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Accounting for Milk Protein in Equivalents by Dye Binding Analysis of Cheese and Whey

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ACCOUNTING FOR MILK PROTEIN EQUIVALENTS BY DYE BINDING ANALYSIS OF CHEESE AND WHEY

by

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Prahlad H. Patel

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ABSTRACT

Accounting for Milk Protein Equivalents by Dye Binding

Analysis of Cheese and Whey

by

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Utah State University, 1969

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Each of thirteen lots of Cheddar cheese was made from 430 lb. of milk, and the protein recovered in the cheese and whey was compared with that in the original milk. Protein determinations on the milk, cheese and whey were made by an acid orange 12 dye binding test, and by Kjeldahl analysis. Similarly fat recovery was determined by using the Babcock fat test on milk, the modified Babcock test on cheese, and the Mojonnier test on whey. The total weight recovered as cheese and whey was 99.0 $\pm$ .5% of the milk weight. Protein recovery was 99.0 $\pm$ .6% by Kjeldahl analysis and 97.7 $\pm$ .6% by the dye binding test. Fat recovery was 97.0 $\pm$ 1%.

Even though Kjeldahl analysis gave better protein accountability than the dye binding method, because of its similarity the dye binding test along with fat testing could be used by cheese factories to account for two economically important milk constituents.

Protein hydrolysis reduced the apparent percent protein in cheese and milk as measured by the dye binding test. However, Cheddar cheese containing 37-38% moisture and cured at 7.2 C was satisfactory for protein accounting up to 30 days of age.

(47 pages)

INTRODUCTION

The protein content of milk is of significant importance from a nutritional standpoint. Milk proteins are of the highest quality, both in digestability and in content of essential amino acids. The protein content of milk is the most important factor affecting the yield of cheese and is a major factor in determining the nutritive value and palatability of many other manufactured dairy products. For these reasons, the pricing of milk can hardly be realistic if protein content is not taken into account.

Until a few years ago no simple and practical method was available for measuring milk protein with sufficient accuracy to use it as a basis for pricing. The Kjeldahl Method, the official method for determining protein, is impractical for routine use because it is costly, complicated, and time-consuming. Research during the last few years on various procedures for measuring protein in dairy and other food products has led to the development of dye-binding methods which are now generally considered superior to other chemical methods for large numbers of samples. Dye-binding procedures have an advantage over Kjeldahl analyses with respect to speed, cost, equipment, training of technicans, and repeatability (4, 5). One disadvantage of the dye-binding method is that results are usually obtained in the form of scale readings on a spectrophotometer, and these are not directly convertible to percent protein. The usual method of conversion is to analyze a series of duplicate samples by a dye-binding method and some other method (most

frequently Kjeldahl). Results of the two methods must be related mathematically to permit rapid conversion of the spectrophotometric readings into percent protein.

Dye-binding techniques have been used for some time to determine the protein content of fluid milk, and a limited amount of work has been published concerning their usefulness for measuring protein in manufactured dairy products. For example, Ashworth (1, 2) and others (17, 28) have shown that dye-binding analysis of various products correlate well with Kjeldahl analysis. The purpose of this study was to investigate whether dye-binding of acid orange-12 by cheese and whey made from the same milk could account for all the protein in the original milk. A second objective was to determine the effect of age of cheese on its dye-binding capacity, and to find a maximum age that cheese might be used for protein accounting.

REVIEW OF LITERATURE

Polar groups in proteins can bind oppositely charged dyes to form insoluble protein-dye complexes. A known excess of dye is required, and the protein content is estimated from the amount of unbound dye (10). The dye concentration can be measured spectrophotometrically (2, 7).

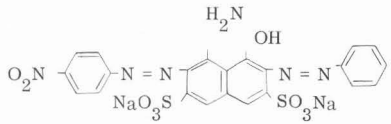
During the early work on protein accounting in grain and dairy products, a relationship was found between bound dye and nitrogen measured by the Kjeldhal procedure (1, 4, 21, 28, 29, 24). Kjeldahl nitrogen in milk was multiplied by 6.38 to give the percent protein. Several writers including Ashworth (1, 2), Ashworth et al. (4), Olsen and Heighes (17), Tarassuk and Abe (25) and Udy (29) showed that this relationship was good enough to justify using dye-binding to indicate the percent protein in the sample.

The need for a rapid routine method for the determination of milk protein has become increasingly evident since increased emphasis has been placed on the importance of the nonfat fraction of milk. Udy (28) first applied the dye-binding procedure to the determination of flour protein. He found that when the binding capacities of the proteins in either whole wheat or wheat flour were taken collectively, the total protein could be characterized by a single binding-capacity value. Although the starch and bran of wheat also bind some dye, the total protein in whole wheat or wheat flour can be conveniently measured by this technique (28). Udy (27) reported 179.5 mg

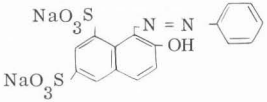
of orange G bound per gram of protein in whole milk, and 182 mg per gram of protein in dry milk.

Dyes used for protein analysis

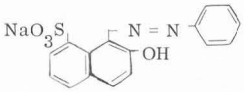
Dyes currently used for protein analysis are amido black 10B, orange G and acid orange 12. Structural formulae (24) for these dyes are as follows:



Amido black 10B mol wt 616.5



Orange G mol wt 452



Acid orange 12 mol wt 349

European workers (7) have preferred Merck's amido black. The use of amido black for protein analysis was first reported by the German scientists Schober and Hetzel (20). The dye content of the commercial product varies from 95 to 97%. Ashowrth et al. (4) specified the use of certified orange G which should assay at least 95% pure. Orange G

and amido black react in the same molar ratio with basic groups in proteins, but amido black, owing to its higher molar absorbancy, gives a more sensitive measurement.

Advantages of orange G over amido black 10B are that the dye dissolves more readily in a simplified buffer system. Filtering is more rapid and it is less likely to give a cloudy filtrate. The commercial dye is also more readily available and can be obtained in pure state.

Structural formulae for the two orange dyes differ mainly in that orange G has two sulfonic acid groups whereas acid orange 12 has only one. Other conditions being the same, one would expect two molecules of acid orange 12 to bind the same amount of protein as one molecule of orange G. Two advantages of acid orange 12 over orange G are that its absorbancy index is greater which allows more precision in measurement, and that the dye binding capacity is about twice that of orange G.

Dye binding capacity (DBC)

Ashworth and Chaudry (3) defined dye binding capacity as the milligrams of dye bound per milligram protein (N x 6.38). Dye binding capacity assumes a stoichiometric reaction between dye and protein, which is true within certain limits.

$$\text{DBC} = \frac{\text{mg of dye bound}}{\text{mg of protein}} = \text{mg of dye/mg of protein}$$

Ashworth and Chaudry (3) reported that DBC was affected by protein concentration and free dye concentration in the supernatant solution.

Ashworth, Seals and Erb (4) reported that whey protein had higher binding values than casein. The milk proteins, casein and β-lactoglobulin, bound 0.34 and 0.58 millimoles of dye respectively per gram of protein. The dialyzable non-protein nitrogen (NPN) fraction in milk reportedly bound no dye (2). This could be true or the NPN could have bound the dye and not precipated with the main protein-dye complex. In this case the soluble NPN-dye complex could have absorbed light at the same wave length as the free dye. At any rate, NPN in milk is included in the dye-binding test only because the test is standardized against total protein as determined by the Kjeldahl procedure which includes NPN. Ashworth (2) reported that the ratio of the milk protein binding factor for acid orange 12 to that of orange G was close to 0.50 which meant that milk proteins had twice the dye binding capacity per mole for acid orange 12 as for orange G. Ashworth and Chaudry (3) gave the following dye binding values for orange G: 178 mg per gram protein for whole milk, 199 mg for α-casein, 170 mg for β-casein and 247 mg for whey protein.

Udy (27) applied the dye binding procedure to the determination of milk protein using orange G dye. He found that, compared with Kjedahl values, the standard error of estimate for the dye method was $\pm$ 0.07% protein in whole fluid milk and $\pm$ 0.42% for spray-dried milk. Steinsholt (23) tested 64 samples of milk preserved with mercuric chloride and 62 unpreserved herd milk samples. The correlation between Kjeldahl protein and absorbancy of the supernatant dye solution was -0.982 for the preserved and -0.975 for the unpreserved samples.

Confidence intervals (0.95 level) for protein by dye binding as compared with Kjeldahl protein were ± 0.12% for fresh samples within the range of 2.8 to 4% total protein, and ± 0.09% for preserved milk samples in the same range. Corresponding confidence intervals for formol titration (by automatic titrator) and for protein calculated from fat content were ± 0.21 and ± 0.37, respectively. Expressed as standard errors of estimate, the error in the dye-binding test was approximately half the above values. Recently, Treece et al. (26) reported a correlation coefficient of 0.98 between the method of Udy (27) and the Kjeldahl procedure. They also stated that seldom did a sample vary as much as ± 0.1 percentage units from the Kjeldahl value, and that approximately two-thirds of the sample fell within ± 0.05 percentage units of the corresponding Kjeldahl value.

Udy (27) published a formula to calculate the percent protein in milk by using orange G dye. After acid orange 12 was tested an addendum was published which gave an extension of the original technique. He also proposed a method for estimating the protein content of manufactured dairy products in general (29). Udy discovered that 1 unit of milk protein binds 0.312 units of acid-orange 12 dye. This factor was based on an average of many samples of milk reacted at room temperature. Later developments showed that the binding equilibrium of protein to dye was a function of temperature (29). One degree fahrenheit was equivalent to 0.005% change in protein. Increased temperatures cause low protein readings. The factor of .312 is based on a 6.33 Kjeldahl factor and a dye-protein reaction at room temperature (77 F).

Effect of pH and dye concentration on dye binding

Orange G dye is a disulfonic acid which binds the basic groups of proteins near pH 2.00. Fraenkel-conrat and Cooper (10) showed that in buffers at pH 2.2 the acid dye, orange G, combined stoichiometric with these basic groups. Schober and Hetzel (20), using dye solutions buffered to various pH values within the range of 3.5 to 1.9, obtained parallel curves when the absorbancy of unbound dye was plotted against the quantity of milk protein added. These workers preferred dyes buffered at pH 2.35. Udy (28) used citrate-phosphate buffer at pH 2.2. Ashworth and Seals (4) employed citric acid alone in orange G dye solutions to give a pH of 2.00. Since the work of Schober and Hetzel (20) indicated high dye binding and an absence of acid hydrolysis of protein at this pH, it appeared a logical choice. They further reported that when amido black 10B was reacted with proteins, dye binding increased in a linear manner as the pH was reduced from 3.5 to 1.9, and that it varied only slightly with temperature between 0 to 40 C. They found that it was unaffected by the addition of lactic acid, lactose, phosphate, citrate or sodium chloride. However, calcium chloride increased dye binding. Schober and Hetzel (20) found that above pH 3.5 precipitation of the milk protein was not quantitative. Lower absorbancies were observed when the pH of the citrate-phosphate buffer was decreased from 2.8 to 1.9. This meant that more dye was bound by the protein at lower pH values. Some dye was precipitated in filtrates from milk treated with trichloracetic acid, indicating that some non-protein nitrogen was bound to the dye. When free amino acids were added

to dye solutions only arginine, at a concentration greater than 1%, changed the absorbancy.

Factors affecting dye binding capacity of milk proteins

The dye binding capacity of milk proteins has been studied by employing experimental procedures and variables which would be common in protein dye-binding tests applied to samples of raw and processed milks. It was assumed that such milks would vary widely in composition and other properties. Udy (27) reported that protein denaturation by heat treatment was the probable reason for the difference in DBC between whole milk and powdered milk. Ashworth and coworkers (3) studied the effect on dye binding of the preservatives H_2O_2, HCHP, $K_2Cr_2O_7$, and $HgCl_2$. They reported that H_2O_2 had no effect on dye binding but could not preserve the milk sample for more than 2 days at room temperature; that HCHO reduced the apparent protein content; that $K_2Cr_2O_7$ increased the apparent protein content at the initial stage and then reduced it after a week of storage at room temperature; and that $HgCl_2$ reduced the apparent protein content very slightly but was the best perservative for milk to be tested by dye-binding. Tarassuk and Abe (24) reported that heating milk enough to induce browning decreased the DBC. The relationship between intensity of browning and DBC was linear. Ashworth (2) reported that commercial sterilization did not appreciably effect the dye-binding capacity of proteins in evaporated milk. Tarassuk and Abe (24) reported that condensation, homogenization (up to 4000 PSI) and heating (up to 90 C for 15 minutes) did not affect dye binding capacity, while mastitis increased dye-binding capacity. Extensive

proteolysis increased the absorbancy of the supernatent dye solution and increased the dye-binding capacity of the proteins not precipitated by 20% TCA. Mass protein analyses by dye-binding (amido black 10B) resulted in somewhat different regression equations for different geographical areas (24). It appeared that the main cause for the differences was that the dye-binding capacity was affected by different protein-dye ratios used by various workers. This relationship between the protein-dye ratio and dye-binding capacity followed a straight line when the dye concentration was between 360 and 320 mg/g protein and the protein concentration in the milk was between 2.10 and 4.8%.

Ashworth (2) reported procedures for using orange G dye to test such products as fortified milk, evaporated and dried milk, and ice cream and sherbet. The dye-binding capacity of cheese products was reduced by the aging process because protein breakdown products bound less dye than intact proteins (2). He also showed that the nonfat solids in milk may be estimated from the protein content by use of a factor of 2.78, which assumes that the nonfat solids to protein ratio is constant. The value of the ratio for the bulk milk coming to a processing plant is sufficiently constant for composition control, although it may vary from one milkshed to another or with the season of the year (8).

Erb et al. (9) reported the correlation coefficient of percent protein to percent SNF was 0.48, 0.62, and 0.58 in 771 Jerseys, 1355 Holsteins, and 710 Guernseys, respectively at monthly intervals. In a similar study on USU dairy herds of 216 Holsteins and 216 Jerseys cattle, LeBaron et al. (14) reported the correlation coefficient of percent protein to percent SNF to be

0.57 and 0.55 respectively. Ashworth (2) found that nonfat powder remained reasonably consistent in its ability to bind dye even after storage periods of a number of years. Ashworth also reported that small molecular weight compounds such as those present in the proteose-peptone fraction of milk and whey react slowly with dyes to form complexes which slowly produce turbid filtrates. Usually this difficulty can be resolved by allowing the reaction mixture to stand overnight at room temperature (25 C) or by using high speed centrifugation. Clear filtrates are evidence of complete separation of the protein-dye complex, although a small amount of turbidity has little effect on the protein determination.

MATERIALS AND METHODS

Protein determination

The nitrogen content of milk, cheese and whey was determined by a semi-micro Kjeldahl method (13). Protein was computed by multiplying N x 6.38 (21).

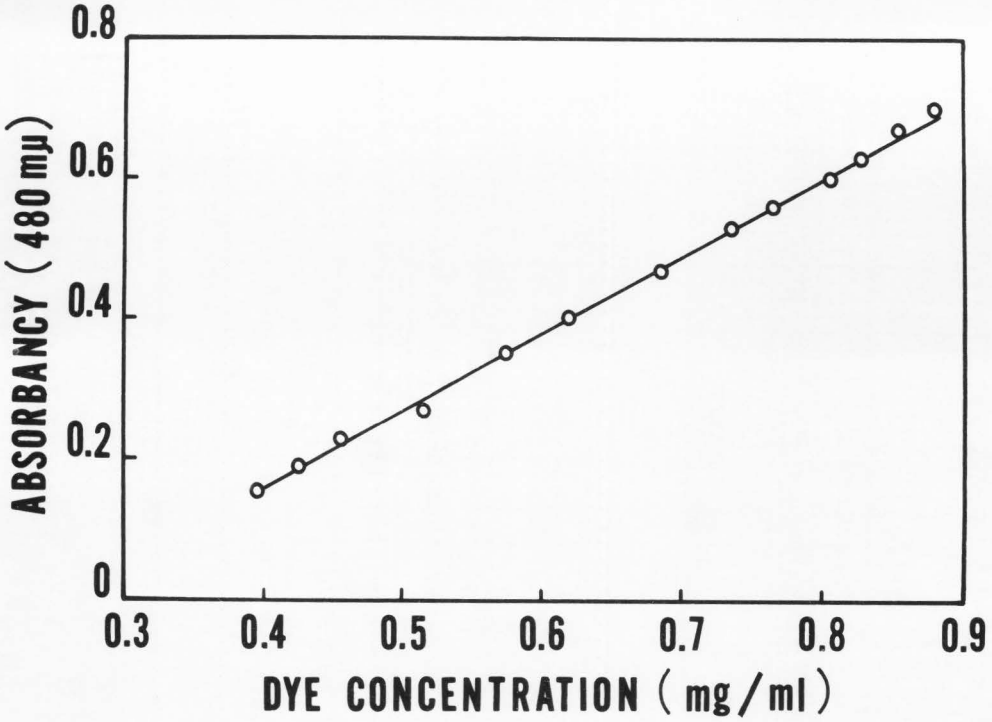
Protein determination by dye binding

Protein determinations were made by the Udy (29) dye-binding method in which acid orange 12 was employed with a Udy color analyzer Model 101. A flow-through cuvette was used which had a light path of approximately 0.30 mm.

Standard curve. A standard curve relating absorbancy at 480 mμ to concentration of acid orange 12 was established on serially diluted dye solutions (Figure 1).

The operating range of this curve was between 0.40 mg/ml to 0.80 mg/ml of dye concentration. Beyond this range inconsistency of results were observed by Ashworth and Udy (2, 29). This curve was used to determine the protein content of milk, cheese and whey. The concentration of unbound dye was read from the standard curve. This value was multiplied by the total sample volume to give the total amount of unbound dye. The bound dye was found by subtracting this amount from the total amount of dye added (52 mg). The dye binding capacity was then calculated as the ratio of dye bound per unit of

Figure 1. Standard curve relating absorbancy at 480 mμ to dye concentration (mg/ml).


0.8
0.6
0.4
0.2
0
ABSORBANCY (480 mμ)
0.3
0.4
0.5
0.6
0.7
0.8
0.9
DYE CONCENTRATION (mg/ml)

protein. From this ratio, its reciprocal was used as a factor for multiplying the amount of dye bound by unknown samples to find the amount of protein present.

Cheese protein was determined by blending 20 g of cheese with 80 g of hot (180 F) 0.05 M NaOH for at least 5 minutes in order to obtain a homogeneous mixture. The mixture was quantitatively transferred to a 100 ml volumetric flask, cooled and made to final volume with distilled water. The protein in this solution was determined by a modification of the acid orange 12 procedure for milk (29).

A calibrated syringe was used to measure 2.24 ml of well mixed cheese solution into a 2 oz. polyethylene sample bottle. The sample weight was determined to within $\pm$ 5 mg. A Udy (29) automatic pipette was used to add 40.44 g reagent dye solution to the sample which was then analyzed for protein by the Udy method for milk protein (29).

The protein content of the cheese was determined by the following formula.

$$\text{Percent protein} = \frac{100\left[V\,Co - (V + v)\,C\right]}{.312\,W} \times 5$$

Where C = concentration of unbound dye in filtrate (mg/ml)

Co = original dye concentration (mg/ml)

V = volume of reagent dye solution

v = volume of sample

W = mg of sample

. 312 = dye binding capacity

5 = constant dilution factor

Whey protein was determined by the Udy (29) method for milk protein except that the sample size was four times as large (8. 98 ml) as for milk samples.

Method of cheese manufacture

All batches of Cheddar cheese were made by following the time schedule recommended by Price and Calbert (18). Their general procedure was modified to the extent that 2% starter was used, and the ripening time was eliminated. An outline of this procedure is given in Table 1.

Thirteen vats of Cheddar cheese were made from pasteurized milk obtained from Utah State University dairy products laboratory. Each vat contained 430 pounds of milk. A mixed strain commercial lactic starter was used throughout the experiment and coagulation of the milk was accomplished by the addition of 90 ml single-strength rennet per 1000 pounds of milk. Single-strength cheese color was added at the rate of 30 ml/1000 pounds of milk. The curd was salted with 2. 90 pounds salt per 1000 pounds of milk.

The rate of acid development in each vat of cheese was carefully noted. Acid development was measured by titratable acidity of the milk or whey and expressed as percent lactic acid, and by pH of the curd which was determined by a DFL (Dairyland Food Laboratory) pH meter with quinhydrone electrode (6). The titratable acidity of the whey and pH of the curd were taken at the time of cutting the curd, draining the whey and milling the curd.

Table 1. Cheddar cheese making record (based on University of Wisconsin Bulletin 464, "Cheddar Cheese from Pasteurized Milk")

Steps in making	Time of each step	Minutes to next step	Temp.	% Acid
Add starter	8:15	0	86°	.16
Add Color	8:15	2	87°	.16
Add Rennet	8:17	12	88°	.165
Coagulation	8:29	18	88°	No test
Cut	8:47	15	88°	.10
Steam on[a]	9:02	30	88°	.10
Steam off[a]	9:32	45	102°	.105
Settle Curd	10:27	30	102°	.12
End Dipping	10:57	15	102°	.14
Pack	11:12	Turn	101°	.17
Pile Two High	11:57	Curd	96°	.25
Pile Three High	12:27	Every	93°	.30
Mill	12:57	15 min.	91°	.40
Salt	13:17	40	89°	No test
Hoop	13:57	20	88°	No test
Press	14:17	30	88°	No test
Dress	14:47	--	No reading	No test

[a]Cooking Schedule =

Minutes from Steam On	0	5	10	15	20	25	30
Temperature °F	88	89	91	93	96	99	102

Curd from each batch was placed in two 20-pound square hoops. The curd was pressed for a minimum of 30 minutes before being removed from the press for dressing. It was then pressed overnight.

The curd was weighed before and after pressing and a sample from each batch was taken for protein, fat, moisture and salt analysis. Cheese blocks were then wrapped with "Parakote" and returned to the hoops for steam sealing. The cheese was cured at 7.2 C for 255 days. Samples were taken for analysis after 1 day and every 15 days thereafter.

Salt analysis of cheese and whey

The ADSA procedure for the determination of salt in cheese was modified according to Silverman et al. (22).

Two grams of cheese, 10 ml of 0.1711 N silver nitrate, 10 ml of nitric acid, and 50 ml of water were placed in a 250-ml Erlenmeyer flask. The mixture was boiled during which time three 5.0 ml portions of saturated potassium permanganate were added. After the cheese was digested, the clear mixture was cooled and 2.0 ml of nitrobenzene and 3.0 ml of ferric ammonium sulfate were added. The flask was shaken and titrated directly with 0.1711 N potassium thiocyanate to a brick-red end point.

Press drippings contained a high salt concentration. Therefore, it was necessary to add 5 ml of 0.1711 N $AgNO_3$ and 5 ml of HNO_3 or to dilute the sample 12.5 times and take a 2 ml aliquot for analysis.

Determination of fat in milk, cheese and whey

The fat content in the milk was determined by the Babcock method (8). Cheese fat was determined by the modified Babcock method (31) and whey fat was measured by the Mojonnier method (16).

Determination of casein in milk

Casein in milk was determined by a formol titration procedure (11).

Determination of moisture in cheese

Cheese moisture was determined by the method of Wilster et al. (31).

Preparation of cheese for analysis

Samples of fresh cheese were pressed through an 8-mesh wire screen and mixed thoroughly in a sample jar. Samples were then used for protein, moisture, fat and salt analysis.

RESULTS

Effect of tryptic digestion on apparent protein in milk

A calibrated syringe was used to measure 2.24 ml of pasteurized milk into eight 2 oz. polyethylene sample bottles. A 280 mg sample of trypsin powder was transferred to a 100 ml volumetric flask and made to final volume with distilled water. Into each of the 8 sample bottles, 0.1 ml aliquot of trypsin solution was added. All bottles were incubated at 25 C. They were removed from incubation at 2-hour intervals at which time the dye solution was added immediately to stop further reaction. From this point on the Udy Dye Method (29) was followed. The nitrogen content in the dye filtrate was determined by Kjeldahl analysis. Then the protein equivalent in dye filtrate was computed by multiplying N x 6.38 (21). The effect of tryptic digestion on the apparent protein content in milk as measured by dye binding is shown in Table 2. The relationship between apparent protein in milk and protein equivalent in the dye filtrate are given in Figure 2. As digestion time increased, the apparent percent protein decreased. Initially the dye binding test indicated $3.47 \pm .02\%$ protein. After 10 hours incubation with trypsin the dye test indicated $1.46 \pm .03\%$ protein. These results were obviously not due to a loss of protein during digestion, but to the effect of protein hydrolysis on the dye binding test.

The validity of Kjeldahl determinations on dye filtrates was tested by running Kjeldahl analyses on 1-ml samples of dye solution to determine whether

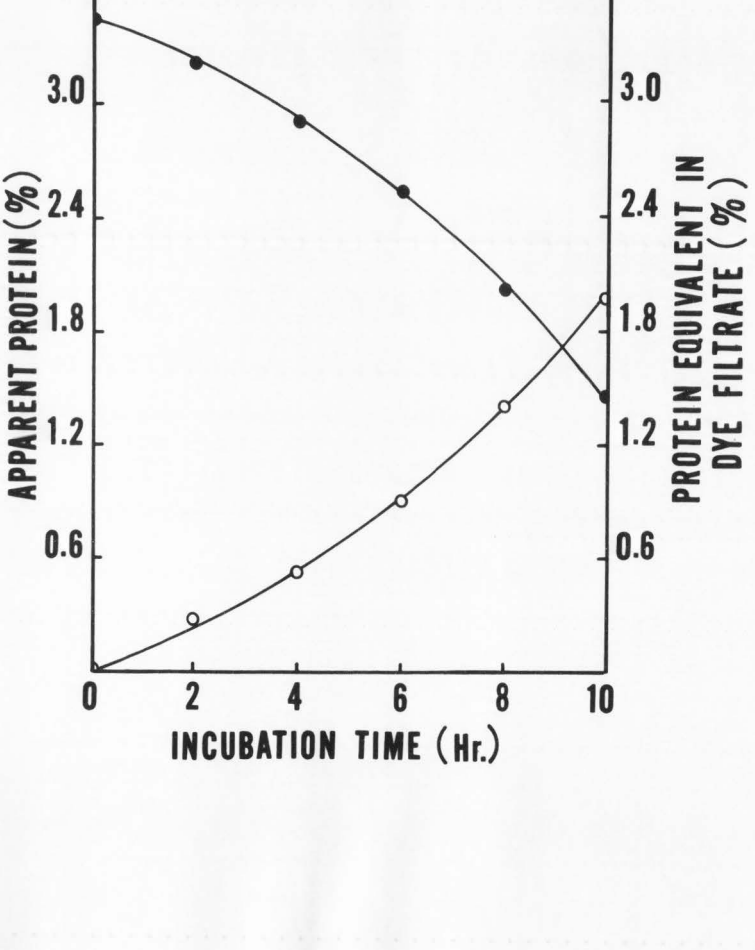
Table 2. Effect of tryptic digestion of milk at 25 C on the apparent percent protein as determined by dye-binding and on the protein equivalent in the dye filtrate as determined by Kjeldahl analysis

Incubation time	Replication	Apparent protein	Protein equivalent in dye filtrate	Total protein equivalent
(hr.)		(%)	(%)	(%)
0	8	3.47 ± .02	0.00	3.47
2	8	3.19 ± .02	0.26	3.45
4	8	2.91 ± .03	0.53	3.44
6	8	2.55 ± .02	0.90	3.45
8	8	2.03 ± .01	1.42	3.45
10	8	1.46 ± .03	1.98	3.44

Figure 2. Change in apparent protein (dye binding) and protein equivalent in dye filtrate during the tryptic digestion of milk.

○——○ Protein equivalent in dye filtrate

●——● Apparent protein


3.6
3.0
2.4
1.8
1.2
0.6
APPARENT PROTEIN (%)
PROTEIN EQUIVALENT IN DYE FILTRATE (%)
0
2
4
6
8
10
INCUBATION TIME (Hr.)

or not nitrogen in the dye itself would be measured, and whether it would interfere with the measurement of soluble nitrogen in dye filtrates. These results are presented in Table 3. Even though acid orange 12 contains nitrogen in its structure, it is evident from the table that this nitrogen does not interfere in Kjeldahl analysis of dye filtrate.

Table 3. Kjeldahl analysis of acid orange 12 dye

	Acid titer (0.0373 N)	
Sample Number	Blank	Sample
	(ml)	(ml)
1	.04	.04
2	.05	.05
3	.04	.04
4	.05	.04

Effect of aging on the apparent protein content of Cheddar cheese

Four 20-lb. blocks of Cheddar cheese were made from different lots of milk and placed in storage at 7.2 C for curing. The cheese contained 38.00, 37.50, 37.00, and 37.25% moisture respectively. Protein determinations on the cheese were made by the dye-binding tests, and the protein equivalent in the dye filtrates was measured by Kjeldahl analysis when the cheese was 1, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 195, 210, 225, 240, and 255 days old. Results of this experiment are presented in Table 4. The relationships

Table 4. Effect of aging four lots of Cheddar cheese at 7.2 C on the apparent percent protein as determined by dye binding with acid orange 12 and on protein equivalent in the dye filtrate

Age	Apparent protein				Protein equivalent in dye filtrate			
(days)	(%)				(%)			
	Lot I	Lot II	Lot III	Lot IV	Lot I	Lot II	Lot III	Lot IV
1	23.07	23.50	22.94	23.00	00	00	00	00
15	23.07	23.50	22.94	23.00	00	00	00	00
30	23.04	23.44	22.90	22.95	.03	.06	.04	.05
45	22.95	23.36	22.81	22.88	.12	.14	.13	.12
60	22.71	23.26	22.78	22.73	.36	.24	.16	.27
75	22.50	22.87	22.10	22.40	.45	.61	.75	.59
90	22.39	22.76	22.09	22.16	.67	.73	.81	.72
105	22.10	22.61	21.93	22.06	.97	.89	1.01	.94
120	21.86	22.40	21.69	21.91	1.21	1.10	1.25	1.09
135	21.41	21.75	21.00	21.19	1.65	1.74	1.93	1.81
150	21.07	21.45	20.78	20.99	1.98	2.05	2.16	2.01
165	20.76	21.08	20.60	20.81	2.31	2.40	2.31	2.19
180	20.43	20.90	20.28		2.64	2.57	2.62	
195	20.00	20.39	19.94		3.05	3.09	2.99	
210	19.48	19.56			3.59	3.91		
225	19.09	19.49			3.98	4.01		
240	19.02	19.40			4.05	4.10		
255	18.82	19.19			4.25	4.31		

between apparent protein in the cheese and protein equivalent in the dye filtrate are given in Figure 3.

The apparent percent protein decreased in all four batches of Cheddar cheese as aging progressed. The percentage of apparent protein in the fresh cheese were 23.07, 23.50, 22.94, 23.00 respectively for batches 1, 2, 3, and 4. The protein percent after 255 days of aging for batch 1 and 2 was 18.82 and 19.19 respectively and for batches 3 and 4 was 19.94 and 20.81 at 195 and 165 days respectively. The sum of apparent protein and protein equivalent in the dye filtrate was compared with the total protein in each batch of cheese. The results presented in Table 5 show that the agreement was very close and that protein not accounted for by dye binding was accurately measured in terms of its equivalents in the dye filtrate. Since the same phenomenon was observed during tryptic hydrolysis of milk it was apparent that the decrease in apparent percent protein during cheese curing was the result of protein hydrolysis. A corresponding increase in protein equivalent in the dye filtrates was obtained during curing. The dye filtrate was completely free of Kjeldahl nitrogen until the cheese was 15 days old but increased as the cheese cured. Accordingly the protein equivalent in the dye filtrate increased to 4.25 and 4.31 in lots 1 and 2 respectively, when aged for 255 days, and to 2.99 and 2.19 for batches 3 and 4 that were aged for 195 days and 165 days respectively. The sum of apparent protein in the cheese and the protein equivalent in the dye diltrate was closely equivalent to the total protein as measured by the Kjeldahl procedure.

Figure 3. Change in apparent protein (dye binding) and protein equivalent in dye filtrate during the aging of cheddar cheese.

o——o Protein equivalent in dye filtrate

●——● Apparent protein

APPARENT PROTEIN (%)
23
22
21
20
19
0
60
120
180
240
AGE (DAYS)
PROTEIN EQUIVALENT IN DYE FILTRATE (%)
0
1
2
3
4

Table 5. Effect of aging four lots of Cheddar cheese at 7.2 C on the total protein equivalent (sum of apparent protein and protein equivalent in dye filtrate) and total protein

Total protein equivalent (%)				Total protein (Kjeldahl) (%)			
Lot I	Lot II	Lot III	Lot IV	Lot I	Lot II	Lot III	Lot IV
23.07	23.50	22.94	23.00	23.71	23.69	23.70	23.25
23.07	23.50	22.94	23.00	23.78	23.71	23.01	23.24
23.07	23.50	22.94	23.00	23.80	23.75	23.15	23.25
23.07	23.50	22.94	23.00	23.75	23.70	23.20	23.24
23.07	23.50	22.94	23.00	23.58	23.71	23.21	23.24
23.05	23.48	22.93	22.99	23.80	23.74	23.17	23.25
23.06	23.39	22.92	22.98	23.90	23.65	23.16	23.30
23.07	23.50	22.94	23.00	23.78	23.68	23.17	23.25
23.07	23.50	22.94	23.00	23.85	23.71	23.17	23.30
23.06	23.49	22.93	23.00	23.87	23.68	23.16	23.31
23.05	23.50	22.94	23.00	23.88	23.71	23.17	23.30
23.07	23.48	22.91	23.00	23.95	23.72	23.16	23.31
23.07	23.47	22.90		23.87	23.76	23.15	
23.05	23.48	22.93		23.81	23.71	23.10	
23.07	23.47			23.82	23.70		
23.07	23.50			23.91	23.81		
23.07	23.50			23.94	23.81		
23.07	23.50			23.97	23.80		

Accountability of milk protein by dye binding of acid orange 12 on cheese and whey

The proposed dye binding method for the determination of the protein in milk, cheese, and whey was compared with the conventional Kjeldahl procedure on each of 9 lots of Cheddar cheese.

A sample of milk was taken from each of the 9 lots of cheese milk and analyzed for protein, fat and casein before the milk was manufactured into cheese.

A 150 ml of sample of whey from each lot was taken for determination of protein and fat. A sample of press drippings was also taken from each of the cheese blocks for protein, fat and salt analysis. Each cheese was analyzed for protein, moisture, fat and salt.

The results in Table 6 show the protein content of milk, cheese and whey as determined by dye binding and the Kjeldahl Method. The total recovery of protein obtained with the Kjeldahl and dye binding methods was 99.00 $\pm$ .6% and 97.7 $\pm$ .6% respectively. It is evident that the differences between these two methods, with respect to total protein recovery, are small but the total recovery of protein by dye binding was less than that obtained by the Kjeldahl procedure.

Analysis of nine lots of milk, cheese and whey for fat and protein

Table 7 shows the percent recovery of milk fat and protein in 9 lots of cheese and whey. The salt content of the cheese and press drippings were subtracted weight to arrive at the recovery figures. It can be seen from the table that average percent recovery of milk fat in cheese and whey was

Table 6. Protein content of milk, Cheddar cheese and whey as determined by Kjeldahl and dye binding (acid orange 12)

			Dye Binding			
Batch	Product	Weight	Replication	Protein	Protein	Protein accounted for
		(lbs.)		(%)	(lbs.)	(%)
1	Milk	438.60	4	3.20 ± .03	14.03	98.07
	Cheese	41.25	6	23.90 ± .61	9.86	
	Whey	390.00	4	1.00 ± .07	3.90	
	Milk - (C + W)				00.27	
2	Milk	438.60	4	3.22 ± .02	14.12	98.51
	Cheese	42.00	6	24.00 ± .59	10.08	
	Whey	391.00	4	0.98 ± .08	3.83	
	M - (C + W)	5.60			00.21	
3	Milk	438.60	4	3.25 ± .03	14.25	97.47
	Cheese	43.00	6	24.05 ± .55	10.34	
	Whey	394.25	4	0.90 ± .09	3.55	
	M - (C + W)	1.35			00.36	
4	Milk	438.60	4	3.25 ± .02	14.25	97.26
	Cheese	42.00	6	24.11 ± .52	10.13	
	Whey	393.00	4	0.95 ± .10	3.73	
	M - (C + W)	3.60			00.39	
5	Milk	438.60	4	3.21 ± .02	14.08	98.08
	Cheese	43.00	6	23.95 ± .49	10.30	
	Whey	394.00	4	00.89 ± .08	3.51	
	M - (C + W)	1.60		00.89 ± .08	00.17	
6	Milk	438.60	4	3.21 ± .01	14.08	97.58
	Cheese	42.75	6	23.84 ± .41	10.19	
	Whey	394.00	4	00.90 ± .05	3.55	
	M - (C + W)	1.85			00.34	
7	Milk	438.60	4	3.26 ± .07	14.30	96.64
	Cheese	42.50	6	24.10 ± .42	10.24	
	Whey	395.75	4	00.91 ± .07	3.58	
	M - (C + W)	2.35			0.48	
8	Milk	438.00	4	3.26 ± .01	14.30	97.34
	Cheese	43.00	6	24.00 ± .48	10.32	
	Whey	394.00	4	0.92 ± .06	3.60	
	M - (C + W)	1.60			0.38	
9	Milk	438.60	4	3.22 ± .02	14.12	98.72
	Cheese	42.75	6	23.92 ± .47	10.23	
	Whey	395.00	4	0.94 ± .03	3.71	
	M - (C + W)	00.85			0.18	
Average						97.7 ± .6

Kjeldahl			
Replication	Protein	Protein	Protein accounted for
	(%)	(lb.)	(%)
4	3.21 ± .01	14.08	98.79
4	24.57 ± .21	9.93	
4	1.02 ± .02	3.98	
		0.17	
4	3.24 ± .01	14.21	99.65
4	24.30 ± .24	10.21	
4	1.01 ± .03	3.95	
		0.05	
4	3.26 ± .01	14.30	99.37
4	24.35 ± .22	10.47	
4	00.95 ± .02	3.74	
		0.09	
4	3.26 ± .02	14.30	98.32
4	24.32 ± .25	10.21	
4	00.98 ± .03	3.85	
		0.24	
4	3.23 ± .01	14.17	99.29
4	24.20 ± .20	10.41	
4	00.93 ± .03	3.66	
		0.10	
4	3.23 ± .01	14.17	99.65
4	24.10 ± .26	10.30	
4	00.97 ± .03	3.82	
		0.05	
4	3.27 ± .01	14.34	97.98
4	24.27 ± .21	10.31	
4	00.95 ± .02	3.74	
		0.29	
4	3.27 ± .01	14.34	98.88
4	24.19 ± .19	10.40	
4	00.96 ± .02	3.78	
		0.16	
4	3.24 ± .01	14.21	99.08
4	24.10 ± .18	10.31	
4	00.98 ± .03	3.77	
		0.13	
			99.0 ± .6

Table 7. Analysis of nine batches of milk, cheese and whey for fat and protein

Batch	Product	Cheese	Whey	Milk	Recovery
		(lbs.)	(lbs.)	(lbs.)	(%)
I	Weight	41.25	390.00	438.60	98.05
	Fat	13.20	1.72	15.13	97.80
	Protein	9.86	3.90	14.03	98.07
II	Weight	42.00	391.00	438.60	98.45
	Fat	13.18	1.85	15.35	97.90
	Protein	10.08	3.83	14.12	98.51
III	Weight	43.00	394.25	438.60	98.42
	Fat	13.20	1.31	15.13	95.90
	Protein	10.34	3.55	14.25	97.47
IV	Weight	42.00	393.00	438.60	99.15
	Fat	13.80	1.49	15.79	96.83
	Protein	10.13	3.73	14.25	97.26
V	Weight	43.00	394.00	438.60	99.37
	Fat	12.90	1.77	15.35	94.90
	Protein	10.30	3.51	1.408	98.08
VI	Weight	42.75	394.00	438.60	99.32
	Fat	13.68	1.50	15.79	96.14
	Protein	10.19	3.55	14.08	97.58
VII	Weight	42.50	393.75	438.60	99.19
	Fat	13.01	1.85	15.35	96.80
	Protein	10.24	3.58	14.30	99.64
VIII	Weight	43.60	394.00	438.60	99.49
	Fat	14.00	1.21	15.79	99.96
	Protein	10.32	3.60	14.30	97.34
IX	Weight	42.75	395.00	438.60	99.52
	Fat	13.25	1.66	13.35	97.10
	Protein	10.23	3.71	14.13	98.72
Total	Weight	382.25	3539.00	3947.40	99.0 $\pm$ .5
	Fat	120.22	14.46	139.03	97.0 $\pm$ 1
	Protein	127.53	32.96	91.69	97.7 $\pm$ .6

97.00 ± 1% whereas the average protein recovery was 97.7 ± 6 %. The average percentage weight recovery in 9 lots of cheese milk was 99.00 ± 6% as shown in Table 8.

Table 8. Analysis of nine lots of Cheddar cheese for fat, moisture and salt

Lot number[a]	Moisture	F/DM	Cheese Salt
	(%)	(%)	(%)
1	38.15	52.0	1.83
2	39.04	53.3	1.84
3	38.00	52.0	1.90
4	37.77	52.5	1.87
5	38.40	52.9	1.80
6	38.10	51.4	1.86
7	37.87	52.8	1.86
8	37.94	51.4	1.89
9	37.81	53.0	1.78

[a]Samples run in duplicate

DISCUSSION

The dye binding capacity of Cheddar cheese decreased with age, and the apparent percent protein also decreased. However, the protein equivalent (NPN) in dye filtrates exhibited a corresponding increase. This suggested that the decrease in percent protein was due to proteolysis which accompanied aging. It was assumed by Ashworth (2) that the non-protein nitrogen fraction in milk bound none of the dye. It must be recognized that these compounds have been determined in fluid milk only because, the dye methods were standardized against total Kjeldahl nitrogen on samples in which the NPN fraction was included. Actually as mentioned by Ashworth (2), the non-protein nitrogen in milk may have bound the dye even though Udy was unable to show a reduction in color in the filtrate. The nitrogen in acid orange 12 was not measured by Kjeldahl analysis. This enabled the Kjeldahl determination of unprecipitated NPN in dye filtrates.

The dye binding method successfully accounted for the protein in Cheddar cheese up to 30 days of age when cured at 7.2 C and when the moisture content of the cheese was 37.00-38.00%. Thereafter the apparent protein content of the cheese decreased, and the protein equivalent in the dye filtrate increased.

Fat accounting has long been used as an indication of processing efficiency in dairy plants. Since cheese yield is as dependent on the casein content of milk as it is on fat, accounting for protein as well as fat should provide a better index of efficiency.

Use of dye-binding test with acid orange 12 on 9 lots of Cheddar cheese and whey was capable of accounting for 97.7 $\pm$ .6% of the original milk protein. At the same time 99.0 $\pm$ .5% of the original milk weight was recovered. The corresponding recovery of fat was 97.00 $\pm$ 1%. Even though Kjeldahl analysis gave better protein accountability (99.0 $\pm$ .6%) than dye binding, results from the dye binding test for proteins were as good as those obtained by current methods of fat accounting. Because of their speed and simplicity, the dye binding test along with fat testing could be used by cheese factories to account for two economically important milk constituents.

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