et al (6) and Rowlin and Schmidt (16) had indicated that complete dissociation of protein groups in the presence of dye was approached at these points. It did not seem advisable to use stronger acid or alkaline buffers, since the advantage of more complete dissociation of protein groups might be overshadowed by the disadvantage of protein breakdown. Thus, for example, protein treated with safranine O at pH 12.0 bound considerably more dye than at pH 11.5. This phenomenon might be attributed to either protein breakdown or physical adsorption of dye. Udy (29) used orange G 12 dye in a stabilizing pH 2.0 buffer system to react with the protein's basic groups that originate from the basic amino acids histidine, arginine and lysine.

Factors Affecting the Dye Binding Capacity of Proteins.

Milk constituents. Udy (28) demonstrated that proteins are the only milk constituents that bind the acid dyes. He based this finding on the fact that an extrapolated straight line relating protein percentage and the concentration of unbound dye passed through the zero of percentage protein and the original concentration of dye. Several findings by Seals (18) confirmed Udy's observations:

a) The dye binding capacity values are the same for dry whole milk and nonfat dry milk from the same lot of whole milk.

b) One milliliter of milk fat from which the curd has been removed by washing with warm water bound no measurable amount of dye.

c) When 0.5 ml of the milk fat was added to one milliliter of

skimmilk containing 35 mg of protein (N X 6.38), the dye binding procedure gave 34.5 mg of protein.

d) Increasing the amount of lactose up to 85 mg per 65 mg of pure casein solution in a two milliliter volume did not change the dye binding capacity of casein.

e) Samples of varying percentages of milk fat tested for protein by both dye binding and macro-Kjeldahl, gave no relationship between the difference in protein level and the percentage fat in milk.

Ashworth et al. (4) observed that doubling the amount of lactose in milk did not affect the amount of dye bound; however, the addition of one percent calcium chloride caused a 25% decrease in the optical density of the supernatant.

Time and temperature. Perman (13) showed that time and temperature had no effect on the amount of dye which could combine with certain proteins in solution. He indicated that within limits of experimental conditions the amount of protein (gelatin or fibrin) combined with dye is independent of time and temperature. Ashworth and Seals (3) found that after mixing the dye and protein, the mixture could be left at room temperature for 14 days without change in the optical density of the unbound dye. Also, they reported that in the temperature range between 46 and 104 F, no significant effect of temperature could be observed.

Heat treatment of samples. It is well known that when some materials are subjected to heat processing (11), the labile, and often limiting amino acid, lysine, is chemically altered and becomes nutritionally unavailable. In these instances, the nitrogen content remains the same, but the amount of found dye is reduced in proportion to the amount of

lysine destroyed. Thus dye binding offers a means of determining both quantity of protein and nutritional value.

Determination of Casein and Whey Proteins by Dye Binding.

Dolby (8) determined milk casein with a dye binding technique using amido black 10B dye. Both total and whey proteins were separated by Rowland's method (15). Total proteins and whey proteins also were estimated by the Kjeldahl method and the casein was figured by difference. The mean error of total proteins, whey proteins and casein was 0.025, 0.044 and 0.019% protein, respectively. On the average whey protein content was slightly lower when measured by dye binding than when measured by Kjeldahl procedures. However, casein values calculated by difference agreed quite closely. Ashworth and Seals (3) estimated serum proteins by acid orange G dye. The casein was removed by isoelectric precipitation and 15 ml of the filtrate were mixed with 25 ml (0.95 mg/ml) of the dye in a centrifuge bottle. The mixture was centrifuged and the absorbance of the supernatant was read in a Beckman model B spectrophotometer at 420 nm. The difference in absorbance between the standard and the sample was taken as a measure of the amount of protein.

Mickelsen and Shukri (12) determined casein from unheated skimmilk by a dye binding technique using acid orange G 12 dye. The whey was separated by clotting unheated skimmilk with veal rennet. The resulting curd was cut, centrifuged and filtered through Whatman #41 paper. Total proteins and whey proteins were determined by dye binding and casein was figured by difference. They reported a correlation coefficient of 0.82 between their method and casein determined by AOAC method No. 1 (5).

Comparison Between Dye Binding and Other Methods Used for Determining Milk Proteins.

In comparison with Kjeldahl, Udy (27) found the standard error of estimate for the dye binding method was ±0.42% for spray dried milk. Treece et al. (25) reported a standard error of estimate of 0.05% protein for orange G and Kjeldahl methods for whole milk from individual cows. When disregarding non-protein nitrogen they observed that the dye method was as accurate as Kjeldahl and took only one-fourth to one-fifth the time. They reported a correlation of 0.98 between the two methods, indicating that orange G dye and Kjeldahl methods were equally accurate. Also, they observed that the standard deviation showed less variation with dye binding than with Kjeldahl. Furthermore, they cited the following advantages: The cost per sample was much less; no handling of strong acids and alkalies were necessary; the method was simple enough to be used routinely; and it was adaptable to field use.

A relatively constant relationship was observed by Dolby (8) between the dye binding values and Kjeldahl proteins in milks of individual cows from three to four days after calving to three to four weeks before the end of lactation. He observed that late lactation milks gave about 5% higher dye values than did normal milks of the same protein content. Treece et al (25) using slight modification of Uye's orange G method, found no significant difference between dye binding and Kjeldahl ($P<0.001$).

Mickelsen and Shukri (12) determined casein content of unheated skimmilk by four methods and reported standard deviations of 0.0612, 0.1412, 0.1918 and 0.9895 for dye binding, AOAC, Rowland's and formol titration methods, respectively.

MATERIALS AND METHODS

Enzymes Used and Their Sources.

The original objectives of this work was to study the effect of various milk clotting enzymes on the accurancy of measuring casein by the dye binding technique developed by Mickelsen and Shukri (12). To achieve this purpose, five commercial milk clotting enzymes were evaluated for their suitability for use in the casein test. The enzymes used were: veal rennet, adult bovine rennet, rennet-pepsin blends, and the milk clotting enzymes derived from the fungi Mucor miehei and Mucor pusillus. All of the enzyme preparations were obtained from commercial rennet suppliers and designated by the supplier as being "single strength."

Preparation of Substrate for Rennet Testing.

Low-heat nonfat dry milk (NDM) was prepared at the Kansas State University milk processing plant and placed in sealed glass jars to prevent moisture accumulation. The NDM samples were kept at minus 5 C until used. Sixty grams of the NDM were transferred to one liter Erlenmeyer flask containing 500 ml of 0.01 M calcium chloride. The mixture was agitated on a mechanical shaker until no visible undissolved NDM was apparent. A crystal of thymol was added as a preservative and the milk was chilled in an ice bath and allowed to equilibrate at 2 C for 20 h before it was used (31).

Measuring Rennin Activity.

Twenty five milliliter aliquots of reconstituted substrate were pipetted into 100 ml wide mouth rennet testing bottles. The bottles were stoppered and placed in a water bath at 30 C until the substrate

temperature reached 30 C. One milliliter of 1:250 water dilution of each of the five enzymes was added to the substrate in triplicate. The substrate containing the added enzyme was placed onto a rennet tester described by Sommer and Matsen (23). The first appearance of visible flecks on the rotating glass surface was taken as the end point. Clotting time was recorded to the nearest second.

Veal rennet was arbitrarily assigned 100 rennet units per ml. The rennet strength of the other four enzymes was determined and compared to veal rennet. Enzyme dilutions were prepared in such a way that they contained the same number of rennet units per ml. The dilutions that gave the same clotting time were 1:250, 1:322, 1:411, 1:474, and 1:514, for veal rennet, rennet derived from Mucor miehei, adult bovine rennet, rennet derived from Mucor pusillus and rennet-pepsin blend, respectively. Dilutions of milk clotting enzymes ten times stronger than those listed in the foregoing (i.e. 1:25, 1:32.2, 1:41.1, 1:47.4 and 1:51.4) were prepared fresh each time they were used in determining casein by dye binding. The more concentrated dilutions were preferred in order to avoid excessive dilution of milk and to decrease the amount of time necessary to achieve a firm clot.

Sources of Milk.

Raw skimmilk used in this study was obtained from the bulk milk tank of Kansas State University milk processing plant after it has been separated to reduce the milkfat to less than 0.15%. Some lots were from the University Dairy herd. Other lots were mixtures of milk from the University Dairy herd and milk from Mid-America Dairymen, Sabetha, Kansas. Raw milk was obtained from the bulk milk tank at the Kansas State

University Dairy barn immediately after the evening milking was finished. The fat test was determined by the Milko-Tester procedure.

Selection of Incubation Temperature and Time for Milk Clotting.

Incubation temperature. Nine different time-temperature combinations were evaluated to determine optimum conditions for clotting milk to be tested for casein content by the dye binding procedure.

Twenty seven samples, 200 ml each of unheated skimmilk were transferred to 250 ml beakers and divided into three groups of equal sample size (9 samples each). The first group was incubated at 23 C (room temperature), the second group at 27 C and the third group at 30 C. One ml of 1:25 dilution of veal rennet was added to each beaker. Equal samples of each group were tested for curd formation by cutting thoroughly with a spatula after 30 minutes, one hour and two hours incubation time.

Incubation time. Nine samples (200 ml each) of unheated skimmilk were incubated at 30 C after adding one milliliter of 1:25 dilution of veal rennet. The samples were divided into three groups tested at 30 minutes, one hour and two hours incubation time. After the designated incubation period, the curd was cut and one sample of each group was transferred to a 60 C water bath for 15 minutes to inactivate the rennet. The whey was then cooled to 30 C, filtered and whey protein concentration determined by dye binding. The other two samples of each group were filtered directly after the curd was cut. One sample of each group was immediately tested for whey protein concentration, the other was left at room temperature for 6 hours and then tested for whey protein concentration by dye binding. The foregoing procedure was repeated for the remaining four enzymes used in the study.

Milk and Enzyme Preparation.

After thorough mixing, 200 ml volumes of unheated skimmilk were pipetted into 250 ml beakers and tempered to 30 C in a thermostatically controlled water bath. During the time samples were tempering, enzyme solutions were diluted with distilled water to predetermined concentrations. One milliliter of each diluted enzyme was added to 200 ml of skimmilk. The mixture was stirred then left undisturbed for one hour to clot.

Whey Separation.

After one hour incubation time, the curd was cut using a spatula. One beaker of each duplicate was covered with polyethylene film to retard moisture loss through evaporation, and transferred to a 60 C water bath for 15 minutes. At the end of the heating period, the temperature of the samples reached 58 to 59 C which was high enough to inactivate the enzymes and inhibit their proteolytic activity on the proteins. The samples then were left to cool to 30 C before filtering.

The unheated sample for each enzyme was filtered immediately after cutting the curd. The whey thus obtained was divided into two portions; one portion was immediately mixed with the acid dye reagent and tested for whey protein content. The other portion was kept at room temperature (23 C) for six hours before it was tested for whey protein content. All samples were filtered through Whatman #41 paper.

Except for the experiment pertaining to reaction time between acid orange G 12 dye and whey proteins, the experimental design followed the outline shown in Figure 1.

Reaction Time.

It has been reported by Ashworth (1) that the reaction between whey

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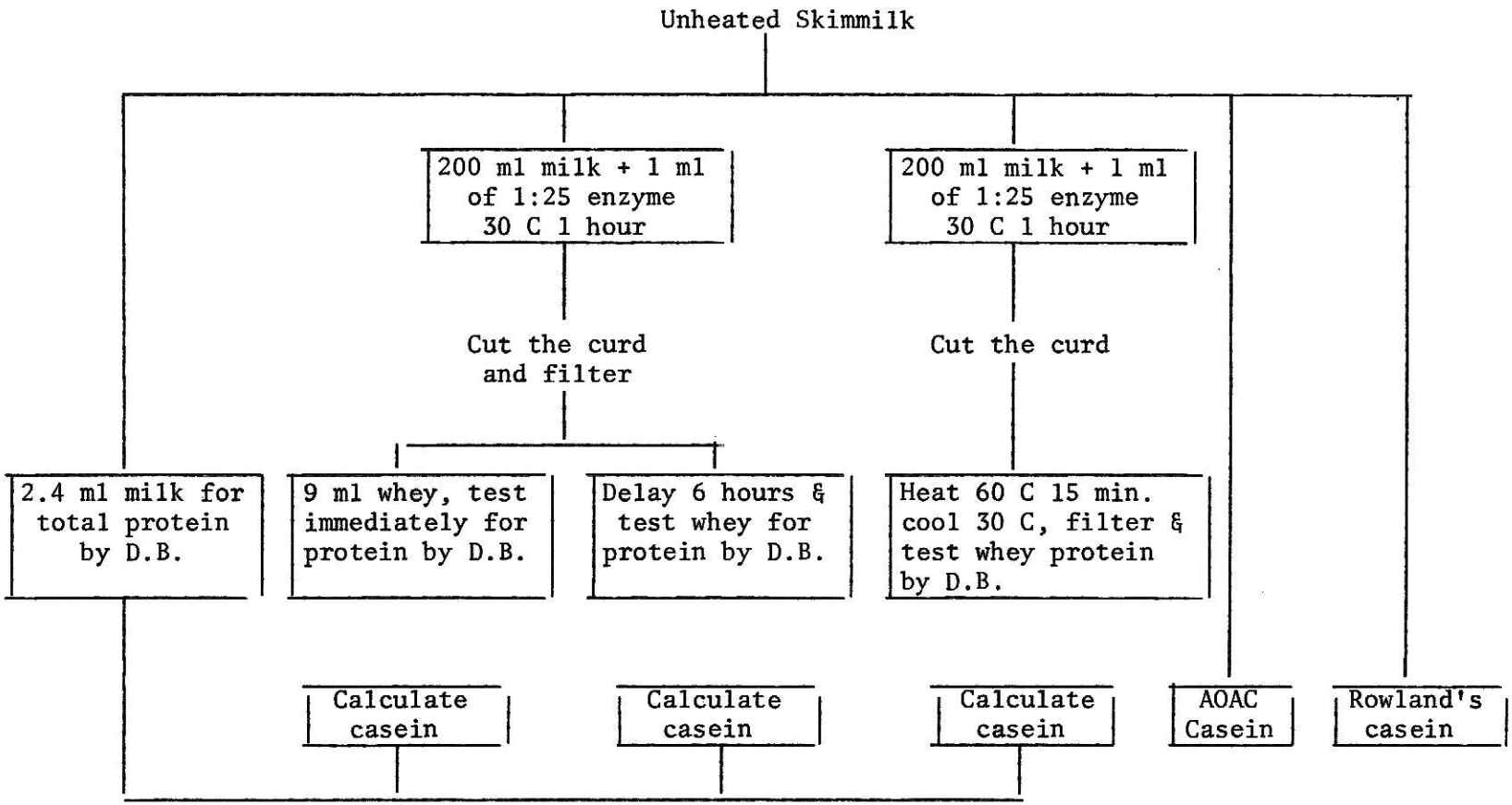

FIG. 1. Flow sheet of the experimental design used to evaluate milk clotting enzymes and procedures for application in the dye binding method for determining casein.

proteins and acid orange G 12 dye proceeds rather slowly to equilibrium. Therefore, an experiment was conducted to study the effect of reaction time between whey proteins and the dye on the outcome of determined casein by dye binding procedures. The whey proteins and dye were allowed to react for zero, two, four, six and 10 hours prior to being tested in the Udy instrument.

Acid Orange G 12 Dye Dilution.

The reagent dye used in this study was acid orange G 12 dye which was received in a concentrated form from the manufacturer (26). The concentrated dye reagent was diluted approximately 10 fold with distilled water. The directions for dilution were provided by the manufacturer.

The one half gallon volume of concentrated dye was warmed in a hot water bath for at least one hour. Then all the dye was carefully transferred to a 20 liter carboy containing about 12 liters of distilled water at 21 C. The half gallon concentrate container was rinsed many times to make sure that all the dye was transferred to the carboy. The dilution was completed by adding distilled water at 21 C up to the 20 liter mark. The incorporated air bubbles were removed by gentle tapping with a plastic stirring tube and then refilled to the calibration mark. The diluted reagent dye was thoroughly stirred for about five minutes and left to be used the following day. The final dilution was adjusted to read exactly 37% transmission when mixed with an equal volume of distilled water and tested in calibrated Udy Analyzer.

Standardizing Udy Analyzer.

Udy Analyzer (Udy Analyzer Company, Boulder, Colorado, Model 101) was used in this study for measuring the percentage transmittance of the

unbound dye remaining from the mixture of the sample tested and acid orange G 12 dye reagent. The analyzer was switched on at least two hours to warm up before it was used. The instrument was adjusted by reference dyes from a five milliliter pipet until it steadily read exactly 42% transmittance while the dye was flowing through the cuvette.

Casein Determination in Skimmilk.

After thorough mixing of the raw skimmilk sample, approximately 2.4 ml was transferred by a syringe into a preweighed two ounce polyethylene dropper bottle. The bottle plus sample weight was determined so that the weight of milk used could be obtained by difference. Then 40.44 (40 ml) ± 0.04 acid orange G 12 dye were added from an automatic pipet. The mixture was vigorously shaken for 30 sec on a Vertix Genie Vibrator (Fisher Scientific) to insure thorough mixing of milk sample and dye. The unbound dye was filtered through a spun glass filter into the cuvette funnel of the Udy instrument. Ten to fifteen drops of filtrate were slowly and carefully dropped into the cuvette until the spectrophometer meter needle gave a steady reading. Percentage transmittance was recorded to the nearest 0.25%, avoiding parallax. Then total milk proteins were calculated using milk calibration tables prepared by the instrument manufacturer.

Whey proteins were determined by the same procedure except that nine ml of whey were tested instead of 2.4 ml of skimmilk. Casein was calculated by subtracting whey protein concentration from total protein content of the unheated skimmilk.

Determination of Casein in Whole Milk.

The foregoing procedure for measuring casein content of skimmilk also was extended to whole milk. As milk fat does not affect the dye binding capacity of milk proteins (18), the amount of milk proteins

replaced by milk fat was compensated for by increasing the volume of sample used to measure total proteins in whole milk by dye binding. Instead of 2.4 ml (for skimmilk), 2.5 ml of unheated whole milk were used. Otherwise, the same procedure used for unheated skimmilk was followed. Combinations of adult bovine rennet and heat treatment of the clotted milk were the variables used for measuring casein in whole milk.

RESULTS AND DISCUSSION

Since milk, compared with other food products, has a very accessible protein system that rapidly interacts with azo dyes, it was considered the most suitable substrate for dye binding procedures.

In order to determine the optimum conditions for casein determination and propose a suitable procedure for accurate and precise results, the effect of several analytical parameters were investigated. Three treatments with each of five milk clotting enzymes were conducted and compared to results obtained by AOAC and Rowland's procedures. The data from the 15 treatments show the effect of each treatment on measuring casein by dye binding techniques.

Selection of Optimum Incubation Temperature and Time for Milk Clotting.

Temperature selection. The group of samples held at 23 C did not clot for up to four hours incubation. The group incubated at 27 C did not clot in 30 minutes; however, a very soft curd was obtained after one and two hours incubation time. The third group, which was incubated at 30 C, gave a soft curd in 30 minutes, and a firm curd was obtained after both one and two hours incubation. Therefore, the 23 and 27 C incubation temperatures were disregarded because they gave clots which were unsuitable for application in the casein determination procedure.

Time selection. The 30 minutes incubation time resulted in a soft curd and milky whey with all enzymes used in the test except rennet-pepsin blend where no clotting occurred. The one hour incubation period resulted in firm curd with all enzymes except rennet-pepsin blend which had a soft curd. After two hours incubation, the rennet-pepsin blend still had a

soft curd; however, the samples clotted by the other four enzymes were firm and started wheying off excessively before cutting, indicating that two hours incubation was longer than necessary. Thus, one hour incubation time was selected as the most suitable time with respect to curd firmness, clarity of whey, time taken to conduct the casein test, and accuracy of measuring casein by dye binding as compared to AOAC procedures. Results obtained are shown in Tables 1 and 2.

Effect of Delayed Whey Test on the Casein Test.

As milk clotting enzymes used in this study were proteolytic, their effect on measuring casein by the dye binding technique was studied. Unheated whey was left for six hours at room temperature (23 C) before acid orange G 12 dye was added to test for whey protein concentration. The results obtained are shown in Table 3. Casein percentage was slightly higher in samples of whey left for proteolytic enzymes to act upon as compared to the same sample tested immediately after filtering. A small but not significant ($P>.05$) increase in casein percentage resulted by holding the mixture for six hours with all enzymes except veal rennet. The reason for this small increase in percentage casein in the delayed test of whey possibly could be explained by whey proteins undergoing degradation by the proteolytic milk clotting enzymes to non protein nitrogen compounds which were not bound by the acid orange G 12 dye. This decrease in whey protein concentration would consequently be added to casein as the latter was calculated by difference. Therefore, the increase in reported casein due to proteolytic activity of milk clotting enzyme should not be considered as casein.

A more significant proteolysis likely would have been obtained if the whey samples were incubated at (37 to 39 C) normal body temperature

TABLE 1. Whey protein concentration at different incubation times and different treatments.

Enzymes	Whey proteins (%) [1]								
	30 minutes incubation			One hour incubation			Two hours incubation		
	1 [2]	2 [3]	3 [4]	1	2	3	1	2	3
Veal rennet	0.9650	0.9214	0.8407	0.8995	0.8867	0.8461	0.8849	0.8791	0.8594
Mucor miehei rennet	1.1474	1.1173	0.8806	0.9486	0.9377	0.8711	0.9026	0.9088	0.8800
Adult bovine rennet	1.0258	1.0094	0.8943	0.9699	0.9310	0.8734	0.9116	0.8744	0.8745
Mucor pusillus rennet	1.3011	1.2470	1.1698	1.0098	0.8347	0.9745	0.8905	0.8474	0.8408
Rennet-pepsin blend	------	------	------	1.0598	1.0604	0.8647	0.9071	0.9079	0.8994

[1] As determined by dye binding of rennet whey using milk and milk product standard conversion table prepared by the Udy analyzer company of Boulder, Colorado.

[2] Whey mixed with the dye and tested immediately after filtering.

[3] Whey left for six hours at room temperature before adding the dye and testing.

[4] Whey heated at 60 C for 15 minutes, cooled to 30 C and tested.

TABLE 2. Effect of different incubation times on results of casein determination by dye binding.

Enzymes	Casein (%)								
	30 minutes incubation			One hour incubation			Two hours incubation		
	1 [1]	2 [2]	3 [3]	1	2	3	1	2	3
Veal rennet	2.3649	2.4085	2.4892	2.4304	2.4432	2.4838	2.4450	2.4508	2.4705
Mucor miehei rennet	2.0973	2.1274	2.3641	2.2961	2.3070	2.3736	2.3421	2.3359	2.3647
Adult bovine rennet	2.2045	2.2209	2.3360	2.2717	2.2993	2.3569	2.3230	2.3559	2.3558
Mucor pusillus rennet	1.9466	2.0007	2.0779	2.2379	2.2732	2.4130	2.3566	2.4003	2.3994
Rennet-pepsin blend	------	------	------	2.1879	2.1873	2.3840	2.3406	2.3398	2.3483

[1] Whey mixed with the dye and tested directly after filtering.

[2] Whey left for six hours at room temperature before adding the dye and testing.

[3] Whey heated at 60 C for 15 minutes to inactivate enzymes.

TABLE 3. Effect of proteolytic activity of milk clotting enzymes on percentage casein when determined by the dye binding procedure.

Enzymes	Casein (%)		
	Whey immediately tested	Whey tested after six hours [1]	Difference (%)
Veal rennet	2.3559[a]	2.3550[a]	- -0.0009
Mucor miehei rennet	2.3301[b]	2.3474[b]	+ +0.0158
Adult bovine rennet	2.3294[c]	2.3371[c]	+0.0077
Mucor pusillus rennet	2.1892[d]	2.2349[d]	+0.0257
Rennet-pepsin blend	2.1446[e]	2.1629[e]	+0.0183

Pairs of means denoted by the same superscript did not differ ($P \leq 0.05$)

[1] Whey left for six hours at room temperature before adding dye and testing for whey protein concentration.

at which milk clotting enzymes normally act. Jenness and Patton (10) found that significant proteolysis occurs in cow's milk at 37 C even when bacterial counts are negligible. Furthermore, Storrs and Hull (24) demonstrated the liberation of the amino acids tyrosine and tryptophan in cow's milk incubated at 37 C for time periods up to six hours. The above observation agreed with the findings of Cole (7) in which he indicated that milk proteins were more extensively proteolyzed by bacterial enzymes at 37 C than they were at 32 or 25 C.

Effect of the Reaction Between Whey Proteins and Acid Orange G 12 Dye.

Results of reaction time on whey proteins determined by dye binding are shown in Table 4. Although the first test at zero hour appeared to give lower whey protein concentration, two-way analysis of variance (20) indicated no difference ($P>0.05$). Therefore, whey proteins can be determined with minimum holding time necessary for the reaction between milk proteins and acid orange G 12 dye to attain equilibrium without incurring significant differences in the casein test. A minimum contact time of 10 min was recommended by Steinholtz (22) provided thorough mixing was assured.

Effect of Heat Treatment of Whey on the Casein Test.

The primary objective of heating rennet whey to 60 C soon after the incubation period was to inhibit the proteolytic activity of the enzymes used for clotting milk samples for measuring casein by dye binding. Generally, it was observed that whey obtained from all heat treated samples was more clear than the corresponding unheated duplicates. However, within the same heat treatment, whey obtained from milk samples clotted by the enzyme derived from Mucor pusillus and rennet-pepsin blend was not as clear

TABLE 4. Effect of time on the reaction between whey proteins and acid orange G 12 dye.

	Whey Proteins (%)				
	0 hours	2 hours	4 hours	6 hours	10 hours
Means[a/]	0.8223	0.8523	0.8559	0.8588	0.8569[b/]

[a/] Means are the average of 10 samples of unheated skimmilk tested in triplicates.

[b/] A solid line under adjacent means indicates homogeneity.

as that obtained from milk samples clotted by the other enzymes used in this experiment.

Percentage casein derived from heat treated samples compared to those obtained from their corresponding unheated duplicates are shown in Table 5. The amount of casein obtained from both heated and unheated treatments of samples of milk clotted by veal rennet, rennet derived from Mucor miehei did not differ ($P>0.05$). However, casein content determined from heated and unheated samples of milk clotted by rennet derived from Mucor pusillus and rennet-pepsin blend differed ($P<0.05$) (20).

Comparison of Different Methods for Measuring casein.

Comparison of procedures. The present procedures recognized generally for measuring casein are the AOAC method No. 1 (16.041) (5), Rowland's (15) and formol titration (32) methods. The AOAC is the standard official method used in the United States; it is accurate and characterized by low standard deviation. However, it is time consuming and involves the use of Kjeldahl procedures with concentrated acid and alkalies. Rowland's method for measuring casein also is accurate with low standard deviation, but it is more time consuming than AOAC because it involves determination of both total and whey proteins by Kjeldahl procedures. Although the formol titration method is simple and rapid, it is not accurate enough to be used for precise work. Results usually are characterized by high standard deviation. Another disadvantage of the formol titration method for milk analysis, is that milk proteins differ in amino nitrogen content, and since their proportions also vary, the factor relating formol titer to protein content is not constant (10).

The dye binding procedure developed by Mickelsen and Shukri (12) for measuring casein is simple, rapid, precise and less expensive. In terms

TABLE 5. Effect of heat treatment on percentage casein as determined by by dye binding.

Enzymes	Casein (%)		
	Unheated	Heated [1/]	Difference (%)
Veal rennet	2.3590[a]	2.3815[a]	+0.0325
Mucor miehei rennet	2.3301[b]	2.3653[b]	+0.0352
Adult bovine rennet	2.3294[c]	2.3769[c]	+0.0475
Mucor pusillus rennet	2.1892[d]	2.3550[e]	+0.1658
Rennet-pepsin blend	2.1446[f]	2.2761[g]	+0.1315

Pairs of means not denoted by the same superscript were different ($P<0.05$).

[1/] Whey heated at 60 C for 15 minutes.

of accuracy, it is characterized by very low standard deviation when compared with AOAC, Rowland's and formol titration methods.

Comparison of results. In this study casein measured by the dye binding procedure was compared to that determined by AOAC method 1 and Rowland's procedure. Results obtained by the three methods are summerized in Table 6. One way analysis of variance indicated that when veal rennet, Mucor miehei rennet and adult bovine rennet were used in the measurement of casein, the results were not different from those obtained by AOAC and Rowland's procedures in the three different treatments (whey tested immediately after filtering, whey tested after six hours and whey heated immediately after cutting curd), ($P<0.05$). However, Mucor pusillus rennet gave results comparable to AOAC and Rowland's procedures in the heated treatment only. The remainder of the treatments using Mucor pusillus and all treatments using rennet-pepsin blend gave different results ($P<0.05$) from those obtained by AOAC and Rowland's methods. Casein test results obtained from the heated treatment of veal rennet produced curd and whey that gave the best results among other methods and treatments. It gave the lowest standard deviation among all treatments and methods (± 0.042) and among other enzyme treatments it resulted in casein percentage closest to that determined by AOAC method. AOAC and Mucor miehei ranked next with respect to lowest standard deviations.

The results obtained from this research obviously indicated that the choice of enzyme to be used for measuring casein by dye binding technique was of prime importance. Veal rennet, Mucor miehei rennet and adult bovine rennet were found satisfactory in all three treatments used. Mucor pusillus rennet was found satisfactory for the casein test only when heat treatment was applied. Rennet-pepsin blend was suitable as a milk clotting enzyme for the casein test.

TABLE 6. Means, standard deviations and significance of differences for several measures of casein in unheated skimmilk as determined by variations in dye binding, AOAC and Rowland's procedures.

Sources of enzymes used	Casein (%) [1] Dye binding			AOAC	Rowland's
	Method A [2]	Method B [3]	Method C [4]		
_____				$2.361^{a,b} \pm 0.043$	
_____					$2.418^{a} \pm 0.076$
Veal Rennet	$2.359^{a,b} \pm 0.049$	$2.355^{a,b} \pm 0.059$	$2.382^{a} \pm 0.042$		
M miehei rennet	$2.330^{a,b} \pm 0.052$	$2.347^{a,b} \pm 0.044$	$2.365^{a,b} \pm 0.047$		
Adult bovine rennet	$2.329^{a,b} \pm 0.067$	$2.337^{a,b} \pm 0.065$	$2.377^{a} \pm 0.051$		
M. pusillus rennet	$2.189^{c,d} \pm 0.155$	$2.235^{c,d} \pm 0.127$	$2.350^{a,b} \pm 0.075$		
Rennin-Pepsin Blend	$2.144^{d} \pm 0.146$	$2.163^{d} \pm 0.138$	$2.276^{b,c} \pm 0.111$		

[1] All means not denoted by the same superscript are different ($P<.05$).

[2] Whey immediately mixed with the dye after filtering and tested.

[3] Whey left for 6 hrs at room temp. before adding the dye and testing.

[4] Whey heated for 15 min at 60 C, cooled to 30 C and tested.

Determination of Casein from Whole Milk by Dye Binding.

Results of the casein test on whole milk are summerized on Table 7. Higher standard deviations were given by both AOAC (±0.111) and dye binding (±0.096) with whole milk than corresponding values for AOAC (±0.043) and dye binding (±0.051)in skimmilk, (Table 6). However, one way analysis of variance indicated no difference ($P<0.05$) between means of casein measured by dye binding and AOAC methods for unheated whole milk. Nevertheless, casein was measured more accurately from unheated skimmilk than from unheated whole milk.

TABLE 7. Comparison of casein test as measured by dye binding and AOAC methods on unheated whole milk.

Method	Casein (%) Means[a]	Standard deviation
Dye binding	2.3736	±0.096
AOAC	2.3696	±0.111

[a]Means were average of 10 samples.

CONCLUSIONS

In summarizing results of this research, the following conclusions were drawn:

1) Casein could be measured accurately by a dye binding procedure when veal rennet, Mucor miehei rennet and adult bovine rennet were used as milk clotting enzymes.
2) Mucor pusillus rennet was satisfactory as a milk clotting enzyme for use in the casein test only when heat treatment was applied to the clotted curd to inactivate the enzyme.
3) Rennet-pepsin blend was not satisfactory as milk clotting agent for measuring casein by dye binding.
4) Rennet whey could be left at room temperature (23 C) for up to six hours without incurring significant difference in casein percentage as measured by dye binding.
5) Whey can be tested accurately for protein concentration by dye binding after minimum reaction time of 10 min necessary to attain equilibrium between milk and acid orange G 12 dye.
6) Casein can be measured accurately from whole milk using the dye binding procedure.

Based on the results obtained from this investigation, the following procedure is recommended for the determination of casein by the dye binding techniques:

1) Determine the protein content of unheated skimmilk by conventional dye binding procedure as follows:
   a) Transfer 2.4 ml of unheated skimmilk into a pre-weighed two-ounce polyethylene dropper bottle.

b) Weigh again to determine net weight of milk used.

c) Add 40.44 ml acid orange G 12 dye from the automatic pipet.

d) Shake thoroughly and leave for 10 minutes, filter through a fitted spun glass filter into the cuvette of calibrated Udy Analyzer.

e) Calculate total proteins of milk using milk calibration tables prepared as outlined in AOAC 16.040 (5).

2) Determine whey protein concentration by dye binding as follows:

a) Warm 200 ml of unheated skimmilk to 30 C in a 250 ml beaker, and add one milliliter of 1:25 dilution of veal rennet. Mix thoroughly and incubate at 30 C for one hour.

b) Cut the resulting curd using a spatula, and filter through Whatman #41 paper.

c) Transfer approximately 9 ml of filtered whey to a preweighed two-ounce polyethylene dropper bottle, and determine whey protein concentration by conventional dye binding as above.

3) Casein is calculated by subtracting the whey protein concentration from total protein content of unheated skimmilk.

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EFFECT OF VARIOUS MILK CLOTTING ENZYMES ON THE DETERMINATION OF CASEIN BY DYE BINDING PROCEDURES

by

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Previous studies in this laboratory have shown that casein can be measured accurately by determining the protein content of unheated skimmilk and rennet whey by conventional dye binding procedures using acid orange G 12 dye. Casein is calculated by subtracting rennet whey protein concentration from that of raw skimmilk.

The primary objectives of this study were to evaluate various milk clotting enzymes to determine their suitability for use in the test to measure casein by the dye binding procedure reported previously from this laboratory.

In this study, five commercial milk clotting enzymes were used: veal rennet, Mucor miehei rennet, adult bovine rennet, Mucor pusillus rennet and a rennet-pepsin blend. Since these enzymes are proteolytic, their influence on the accuracy of measuring casein by the dye binding procedure was studied. Each enzyme was evaluated in three different procedures as follows:

1) Rennet whey was tested for protein concentration by dye binding immediately after separating it from the curd by filtration.
2) After filtering, rennet whey was incubated at room temperature for six hours before adding the dye and testing for whey protein concentration.
3) Rennet whey was heated at 60 C for 15 minutes immediately after filtering to inhibit enzyme action, cooled to 30 C and tested for whey protein content.

Also, casein was determined for comparison purposes by AOAC and Rowland's methods.

An experiment also was designed to study the effect of time on the reaction between whey proteins and acid orange G 12 dye. Whey obtained from unheated skimmilk clotted by veal rennet was tested for whey protein content at zero, two, four, six and ten hours contact time between the whey sample and acid orange G 12 dye.

The results obtained from this work indicated that veal rennet, Mucor miehei rennet and adult bovine rennet were satisfactory as milk clotting enzymes for measuring casein by dye binding as compared to casein percentages obtained by AOAC and Rowland's methods. However, Mucor pusillus rennet gave results comparable to AOAC and Rowland only when heat treatment of whey was applied. Rennet-pepsin blend was not satisfactory as a milk clotting enzyme for use in the procedures to measure casein by dye binding.

Results of reaction time on whey proteins determined by dye binding indicated no difference ($P>0.05$) between zero hour and subsequent holding times.

The dye binding procedure also was extended to whole milk. Unheated whole milk was tested for casein content by dye binding and AOAC procedures. One way analysis of variance indicated no difference ($P>0.05$) between casein measured by dye binding and AOAC method I.