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THE DYE BINDING OF MILK PROTEINS.

TARASSUK, N.P., ABE, N., MOATS, W.A. 1 OF 1

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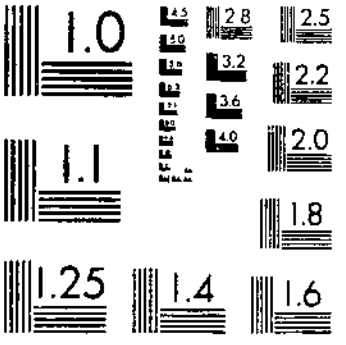


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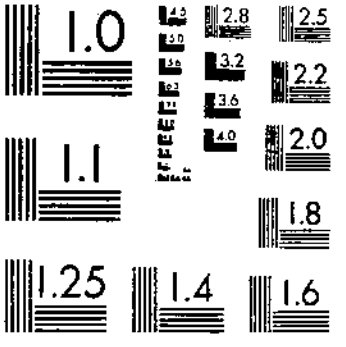


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# THE DYE BINDING of MILK PROTEINS



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Washington, D.C. Issued January 1967

# THE DYE BINDING OF MILK PROTEINS

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## INTRODUCTION

From a nutritional standpoint, the protein content of milk is of outstanding importance. Milk proteins are of the highest quality, both in digestibility and in content of essential amino acids. Protein content is a reliable indication of the content of calcium and phosphorus in milk. In addition, the protein content of milk is the most important factor affecting the yield in cheesemaking and is a major factor in determining the nutritive value and palatability of nearly all 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 determining protein with sufficient accuracy to use it in pricing milk. The Kjeldahl method, the official method of determining protein, is impractical for routine use, because it is so costly, complicated, and time-consuming. In the last 30 years, many approximative methods (based on diverse principles) have been proposed; the more important ones were reviewed by Booy and coworkers in 1962 (*5*).[1] Of all methods published, only two—alkali steam distillation and dye binding—are accurate and simple enough to be used on a large scale for pricing milk.

The Kofrányi method (*17*), which is based on the liberation by alkali steam distillation of ammonia in proportion to the quantity of protein, was found to be more reliable than the formal titration method and was the first to be adopted for mass analysis of milk samples for protein content (*19*). Now the Kofrányi method has been superseded by the simpler dye-binding methods (*21*, *22*).

The development of dye-binding methods, the study of which began in 1925, has depended on the quantitative aspects of the binding of dyes by proteins (*9*). Polar groups in proteins can bind dyes of opposite charge. Milk proteins, on the acid side of the isoelectric point, carry an excess positive charge and act as large cations. When a negatively charged dye (an acid dye) is introduced into the solution, it combines with protein, forming

[1] Italic numbers in parentheses refer to Literature Cited, p. 42.

an insoluble protein-dye complex. Excess dye is required for the precipitation of protein to be complete. The protein content is estimated from the amount of free dye (not bound), which can be determined by measuring the optical density of the supernatant.

The dye-binding procedure appears to be a quick and reliable method for the routine determination of protein in normal milk (*2, 3, 7, 22, 27, 28, 36*). Yet to be overcome are some of the limitations of the dye-binding method. Also, there is a need for standardization of the method so that the same procedure can be used in all laboratories. Opinions differ as to the choice of a dye. The principal dyes used for the test have been two acid dyes, orange G and amido black 10B. It has been shown that amido black gives much more sensitive optical indication of protein content than orange G (*7*). On the other hand, Ashworth and Chaudry (*2*) claim that orange G shows more stoichiometry in its reaction with milk proteins than amido black. The apparently greater stoichiometry of orange G may instead reflect its lower sensitivity. European laboratories (*21, 23*) are using amido black 10B for mass protein testing, with what appears to be great success.

In Holland, the dye-binding method with amido black 10B for protein determination has been used on a large scale since 1958, both for milk pricing and guidance in breeding. In 1964 in the province of Friesland, all milk produced was paid for on the basis of protein and fat content. In other regions, milk for manufacturing was paid for on the basis of protein and fat, and market milk on the basis of fat alone. Protein determinations are made in large central laboratories, where the amido black test is practically completely automated.

The procedure used in the central laboratory of The Association of Cooperative Dairies, Zutphen, Holland, consists of the following phases:

(1) Pipette 1 ml. of milk (96 samples are taken simultaneously with an assembly of Cornwall-syringe pipettes).

(2) Add 20 ml. of amido black solution (automatic).

(3) Mix milk and dye (by introducing a stream of compressed air for 1 min.).

(4) Separate the protein-dye complex (samples in racks are centrifuged in a specially built, large centrifuge).

(5) Read the optical density of the supernatant and convert the reading directly into percentage of protein. Both these steps are fully automated, which makes possible the protein determination of 2,000 samples per hour.

Every 12th sample is a control (protein content determined by Kjeldahl). Twenty-five percent of all samples are run in duplicate. The maximum tolerance of deviation from the Kjeldahl test is $\pm 0.12$ percent. The all-inclusive cost of the test is 0.1244 guilder per sample, which is equivalent to about 3.5 cents. The test is run on producer's composite milk samples preserved with $HgCl_2$.

The Zutphen laboratory—with a personnel of 12 girls, 2 technicians with college degrees, and 1 supervisor—ran 3 million test samples in a year of 235 working days. This experience with dye binding in Holland has been described in detail to illustrate the

feasibility of routine, large-scale protein determination by the dye-binding method.

This bulletin consists of a review of the literature and a detailed study of the binding of dye by milk proteins. The topics covered include an evaluation of the accuracy, reproducibility, and sensitivity of the dye-binding reaction; binding by individual proteins; effect of processing and preservatives; abnormal milks; and results from field tests.

## LITERATURE REVIEW

With the increase in emphasis on the importance of milk protein, the demand has grown for simple and rapid methods for protein determination. Several methods have been studied for this purpose. Developments in this field have been reviewed briefly by Raadsveld (*23*), Dolby (*7*), and recently, Booy and coworkers (*5*). Among the many methods, the one thought to be the most suitable at present is dye binding.

### Dyes Used

The dyes currently used for dye binding are orange G and amido black 10B. Acid orange 12 has been recommended by Udy (*34*). Figure 1 shows the structural formulas of these dyes.

In 1944, Fraenkel-Conrat and Cooper (*9*) reported that orange G is bound almost quantitatively by several proteins at pH 2.2. Udy first applied orange G to determine the protein content of

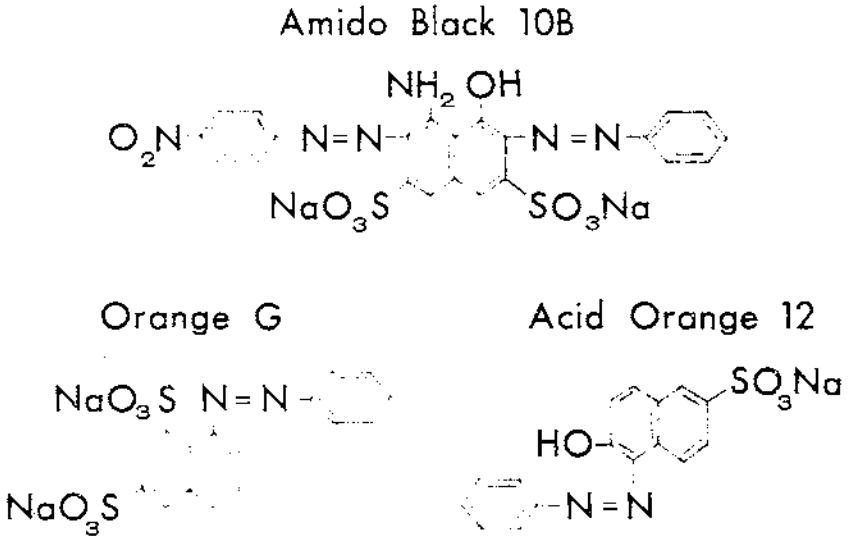


FIGURE 1.—Dyes most frequently used in dye binding.

wheat (*31*), and later applied the method to milk and powdered milk (*32*). Additional work with the orange G method has been reported by Ashworth and coworkers (*3*).

The amido black method was first reported by the German scientists Schober and Hetzel (*27*). The accuracy of the amido black method for determination of the protein in milk was extensively studied by Steinsholt (*29*). Raadsveld (*22*) and Posthumus (*21*) modified the method to make it suitable and practical for mass protein testing of producers' milk in Holland.

The procedures of the dye-binding test used and recommended by various workers vary greatly. These variations are briefly reviewed here.

## Amount of Sample

When using the orange G method, most workers have used for the test 1.5 ml. of milk. Ashworth indicated, more precisely, 1.500± 0.003 ml. (*3*). The dilution of milk samples is suggested by some workers (*7*, *28*). When the amido black method is used, it has been claimed that diluted samples give more accurate results (*7*, *27*, *28*, *29*). A certain type of syringe used by Raadsveld gave good results without any dilution of milk samples (*22*). The standard deviation of that syringe in measuring 0.5 ml. was only 0.005 ml. Others have used 1 ml. (*11*) and 0.95 ml. (*21*) of milk sample.

## Buffer Solution and Dye Concentration

To make the precipitation of the protein dye complex complete, the protein must be treated with excess dye in acidic solution. For the results to be reproducible, the pH of the reaction mixture must be rigidly controlled, which is accomplished by dissolving the dye in acidic buffer solution.

Fraenkel-Conrat and Cooper (*9*), working with egg albumen and several other proteins, including casein, and using citrate-phosphate buffer, reported that protein dissociation was complete at pH 2.2. An acid solution of lower pH caused a breakdown of protein. The amount of dye bound per unit amount of protein was independent of protein or dye concentration, provided that protein concentration was within the limits of 0.08 to 0.2 percent and dye concentration was within 0.05 to 0.1 percent.

Citric acid buffer of pH 2.0 was used by Ashworth and coworkers (*3*). Shiga and coworkers (*28*) used a 0.1 *M* HCl-sodium citrate buffer at pH 2.2 and 25 ml. of buffer-dye solution (1.0 g. of orange G per liter). Dolby (*7*) used a 0.3 *M* citric acid buffer; his dye concentration was also 0.1 percent, but he used 10 ml. of buffer-dye solution.

All workers using the amido black method reported using citrate-phosphate buffer. Schober and Hetzel (*27*) dissolved 0.6165 g. of amido black 10B in 1 liter of 0.1 *M* citric acid phosphate buffer of pH 2.8 (19.212 g. citric acid and 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ dissolved in 1 liter of water). Twenty ml. of the

buffer-dye solution was used in the experiments. They reported that the protein-dye complex precipitation was incomplete above pH 3.5. Shiga and coworkers (*28*) used the same buffer-dye solution and reported that the amount of dye bound decreased slightly with an increase in pH. When the pH was more than 4.0, precipitation of the protein-dye complex was incomplete and measurement of the relation between pH and the amount of dye bound became impossible. Raadsveld (*22*) and other workers (*11, 21*) used a buffer-dye solution in which 0.4400 g. of amido black 10B was dissolved into each liter of citrate-phosphate buffer of pH 2.35 (15.850 g. of citric acid and 2.078 g. of $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 1 liter of water). Vanderzant and Tennison (*35*) reported using 0.6 g. of buffalo black dissolved in 1 liter of citrate-phosphate buffer of pH 2.35. They later reported that a dye concentration of 0.342 g. per liter of buffer solution was enough to complete precipitation of the protein-dye complex in 0.5 ml. of milk (*36*). Dolby (*7*) dissolved 0.6165 g. of amido black 10B in 0.3 *M* citric acid buffer and used 10 ml. of buffer-dye solution for the precipitation of protein in a diluted milk sample equivalent to 0.25 ml. of undiluted milk.

Herrington (*13*) reported that phosphoric acid buffer was superior to citrate buffer because citrates are attacked by some micro-organisms, which changes the pH and causes errors in protein determinations. Some workers recommended using mold inhibitors such as thymol and propionic acid.

Commercially prepared dyes are not 100 percent pure. The best grade of amido black 10B available is only 86 percent pure. In addition, it is hygroscopic and changes in moisture content on storage. Raadsveld (*23*) recommended standardizing buffer-dye solution by optical-density measurements. The use of the absorbancy index (absorbancy index $K = A/C$, where $A$ = absorbance in a 1-cm. cuvette and $C$ = dye concentration) as a criterion of the purity of dye was suggested by Ashworth and Chaudry (*2*).

## Method of Mixing Sample Buffer-Dye Mixtures

Fraenkel-Conrat and Cooper (*9*) reported that 20 hours of shaking was necessary to complete the reaction between orange G dye and the protein. The long reaction time was supposedly caused by their use of a very small amount of buffer solution (only 1 ml.). Udy (*32*) shortened the shaking time to 5 minutes; this method was used by later workers (*30*). Ashworth and coworkers (*3*) reported shaking the mixture 15 seconds. Schober and Hetzel (*27*) stirred the mixture with a mechanical stirrer for 10 minutes. Posthumus (*21*) used air bubbling for 1 minute instead of mechanical stirring. Shiga and coworkers (*28*) added buffer-dye solution while shaking the mixture and allowed it to stand for at least 3 minutes. Vanderzant and Tennison (*35, 36*) studied this problem more precisely and found no difference between the shaken and unshaken mixtures. This finding indicates that the reaction between dye and protein is fast enough so that no additional shaking or stirring is necessary if the dye and milk are properly mixed.

## Method of Separating Protein-Dye Complex

The precipitate from the reaction mixture is obtained by filtration or centrifugation. Most workers prefer the centrifugation method as more rapid and suitable, especially in testing a large number of samples. A centrifuge with a capacity of 96 samples has been introduced in Holland (*23*). Various centrifuging speeds have been reported, ranging from 1,500 to 4,000 r.p.m.

Ashworth and coworkers (*3*) claimed that with the orange G method the filtration procedure is superior. The protein-dye complex formed is presumably of lower density with orange G than with amido black and cannot always be separated completely by centrifugation. A very small amount of dye is absorbed by the filter paper, but the amount is so small that the difference in results between filtration and centrifugation is negligible (*28*). The evidence that the protein-dye complex formed with amido black is more dense and therefore more easily separated by centrifugation was one of the reasons for claiming the superiority of the amido black method over the orange G method (*27*, *29*, *36*).

## Measurement of Optical Density

The concentration of unused dye is determined by measuring the optical density of the supernatant of the protein-dye reaction mixture.

For optical measurement to be applicable, the solution must obey Beer's law. The solutions of both orange G and amido black satisfy this requirement under the experimental conditions (*3*, *29*, *31*).

The amount of dye added to the sample is in considerable excess, and therefore the supernatant must be diluted twentyfold or more for measurement of optical density in a usual 1-cm. cuvette. Udy (*33*) reported using a special short-light-path cell that had a light path of 0.007 inch. With this cell he could measure the optical density of the supernatant without any dilution. This type of cell has been used by other workers also.

The maximum absorption of orange G has been reported to be at 485 m$\mu$ by Udy (*31*), and from 430 to 480 m$\mu$ by others (*28*). Maximum absorption for amido black 10B is reported to be at 610 to 620 m$\mu$ (*7*, *29*).

## Accuracy of Dye-Binding Method

Numerous workers (*2*, *7*, *22*, *28*, *29*, *36*) have compared the accuracy of the dye-binding method against the official Kjeldahl method. All have reported a linear relation between the two. Table 1 gives regression equations expressing the relation between percentage protein by the Kjeldahl method and optical density.

The equations differ somewhat because of different experimental procedures. These data would be much more valuable if different laboratories could agree to use the same procedure. However,

TABLE 1.— *Comparison of dye-binding methods with Kjeldahl method for milk protein determination*

| Number of samples | Regression equation [1] | Equation for- | Standard error of estimate | Correlation coefficient | Reference |
|---|---|---|---|---|---|
| | | | *Percent* | | |
| 62 | $P = -3.132\ D + 4.738$ | Amido black 10B | ±0.12 | −0.982 | (*29*) |
| 80 | $P = -2.101\ D + 5.19$ | Amido black 10B | ±0.12 | −0.992 | (*22*) |
| | $X = 0.063\ P + 0.013$ | Amido black 10B | ±0.083 | −0.98 | (*28*) |
| 190 | $P = -3.68\ D + 5.07$ | Amido black 10B | ±0.12 | −0.963 | (*56*) |
| 36 | $P = 6.90\ X + 0.09$ | Amido black 10B | ±0.07 | | (*7*) |
| 36 | $P = 20.6D + 0.25$ | Orange G | ±0.07 | | (*7*) |
| 132 | $P = 1.72D + 4.70$ | Amido black 10B | | −0.912 | (*2*) |

[1] $P$ = percentage of protein; $D$ = optical density; $X$ = dye bound (change in optical density due to dye binding by protein).

there is no doubt as to the high accuracy of either the amido black or the orange G method, as can be seen from the numerical value of standard deviations and correlation coefficients. This review of the literature of the dye-binding test has been limited almost exclusively to mixed samples of fresh raw milk.

## Dye-Binding Capacity

Udy (*32*) reported 179.5 mg. of orange G bound per gram of the protein of whole milk, and 182 mg. for the protein of dry milk. He indicated that the reason for the slight difference might be denaturation of proteins by the heat treatment. Ashworth and Chaudry (*2*) gave the following 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.

For amido black 10B, Hadland and Johnsen (*11*) reported 325 mg. of dye bound per gram of protein of fresh milk, 300 mg. for cheese whey, and 220 mg. for ripened cheese. Values obtained by Vanderzant and Tennison (*36*) were 266 mg. for fresh milk, 279 mg. for α-casein, 212 mg. for β-casein, 202 mg. for γ-casein, and 288 mg. for lactalbumin.

There is a considerable variation in dye-binding capacity (*DBC*) reported by different authors. One of the reasons is undoubtedly a variation in the purity of dyes used.

Rosenberg and Klotz (*25*) theorized multiple equilibria in dye binding. A series of equilibrium relations must be considered between protein and dye, and it is possible that more than one dye molecule can be bound to one protein molecule when the concentration of dye present is in large excess.

Ashworth and Chaudry (*2*) defined *DBC* as the milligrams of dye bound per unit of weight of protein. According to them, *DBC*

was affected by both protein concentration and free dye concentration in the supernatant solution. They stated that the reaction between protein and dye was not a simple stoichiometric reaction. Results were unreliable when the ratio of total dye to protein became excessively large. Working with normal milks, Dolby (*7*) reported, contrary to the finding of Ashworth and coworkers (*3*), no increase in the *DBC* of protein as the protein content of the milk increased.

## Other Factors Affecting Dye Binding

The amount of dye bound was slightly different at different temperatures (*27*), but the difference was so small that the effect of temperature could be disregarded (*28*). Udy (*32*) reported that protein denaturation by heat treatment was the probable reason for the difference in *DBC* between whole milk and powdered milk. Alais and coworkers (*1*) also suggested that the binding of dye might be affected by heating the milk. Vanderzant and Tennison (*36*) reported no significant effect from pasteurization by holding or by the HTST (high temperature-short time) method.

Data are scanty and inconclusive on the accuracy of the dye-binding test on milk from individual cows and atypical milks. Raadsveld (*23*) indicated that testing laboratories in Holland consider the test accurate for mixed milk as well as for milk of individual cows. Only colostrum and milk of the very last days of lactation gave abnormal deviations. Vanderzant and Tennison (*36*) indicated that milks from cows with acute mastitis were frequently outside of the normal deviation from results with the Kjeldahl method.

Ashworth and coworkers (*3*) studied the effect on dye binding of the preservatives $H_2O_2$, HCHO, $K_2Cr_2O_7$, 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 lowered the apparent protein content; that $K_2Cr_2O_7$ increased the apparent protein content at the initial stage and then lowered it after a week of storage at room temperature; and that $HgCl_2$ lowered the apparent protein content very slightly but was the best preservative for the dye-binding test. Successful use of $HgCl_2$ was also reported by others (*22*, *29*). According to Vanderzant and Tennison (*36*), milk samples could be stored for the dye-binding test as long as 7 days at 40° to 45° F., with or without $HgCl_2$ or $K_2Cr_2O_7$ as preservatives.

## Comparison of Orange G and Amido Black Methods

The amido black method was found to be more sensitive than the orange G method (*7*, *28*). The ratio of sensitivities was 4.3/1.2 (*28*). Moreover, the amido black reacted more quickly with the protein, and this protein-dye complex, being more dense, was more easily removed by centrifuging (*27*, *29*, *36*).

# EXPERIMENTAL PROCEDURES

## Determination of Protein in Milk

### Dye-Binding Methods

#### *Amido Black 10B Method*

After preliminary study of several procedures, the authors adopted the Raadsveld method (*22*) with minor modifications. The principal modification was that optical density was measured at 615 mμ (maximum absorption) instead of at 570 to 590 mμ.

*Reagent.*—Amido black 10B, electrophoresis grade.[2]

*Buffer solution of pH 2.35.*—This solution was prepared from 1.674 g. of $Na_2HPO_4$ (anhydrous) and 17.371 g. of citric acid. $H_2O$ per liter of solution (or 826 ml. of 0.1 *M*-citric acid and 59 ml. of 0.2 *M*-$Na_2HPO_4$ and 115 ml. $H_2O$). The pH was adjusted carefully.

*Buffer-dye solution.*—0.4400 g. of amido black 10B dye was added to 1 liter of the citrate-phosphate buffer. The solution was mixed thoroughly and allowed to stand for 24 hours with occasional stirring before standardization. The buffer-dye solution was standardized by a spectrophotometer at 615 mμ. A 100-to-1 dilution of this solution has an optical density of 0.320 ± 0.005 with a Coleman Junior 6A spectrophotometer (10- by 75-mm. cuvette). This instrument was standardized at 610 mμ against a Coleman No. 6-400 didymium calibration standard.

*Method of analysis.*—At room temperature, 0.5 ml. of milk was transferred to a centrifuge tube and exactly 20 ml. of the buffer-dye solution was added.[3] The mixture was stirred for 30 seconds with a mechanical stirrer and then centrifuged for 10 minutes at 2,500 r.p.m.

Most of the supernatant from the centrifuge tube was decanted, and 1 ml. of this supernatant was diluted with 25 ml. of distilled water.[4]

The optical density of this diluted supernatant was measured against distilled water in a 1-cm. cuvette at a wavelength of 615 mμ.

#### *Orange G Method*

The orange G procedure used was essentially that used by Ashworth, except that 0.5 ml. of milk sample and 20 ml. of buffer-dye solution were used in place of 1.5 ml. of milk and 25 ml. of buffer-dye. Both filtration and centrifugation were tried and compared

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[2] Available from E. Merck A.G. Darmstadt, Germany.

[3] The buffer-dye solution was added with a 20-ml. automatic pipet from AB Ljungberg & Co., Stockholm, Sweden; the pipet's standard deviation is 0.0104 (see appendix table 10).

[4] The distilled water was added with a 25-ml. automatic pipet from AB Ljungberg & Co., Stockholm, Sweden; this pipet's standard deviation is 0.0046 (see appendix table 10).

for separation of the protein-dye complex. Optical density was read at 480 mμ. In another experiment, in which the sensitivity of orange G and amido black 10B were compared, 1 ml. of milk sample and 20 ml. of the orange G dye were used, giving nearly the same protein-to-dye ratio as that used by Ashworth and coworkers (*3*).

### *Calculation of Dye-Binding Capacity*

(1) The milligrams of dye left in the supernatant were calculated from the equation:

$$\text{Milligrams of dye} = \frac{E_2}{E_1} \times \frac{\text{dye concentration of buffer-dye solution}}{100} \times D.F.$$

where:

$E_1$ = optical density of 1:100 diluted buffer-dye solution
$E_2$ = optical density of diluted supernatant
$D.F.$ = dilution factor

(2) Protein was calculated from Kjeldahl nitrogen values as follows:

Protein percentage = (total nitrogen − nonprotein nitrogen percentage) × 6.38
Grams of protein = weight of sample × protein percentage

(3) Dye-binding capacity was calculated from the equation:

$$DBC = \frac{\text{milligrams of dye in 20 ml. buffer-dye solution} - \text{milligrams of dye left in supernatant}}{\text{grams of protein}}$$

$$= \text{milligrams of dye/grams of protein}$$

## Kjeldahl (Semi-Micro) Method

The following modification of Rowland's Kjeldahl method (*26*) was used.

### *Kjeldahl Reagent*

One thousand milliliters of concentrated chemically pure $H_2SO_4$ low in nitrogen was added slowly with stirring to a solution prepared by dissolving 25 g. of $CuSO_4 \cdot 5H_2O$ and 200 g. of anhydrous $Na_2SO_4$ in 1,200 ml. of double-distilled water. After the solution was cooled to room temperature, 20 ml. of selenic acid was added, and the total volume was brought to 2,500 ml. with double-distilled water.

### *Receiving Solution*

Ten ml. of 0.1-percent methyl red and 20 ml. of 0.1-percent bromcresol green in ethyl alcohol were added to each 2 liters of a 2 percent boric acid solution.

*Total Nitrogen*

Ten ml. of milk sample was weighed and diluted with double-distilled water to 100 ml. in a volumetric flask. Triplicate analyses for total nitrogen were made on 10-ml. aliquots of the diluted milk samples.

*Nonprotein Nitrogen*

Ten ml. of milk sample was weighed into a 125-ml. Erlenmeyer flask. Twenty ml. of 20-percent trichloroacetic acid and 10 ml. of double-distilled water were added to the sample with continuous shaking. After the contents remained undisturbed for 5 minutes, they were filtered through a Whatman No. 12 folded filter paper. Three 10-ml. samples of the filtrate were pipetted into three Kjeldahl digestion flasks. Two or three glass beads and 8 ml. of Kjeldahl reagent were added to each flask.

*Digestion of the Sample*

After a few drops of antifoam were added, the samples were digested for 1 hour on the digestion rack. Ten ml. of the double-distilled water was added when the flasks were cooled to room temperature.

*Distillation*

After each flask received 17 ml. of 30-percent NaOH, the contents were distilled for 12 minutes. The distillate was received into 20 ml. of receiving solution. The boric acid solution was titrated with 0.01 *N* HCl solution until a faint pink color appeared.

## Preparation of Protein Fractions

The butterfat was separated from mixed herd milk. All casein fractions were prepared from the skim portion. The skim milk was dialyzed against a large volume of distilled water for 72 hours at 2° C. and then freeze-dried.

### Acid Casein

Acid casein was precipitated at pH 4.6 with addition of 1 *N* HCl. The precipitate was washed three times with distilled water and then dissolved with dilute NaOH solution, raising the pH to 8.5. The casein was reprecipitated at pH 4.6. The procedure was repeated four times. Finally, the casein precipitate was washed three times with distilled water and dried with ethyl alcohol followed by ethyl ether.

$\alpha$-, $\beta$-, and $\gamma$-caseins were prepared by the urea method of Hipp and coworkers (*14*).

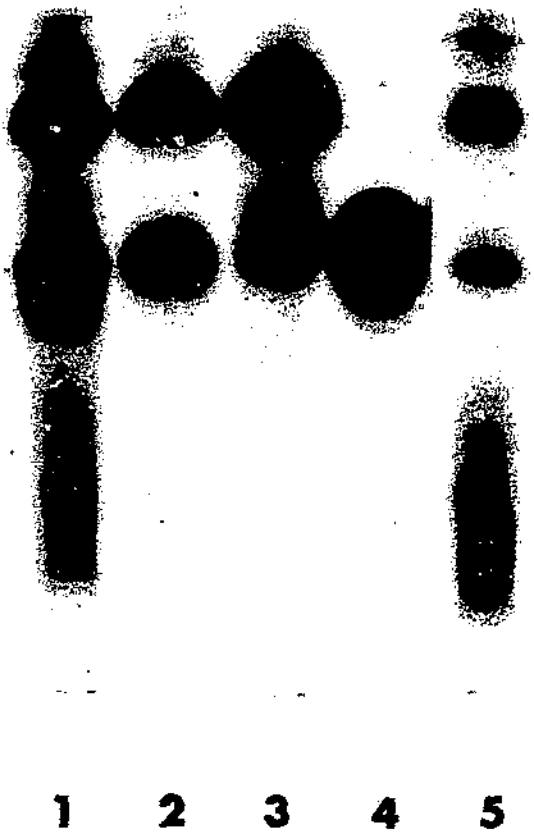


FIGURE 2.—Starch-gel electrophoresis of casein fractions: 1—dialyzed freeze-dried skim milk; 2—acid-precipitated casein; 3—α-casein; 4—β-casein; 5—κ-casein.

## α-Casein

Acid casein was dissolved in 6.6 *M* urea, and the α-fraction was precipitated at a urea concentration of 4.6 *M* by dilution with distilled water. The α-casein was suspended in distilled water and dialyzed against distilled water for 72 hours at 2° C. and then freeze-dried. Starch-gel electrophoresis in 7 *M* urea at pH 8.6 showed some contamination with β-casein (fig. 2).

## β-Casein

The portion that precipitated in 3.3 *M* urea was discarded. β-casein was precipitated by further dilution to a urea concentration of 1.7 *M*, and the pH was then adjusted to 4.7. Purification

was done by reprecipitating twice from 4.6 *M* urea solution. Each time, the portion precipitated at a concentration of 1.7 *M* was collected. The final precipitate was washed with distilled water three times, dialyzed, and freeze-dried. Starch-gel electrophoresis in 7 *M* urea at pH 8.6 showed no contamination with other fractions (fig. 2).

### γ-Casein

The portion soluble in 1 *M* urea but insoluble in 1.6 *M* ammonium sulfate was collected. The precipitate was dissolved in dilute NaOH solution and brought to a pH of 4.7 at 20° C. to precipitate the impurities. The filtrate was dialyzed against distilled water for 72 hours at 2° C. and then freeze-dried. The starch-gel electrophoresis pattern was identical to the one for γ-casein obtained with Hipp's method as shown by Groves and coworkers (*10*).

### Ca-Sensitive and Ca-Insensitive α-Casein

Fox's method (*8*) was used to prepare these fractions. Purified α-casein was dissolved with dilute NaOH (protein concentration was about 2 percent) and the solution was cooled to 5° C. $CaCl_2$ was added to bring the concentration of $CaCl_2$ in the solution to 0.15 *M*. The pH of the solution was then adjusted to 8.3. The precipitated Ca-sensitive fraction was collected by centrifuging, washing with water, and drying with ethyl alcohol and ethyl ether.

The supernatant was warmed to 30° C. and adjusted to pH 4.7. The precipitated Ca-insensitive α-casein was centrifuged, washed twice with distilled water, and dried with organic solvents.

### κ-Casein

κ-casein was prepared by the method of Cheeseman (*6*). To remove Ca-sensitive α-casein, 0.2 *M* $CaCl_2$ was added to casein solution to pH 7.0 at 4° C. The solution was warmed up to 37° C. and then kept standing for 1 hour. The precipitate was centrifuged out. The supernatant solution was kept overnight at 4° C. and κ-casein was precipitated at that temperature and at pH 4.6 with acetic acid. The κ-casein was washed twice with distilled water and dried with organic solvents. The purity of this fraction is shown by starch-gel electrophoresis in 7 *M* urea at pH 8.6 (see fig. 2).

### Whey Protein

Acid whey was kept in the cold (about 0° C.) overnight at pH 4.6, and then filtered. The filtered whey was dialyzed against a large volume of distilled water for 72 hours in the cold and then freeze-dried.

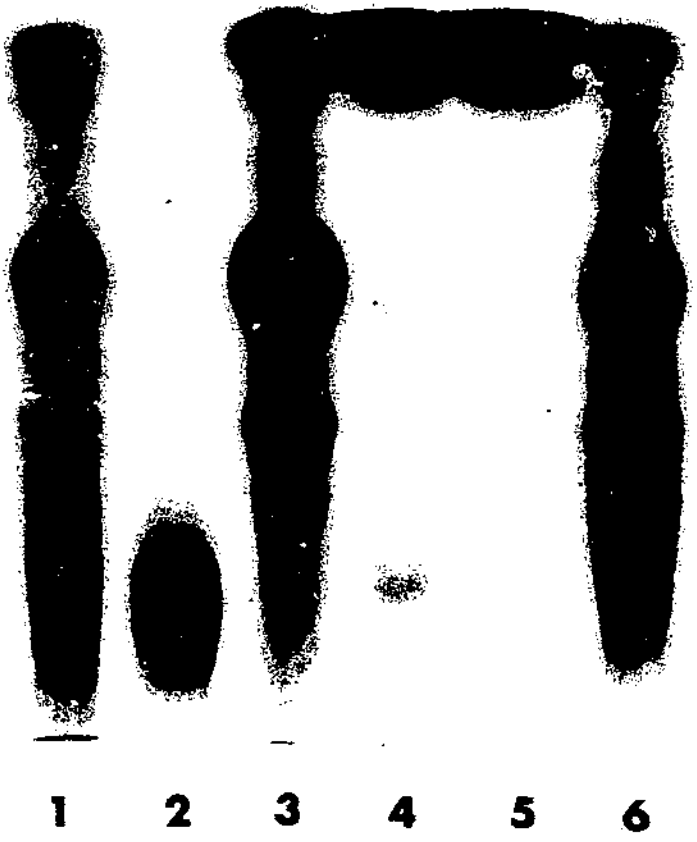


FIGURE 3.—Starch-gel electrophoresis of whey protein fractions: 1—whey protein; 2—globulin; 3—albumin; 4—α-lactalbumin; 5—α-lactalbumin; 6—β-lactoglobulin.

## Lactoglobulin Fraction

Lactoglobulin was precipitated at half-saturation of ammonium sulfate from acid whey at pH 6.0. The precipitate was dissolved in 0.02 *M* phosphate buffer of pH 7.0 and then dialyzed against the same buffer solution for 72 hours. The dialyzed solution was applied to a DEAE column (9.0 by 5.5 cm.) and chromatographed by the stepwise method described by Yaguchi and coworkers (*38*). The effluent of the peak *b* was collected as the lactoglobulin fraction, dialyzed against distilled water, and then freeze-dried. The starch-gel electrophoresis pattern showed only one band, and it is presumed that the fraction was pseudoglobulin (fig. 3).

### α-Lactalbumin and β-Lactoglobulin

The filtrate from the separated globulin fraction of whey was saturated with ammonium sulfate, and the lactalbumin fraction was precipitated. The precipitate was dissolved in phosphate buffer of pH 7.0 (0.02 *M*) and dialyzed against the same buffer. The dialyzed solution was chromatographed on a DEAE column (8.0 by 5.5 cm.) in the same manner as the globulin preparation. The effluent of peaks *c*, *d*, and *e*, comprising primarily the α-lactalbumin fraction,[5] was dialyzed against distilled water and freeze-dried. The β-lactoglobulin fraction was prepared by treating the effluent of peaks *f* and *g* in a similar manner. The starch-gel electrophoresis pattern showed some contamination of α-lactalbumin with β-lactoglobulin, and some admixture of α-lactalbumin in the β-lactoglobulin preparation (fig. 3).

### Starch-Gel Electrophoresis Method

Essentially the same starch-gel electrophoresis method described by Wake and Baldwin (*37*) was used. The only difference was that the stained gel was washed with 2-percent acetic acid (by volume) instead of 10-percent glycerol.

## STUDY OF FACTORS AFFECTING DYE BINDING

### Spectral Absorption Curves of Amido Black 10B and Orange G

One ml. of each buffer-dye solution was diluted to 100 ml. with distilled water. Optical density at each interval of 10 mμ in the range from 400 to 700 mμ was measured with a Coleman 6A Jr. spectrophotometer. Absorption was maximal between 610 and 620 mμ for each amido black 10B and at 480 mμ for orange G (fig. 4). The curve for amido black shows a second small peak at 320 mμ. The pH of the dye solution was found to have no effect on maximum absorption. Wavelengths of 615 and 480 mμ, respectively, were adopted for amido black 10 B and orange G and used in determining optical densities in all subsequent experiments.

### Comparative Sensitivity of Amido Black 10B and Orange G

Milk samples of high, medium, and low protein content were diluted with distilled water to get various protein concentrations ranging from 2.74 to 4.02 percent. The samples were analyzed by three methods: (1) the amido black 10B method, (2) the orange G centrifugation method, and (3) the orange G filtration method. Amido black is more sensitive than orange G (fig. 5). The difference in the results from filtration and centrifugation was not significant.

[5] Obtained from R. Jenness, University of Minnesota.

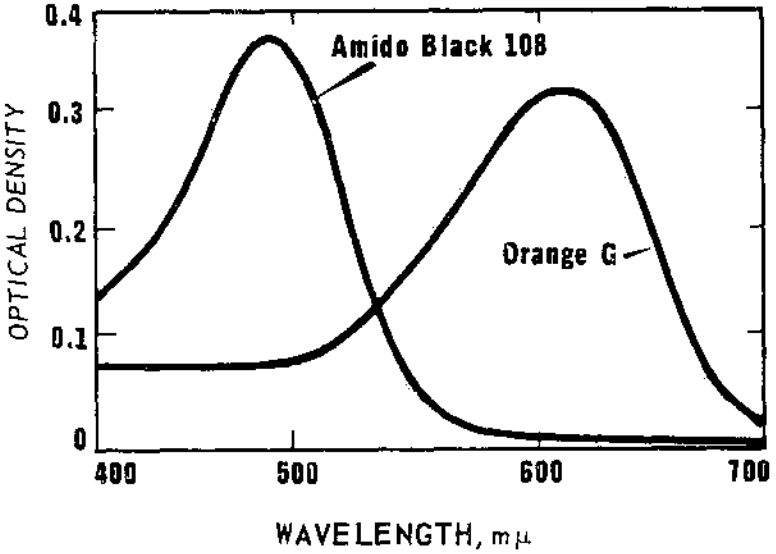


FIGURE 4.—Special absorption curves, amido black 10B and orange G.

TABLE 2.—*Replicated analysis of a milk sample containing 3.19 percent protein (Kjeldahl)*[1]

| Analysis | Protein found with amido black 10B [2] | $d$ | $d^2$ | Protein found with orange G [2] | $d$ | $d^2$ |
|---|---|---|---|---|---|---|
| | *Percent* | | | *Percent* | | |
| 1 | 3.19 | −1 | 1 | 3.19 | 0 | 0 |
| 2 | 3.19 | −1 | 1 | 3.19 | 0 | 0 |
| 3 | 3.20 | 0 | 0 | 3.23 | +4 | 16 |
| 4 | 3.20 | 0 | 0 | 3.19 | 0 | 0 |
| 5 | 3.20 | 0 | 0 | 3.19 | 0 | 0 |
| 6 | 3.19 | −1 | 1 | 3.15 | −4 | 16 |
| 7 | 3.20 | 0 | 0 | 3.19 | 0 | 0 |
| 8 | 3.21 | +1 | 1 | 3.23 | +4 | 16 |
| 9 | 3.19 | −1 | 1 | 3.19 | 0 | 0 |
| 10 | 3.19 | −1 | 1 | 3.19 | 0 | 0 |
| Total | 31.96 | −4 | 6 | 31.94 | +4 | 48 |
| Average | 3.20 | | | 3.19 | | |

[1] $d=(X_1-X)\times 10^2$

[2] Regression equations used to calculate percentage of protein:

Amido black: $X=-4.4893Y+5.111$

Orange G: $X=-39.53Y+16.23$

Where $X$ = percentage of protein
and $Y$ = optical density.

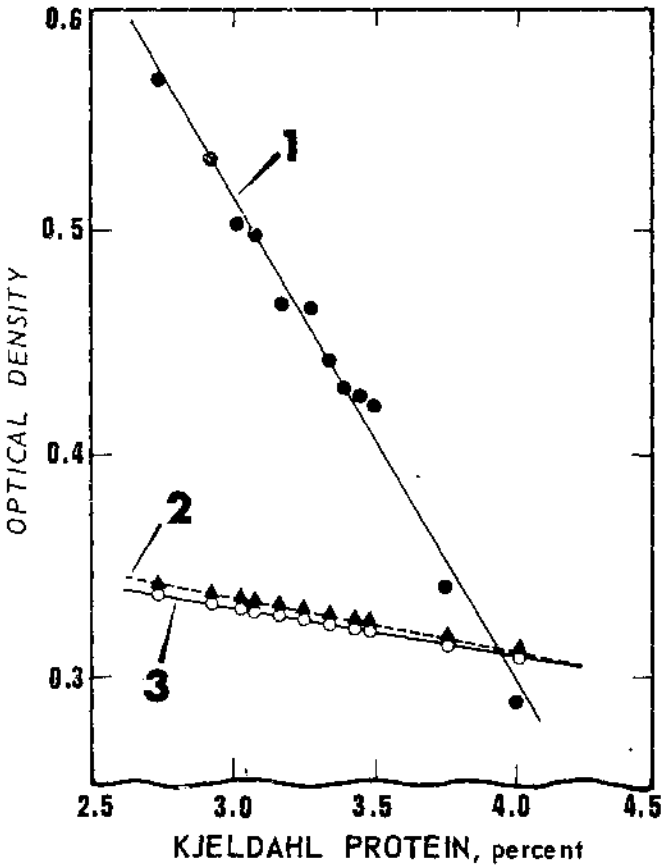


FIGURE 5.—Relation between Kjeldahl protein and optical density. Curves are for amido black (1), orange G (2, centrifugation), and orange G (3, filtration).

The accuracy of the dye-binding test was not affected significantly by whether optical density was measured by the Beckman DU or Coleman 6A Jr. spectrophotometer. The Coleman instrument, which required less time, was therefore used in all subsequent experiments.

## Reproducibility of Amido Black 10B and Orange G Methods

Ten separate analyses were made on the same milk sample with amido black 10B and orange G methods. The protein content of the sample as determined by the Kjeldahl method was 3.19 percent. The results are shown in table 2. Both methods gave excellent reproducibility. Because of its higher sensitivity, the amido black 10B method was used in all of the following experiments, unless stated otherwise.

## Effect of Processing Treatments on Accuracy and Reliability

### pH of Buffer Solution

The same amount of amido black 10B (440 mg.) was dissolved in 1-liter amounts of buffer solutions of pH 1.75, 2.00, 2.35, 2.50, 2.75, 3.00, 4.00, and 5.00. The raw skim milk sample was analyzed with these eight different buffer-dye solutions. The protein-dye reaction was affected greatly by pH (fig. 6). The higher the pH, the less dye was bound by the proteins; at pH 5 almost no protein-dye complex was formed. This fact indicates that the pH of the buffer-dye solution should be adjusted very carefully.

Fraenkel-Conrat and Cooper (*9*) reported that a pH below 2.2 may cause a breakdown of protein by hydrolysis. For the analysis of milk protein, Raadsveld (*23*) and other workers in Europe (*11, 21, 29*) chose a pH of 2.35, which appears to be optimum for the dissociation of ionizable protein groups without acid hydrolysis. This pH was adopted for the procedure used in the experiments described in this bulletin.

### Time of Mixing

The milk sample was pipetted into a centrifuge tube and dye solution was added to the milk. The sample-dye mixture was stirred by a mechanical stirrer for 0, 0.5, 1, 2, 3, 5, 10, and 15 minutes. After the protein-dye complex was removed by centri-

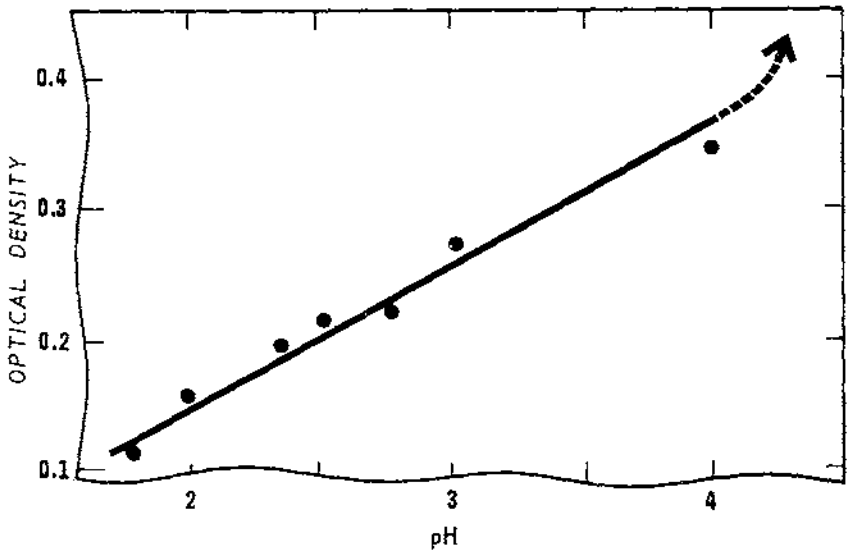


FIGURE 6.—Effect of pH on dye binding; amido black 10B.

TABLE 3.—*The effect of time of mixing on dye binding*

| Time of mixing (*minutes*) | Optical density of dye remaining in supernatant | |
|---|---|---|
| | Amido black 10B | Orange G |
| 0 | 0.445 | 0.330 |
| 0.5 | .442 | .331 |
| 1 | .445 | .329 |
| 2 | .444 | .328 |
| 3 | .444 | .328 |
| 5 | .443 | .327 |
| 10 | .442 | .328 |
| 15 | .441 | .328 |

fuging, optical density was measured. The results are shown in table 3. No significant difference in optical density was found between unstirred samples and those stirred for 15 minutes. The data indicate a very rapid reaction between protein and dye. When, however, milk is added to the dye solution instead of dye solution added to the milk, more mixing time is required. The volume ratio of milk to dye solution is 1:40. Under these conditions, adding milk to dye solution at a pH below the isoelectric point causes partial acid coagulation of casein. Completion of the reaction between protein and dye requires that the coagulated aggregates be dispersed by stirring.

## Reversibility of Protein-Dye Reaction

The reversibility of the protein-dye reaction was tested by suspending the precipitated protein-dye complex in the same buffer solution used in the buffer-dye solution and dialyzing against this buffer for 48 hours at room temperature. No dye was dialyzed out. This fact indicates the irreversibility of the reaction between protein and dye at the pH of the reaction. Additional experiments with dialysis of protein-dye complex at higher pH showed that the dye was dialyzed out at a pH above 5.0.

In the presence of urea in concentrations of 0.2 *M* or above, the dye was dialyzed out at pH 2.35, the chosen optimum pH for dye-protein complex formation. The evidence indicates that hydrogen bonding is involved in the formation of the dye-protein complex.

## Temperature

The optical density of the supernatant was not changed significantly by temperature changes within the range of 10° to 42° C.

## Stability of Buffer-Dye Solution

In repeated trials no change in optical density was observed when the buffer-dye solution was aged for one month. It is concluded that the solution is stable if there is no gross environmental contamination by molds.

## Use of Preservatives

The effect of three preservatives was tested on identical samples of mixed-herd raw milk. Both controls and samples with added preservative were kept at 5° C. and at room temperature. Analyses were made daily for 15 days.

The preservatives and their concentrations were:

(1) Formaldehyde (36.6 percent); 2 drops/200 ml. of milk.
(2) $HgCl_2$: 100 mg./200 ml. of milk.
(3) $K_2Cr_2O_7$: 100 mg./200 ml. of milk.

The results were summarized in table 4. $K_2Cr_2O_7$ significantly lowered initial optical density below that of the control. Since $K_2Cr_2O_7$ is a strong oxidizing agent, it is very likely that some of the amido black was oxidized. Formaldehyde added at the rate of 1 drop/100 ml. of milk [much less than the amount recommended by the A.O.A.C. (4)] had no effect on initial optical density, but with storage the results became erratic. Formaldehyde is known to react with protein, and the reduction in dye bound as formaldehyde concentration is increased (fig. 7) suggests that it may compete with the dye for $NH_3^+$ sites of the protein. Table 4 shows that $HgCl_2$ can be used to preserve milk for the dye-binding test, even though added $HgCl_2$ slightly lowered the initial optical density. The difference was within experimental error, however.

TABLE 4.—*Effect of preservatives on dye binding*

| Storage temperature and time (*days*) | Optical density of dye remaining in supernatant [1] [2] | | | |
|---|---|---|---|---|
| | No preservative | HCHO | $HgCl_2$ | $K_2Cr_2O_7$ |
| Storage at 27° C.: | | | | |
| 0 | 0.455 cd | 0.455 cd | 0.447 def | 0.429 g |
| 3 | .449 def | .474 ab | .451 cde | .435 fg |
| 7 | | .478 a | .451 cde | .432 g |
| 10 | | | .451 cde | .436 efg |
| 15 | | | .457 cd | .447 def |
| Storage at 5° C.: | | | | |
| 0 | 0.455 cd | 0.455 cd | 0.447 def | 0.429 g |
| 3 | .451 cde | .458 cd | .448 def | .428 g |
| 7 | .450 def | .460 bcd | .447 def | .429 g |
| 10 | .451 cde | .462 bcd | .449 def | .426 g |
| 15 | | .466 abc | .452 cd | .431 g |

[1] Average of three samples of 3.31, 3.33, and 3.31 percent protein.

[2] Means followed by the same letter are not significantly different at the 5-percent level according to Duncan's multiple range test.

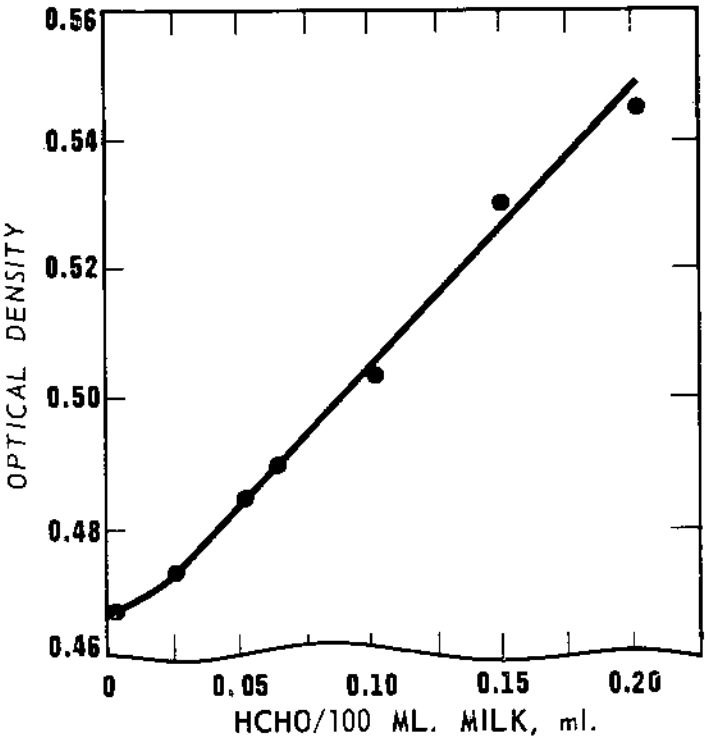


FIGURE 7.—Effect of formaldehyde concentration on dye binding; amido black 10B.

## Heating

Mixed-herd milk was subjected to various temperature-time heat treatments commonly used in processing milk. The heat-treated samples were analyzed for protein content by both the amido black and the Kjeldahl method. No significant difference was observed between the dye-binding capacity of heat-treated and control samples (table 5). Thus, the dye-binding method can be used for protein determination of pasteurized milk and milk subjected to other common processing heat treatments.

A sterilization heat intense enough to cause browning lowers the *DBC* of the milk proteins. Skim milk samples were heated to 120° C. for 10 minutes and for 8 minutes. The *DBC* of unsterilized and sterilized milk samples is shown in table 6. It is well known that the browning reaction in milk is due primarily to the Mailliard reaction of aldo-sugars with amino groups of proteins. The lowering of *DBC* by the browning reaction is to be expected. To study the relation between *DBC* and the intensity of the brown color, 350 ml. of condensed skim milk (32.11 percent total solids) was diluted with 150 ml. of distilled water and heated at 120° C. for 0,

TABLE 5.—*Effect of heat treatment on dye-binding capacity*

| Heat treatment | | *DBC* [1] [2] (amido black 10B) |
|---|---|---|
| Temperature (° C.) | Time | |
| | | *Mg. dye/g. protein* |
| Not heated | | 338.65 a |
| 63 | 30 min. | 340.10 a |
| 74 | 15 min. | 339.15 a |
| 85 | 15 sec. | 339.50 a |
| 93 | 1 sec. | 335.95 a |
| 88 | 15 min. | 338.88 a |

[1] Average of two milk samples.
[2] Means followed by the same letter are not significantly different at the 5-percent level, according to Duncan's multiple range test.

TABLE 6.—*Effect of sterilization of milk on dye-binding capacity*

| Sample | Dye-binding capacity [1] | | | Intensity of brown color of heated sample [2] |
|---|---|---|---|---|
| | No heat treatment | Heated at 120° C. for— | | |
| | | 10 minutes | 8 minutes | |
| | *Mg./g.* | *Mg./g.* | *Mg./g.* | |
| 1 | 334.7 | 322.8 | | Slight. |
| 2 | 330.7 | 321.3 | | Do. |
| 3 | 335.2 | 328.7 | | Do. |
| 4 | 333.8 | | 328.7 | Very slight. |
| 5 | 333.6 | | 326.9 | Do. |

[1] Milligrams of dye per gram of protein; amido black 10B.
[2] Visually observed.

2, 4, 6, 8, and 10 minutes. After the sample was cooled to room temperature, further dilution was made to reduce the concentration of total solids to that of normal skim milk. The brown color was measured by a colorimetric method with Colormaster (a differential colorimeter of Mecco Manufacturers Engineering and Equipment Corp.). Figure 8 shows the straight-line relation between reflectance and optical density at 615 mμ.

## Condensing

Condensed skim milk (3:1) was prepared from freshly separated skim milk with a small milk-condensing apparatus (10-lb. capacity) at 55° C. under a vacuum of 28 inches Hg. The condensed milk was diluted with distilled water to the original skim

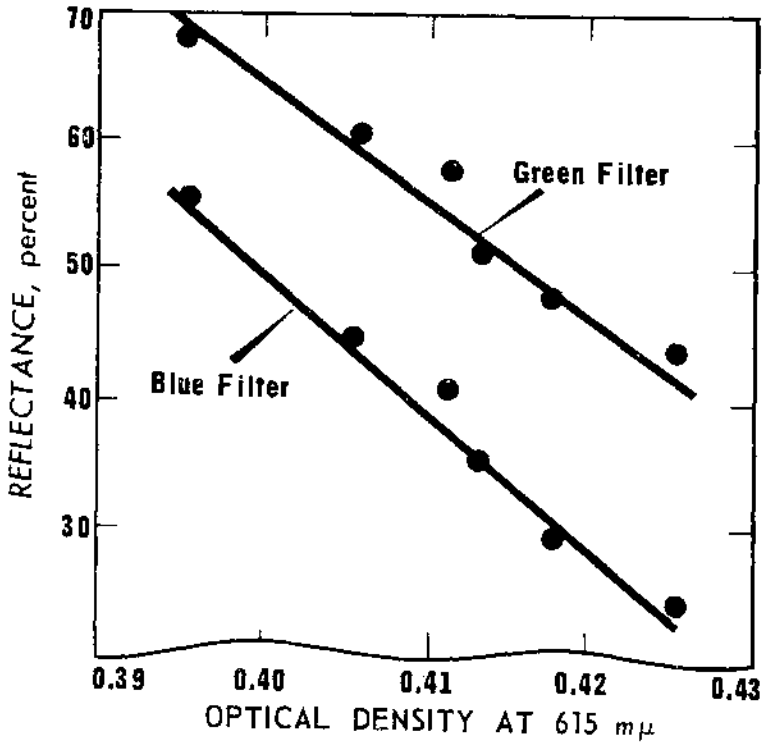


FIGURE 8.—Relation between brown color (inversely proportional to reflectance) and optical density.

milk concentration. Both skim milk and diluted condensed milk were analyzed by the amido black and Kjeldahl methods. The average *DBC* calculated from two independent experiments was 334.2 mg. dye per gram of protein for skim milk and 335.9 mg. dye per gram of protein for diluted condensed milk. The process of condensing had no effect on *DBC*.

## Homogenization

Identical milk samples were warmed to 60° C. and homogenized at pressures ranging from 1,500 to 4,000 p.s.i. The *DBC* of unhomogenized and homogenized samples is shown in table 7. Even at the very high homogenization pressure of 4,000 p.s.i. no change was found in *DBC*.

## Fat Content

It was thought that a dye might be preferentially adsorbed on the fat globules and form a complex with a fat globule membrane material. If this were true, fat content would affect the *DBC*. Samples with a range of fat content were prepared by varying the

TABLE 7.—*Effect of homogenization on dye-binding capacity* [1]

| Homogenization pressure (*p.s.i.*) | *DBC* [2] |
|---|---|
| | *Mg. dye/g. protein* |
| 0 | 342.7 a |
| 1,500 | 340.5 a |
| 2,000 | 342.2 a |
| 2,500 | 341.0 a |
| 3,000 | 341.8 a |
| 3,500 | 340.7 a |
| 4,000 | 342.5 a |

[1] Average of two milk samples, warmed to 60° C.
[2] Means followed by the same letter are not significantly different at the 5-percent level, according to Duncan's multiple range test.

proportions of skim and cream taken from a single lot of milk (table 8). The data show that the *DBC* of milk protein increases slightly as fat content increases from 0 to 4.10 percent; there is no further increase in *DBC* as fat content goes up to 10.50 percent. At a higher concentration, some fat remains in the supernatant after centrifugation and affects the optical density reading. Normally, fat is entrapped in the protein-dye complex, leaving the supernatant clear and free of fat.

## Surface Tension

*DBC* was not affected when the surface tension of skim milk was lowered, by the addition of Tween 20, from 51.8 to 35.8 dynes/cm. or when that of whole milk was lowered from 46 to 35 dynes/cm.

TABLE 8.—*Effect of fat content on dye-binding capacity*

| Sample [1] | Fat | Protein [2] | *DBC* [3] |
|---|---|---|---|
| | *Percent* | *Percent* | *Mg. dye/g. protein* |
| 1 [4] | 4.8 | 3.47 | 303.3 |
| 2 [5] | 0 | 3.73 | 289.9 |
| 3 | 1.15 | 3.67 | 291.4 |
| 4 | 2.20 | 3.63 | 290.8 |
| 5 | 3.10 | 3.62 | 294.6 |
| 6 | 4.10 | 3.46 | 300.9 |
| 7 | 10.50 | 3.34 | 297.8 |

[1] Skim milk and cream, except as indicated.
[2] Kjeldahl.
[3] Amido black 10B method.
[4] Original milk.
[5] Skim milk.

### Proteolysis

Freshly separated skim milk was pasteurized for 15 seconds at 75° C., adjusted to pH 7.5 with diluted NaOH, and warmed to 40°. To 200 ml. of the skim milk was added 200 mg. of pancreatin powder; the mixture was then stirred with a magnetic stirrer until the pancreatin was completely dissolved. The samples were incubated in a constant-temperature water bath at 40°, and samples were removed for analysis every 10 minutes beginning just after the pancreatin was completely dissolved in the milk. To prevent further progress of proteolytic reaction, dye solution was added to each sample as soon as it was removed from the water bath. Both total nitrogen and nonprotein nitrogen were determined by the Kjeldahl method. For nonprotein nitrogen determination, 20 percent trichloroacetic acid was immediately added to the sample. Progressive proteolysis increases *DBC* because of a decrease in true protein content, as shown by the Kjeldahl protein analysis. From the results of experiments to be described subsequently, this increased *DBC* is thought to be due to the decrease in the protein-dye ratio in the reaction mixture. With this lowering of protein content by proteolysis an increase in the optical density of the supernatant is to be expected. This relation is shown in figure 9.

From the data on Kjeldahl protein in this experiment, the extent of proteolysis was much greater even after only 10 minutes of incubation with pancreatin than would normally occur in milk aged for as long as 2 to 3 weeks if kept at about 0° C. It was repeatedly observed in other experiments in this study that a very slight proteolysis may take place when milk is aged without significantly affecting the accuracy of the amido black 10B dye-binding test.

## Stability of the Protein-Dye Bond

The stability of the protein-dye bond as affected by pH and urea solutions was investigated in the following manner.

The protein-dye complex was washed with pH 2.35 citrate-phosphate buffer to remove unbound dye. Different portions of the complex were then suspended in buffer solutions (citrate-phosphate at pH's of 2.0, 3.0, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0) and then dialyzed against these same buffer solutions for 48 hours at room temperature. The effect of pH on the stability of the protein-dye complex is indicated in the following tabulation:

| *pH of buffer* | *Dye dialyzed out* |
|---|---|
| 2.0 | No |
| 3.0 | No |
| 4.0 | No |
| 5.0 | Some |
| 5.5 | Yes, slowly |
| 6.0 | Yes |
| 7.0 | Yes |

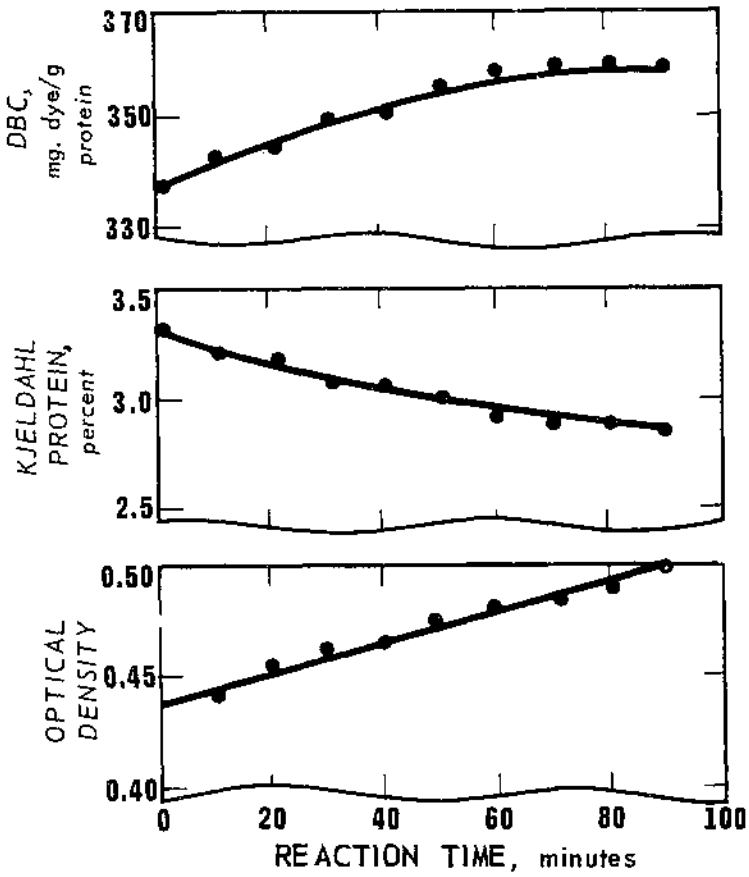


FIGURE 9.—Change in optical density, percentage of protein, and *DBC* with progressive proteolysis.

The reaction between the protein and the dye at the pH of the reaction was irreversible, but when the pH was increased to 5.5 the dye was liberated from the protein-dye complex and dialyzed out. When urea was added to the buffer, this liberation took place at pH 2.35. The effect of urea on the stability of the protein-dye complex is indicated in the following tabulation:

| *Urea concentration in pH 2.35 buffer* | *Dye dialyzed out* |
|---|---|
| 1 *M.* | No |
| 2 *M.* | Yes |
| 4 *M.* | Yes |

# RELATION BETWEEN PROTEIN MEASUREMENT BY DYE-BINDING AND KJELDAHL METHODS

The accuracy of the dye-binding test with amido black 10B was studied on milk from a large number of individual producers from three different areas in California. The areas differ in climate, feeding practices, and the protein content of the milk. A limited study was also made on mastitic milk.

Samples of producer's milk, identical to those taken for the fat test were refrigerated and shipped to the laboratory. At time of analysis the samples were 2 to 4 days old. Separate experiments established that the dye-binding test was unaffected by an increase (up to 0.05 percent) in titratable acidity that might have taken place during shipping. Titratable acidity was determined in all samples suspected of having developed acidity.

## Mastitic Milk

Milk from mastitis-affected udders of four cows was studied. The mastitic infection, experimentally induced, was relatively mild as determined by the California mastitis test. The Kjeldahl nitrogen on all samples was run in triplicate, and, when necessary, the determinations were repeated in duplicate. The dye-binding tests were made in duplicate.

The correlation coefficient between Kjeldahl protein and dye binding was only 0.681 for mastitic milk. This relatively low correlation is to be expected, since the ratio of various protein fractions, particularly those of whey, is quite different in mastitic milk from that in normal milk. Different types of proteins present in milk have different specific binding capacities for the dye.

## Mixed-Herd Milk [6]

In the sample of 440 observations of mixed-herd milk from three different areas in California, the relation between optical density as determined by dye-binding and protein content as determined by Kjeldahl test was as follows:

$$Y = 5.155 - 4.289X$$
$$R^2 = 0.916$$
$$S.E. = \pm 0.093$$

Where $Y$ is the protein content by Kjeldahl and $X$ is the optical density. This equation "explained" 91.6 percent of the variance

[6] This section prepared by A. C. Manchester, agricultural economist, Marketing Economics Division, Economic Research Service, U.S. Department of Agriculture, on the basis of a statistical analysis by Elsie D. Anderson, formerly analytical statistician with the same Division.

in the protein content by Kjeldahl. The standard error of estimate was ± 0.093 percentage points.

Both of the measures are averages of several observations—two measurements of optical density and three of Kjeldahl nitrogen. The measurements are not completely reproducible, of course. The standard deviation about the mean of each sample was:

| | *Standard deviation* | *Mean* |
|---|---|---|
| Optical density | 0.0031 | 0.3989 |
| Kjeldahl nitrogen | .0042 | .5330 |

In other words, two-thirds of the measurements would be within one standard deviation of the mean for each sample.

The observations came from three different areas in California—246 samples from the Sacramento area, 50 from Fernbridge, and 144 from Petaluma. Analysis of the data from each of these areas yielded somewhat different results (table 9).

The equations for herd milk in the Fernbridge and the Petaluma areas are homogeneous. That is to say, statistically there is no significant difference between them. That for the Sacramento area is significantly different in a statistical sense from those for the other two areas. The observations for the three areas are plotted in figure 10.

In an effort to determine why different equations were obtained in the three areas, a large number of regression analyses were performed, including the use of various curvilinear forms. On the

TABLE 9.—*Statistical data for mixed-herd milk*

| Source of samples | Samples | Coefficient of determination [1] | Regression statistics | | |
|---|---|---|---|---|---|
| | | | Constant term | Regression coefficient (with its standard error) [2] | Standard error of estimate [3] |
| | *Number* | *Percent* | | | |
| Sacramento | 246 | 90.6 | 5.6629 | −5.3445 (±0.1104) | ±0.077 |
| Fernbridge | 50 | 91.7 | 5.3128 | −4.6845 (± .2040) | ± .082 |
| Petaluma | 144 | 93.0 | 5.1713 | −4.4893 (± .1031) | ± .064 |

[1] Coefficient of determination is the percentage of variation in milk protein percentage associated with variation in optical density.

[2] Average increase or decrease in percentage of milk protein that occurs with each increase of one in optical density. The figures in parentheses indicate the limits of change to be expected in the regression coefficients two times out of three from repeated sets of samples.

[3] Indication of the agreement expected between a percentage of protein estimated from the equation and the true protein percentage. Estimated values can be distributed about the true values as follows: within one standard error for two out of three estimates, within two times the standard error for 19 out of 20 estimates; within three times the standard error for 99 out of 100 estimates.

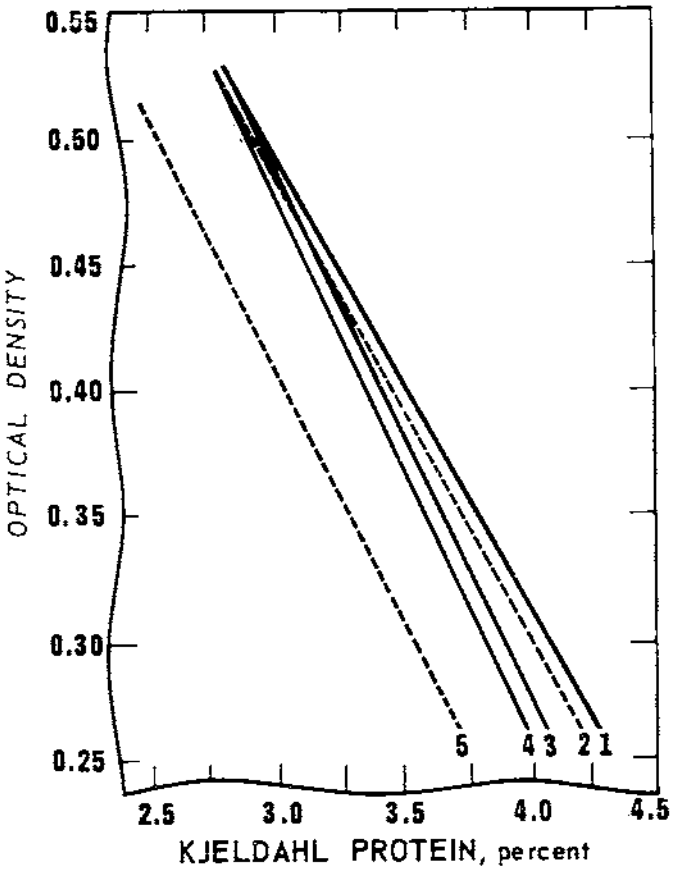


FIGURE 10.—Graph of regression lines for different categories of milk: from Sacramento area (1); from individual cows (2); from Fernbridge area (3); from Petaluma area (4); mastitic milk (5).

basis of the evidence that there is a difference in *DBC* at different levels of protein, an analysis of the relation between optical density and Kjeldahl protein was run at three different levels of protein and no significant differences between these protein levels were found. None of the curvilinear forms gave better results than the linear.

An analysis which divided all 440 observations into three groups on the basis of the level of optical density yielded coefficients of determination ($r^2$) of 0.646, 0.514, and 0.773 for the three groups. The standard errors of estimates for these groups were $\pm$ 0.064, $\pm$ 0.109, and $\pm$ 0.099. Thus, use of three different regressions for different levels of optical density would yield "better" results for only about one-third of the range at the lowest level of protein.

More damaging than this fact, however, was the observation that the regressions obtained by this method were not logically related. When plotted, the three regressions would form a saw-toothed line for which no logical explanation seems possible. Total range of protein content was rather narrow, 94 percent of the samples being between 3 and 4 percent protein, which made fitting the data to a straight line more difficult.

One of the additional regressions fitted did seem to improve the measurements slightly. When the butterfat percentage of the sample was included as a separate independent variable, it increased the predictive power of the equation slightly:

$$\begin{aligned} Y &= 4.126 - 3.214X_1 + 0.144X_2 \\ R^2 &= 0.934 \\ S.E. &= \pm 0.082 \end{aligned}$$

Where $Y$ is protein by Kjeldahl, $X_1$ is the optical density, and $X_2$ is the butterfat percentage. The slightly curvilinear relation between protein content and butterfat content known from much previous work appeared to improve the measurement slightly. This is consistent with the experimental observation that butterfat content did have a small effect on *DBC* (table 8).

There are a number of possible sources of variation, which may account for the somewhat unsatisfactory fit. These include season of the year—the Sacramento area observations were from July 26 to September 2; Fernbridge, October 2 to October 16; and Petaluma, October 16 to December 6—and breed. The Fernbridge area had predominantly Jersey and Guernsey; the Petaluma area had about 50 percent Jersey and Guernsey and 50 percent Holstein; and the Sacramento area had almost exclusively Holstein. It is possible that these and other factors may cause variations in the nonprotein nitrogen content or in the relative amounts of various proteins that differ in dye-binding capacity.

## STOICHIOMETRY OF DYE-BINDING METHODS

It has been commonly assumed that, within certain limits of protein and dye concentrations, the reaction between protein and dye is stoichiometric. Dolby (7) reported no *DBC* change with normal protein variation, but Ashworth and coworkers (3) found the contrary. The data of mass protein analysis suggested that the difference in protein content between milks from different areas may be a significant factor in the difference in *DBC* indicated by the regression equations for the different areas. Therefore, the following experiment, was designed to study the relation between *DBC* and protein-dye concentration in the reaction mixture.

Six samples with protein contents ranging from 1.011 to 6.003 percent were prepared from condensed skim milk (12.2 percent protein concentration) by dilution with distilled water. The *DBC* was determined at six levels of dye concentration. Figure 11

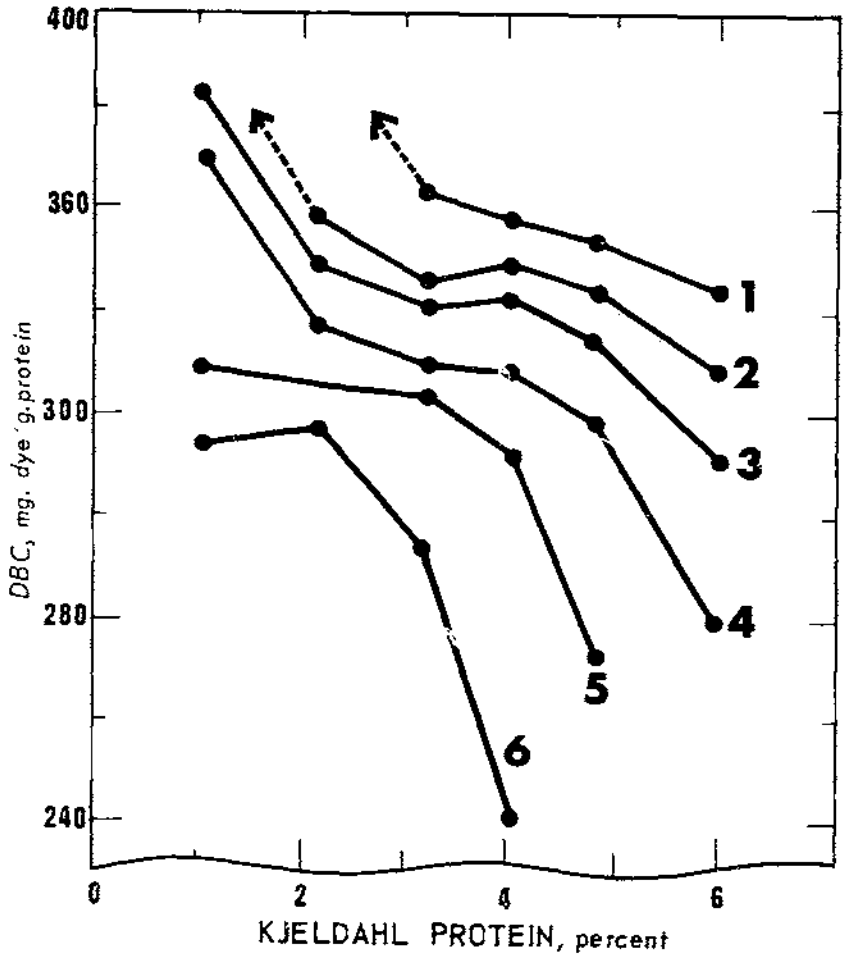


FIGURE 11.—Relation between Kjeldahl protein and *DBC* at various levels of dye concentration. The dye concentration (in milligrams per liter of buffer solution) is constant for each curve: 1—680; 2—581.5; 3—506.5; 4—440.0; 5—341.5; 6—249.0.

shows the relation between *DBC* and Kjeldahl protein percentage for this series. It is clearly shown that at each level of dye concentration, the *DBC* is decreased by an increase in the protein content. However, it should be noted that at the dye concentration level of 440 mg./liter (the concentration used in the amido black method), the extreme difference in the protein content of normal milk, from 2.8 to 4.5 percent, will give a difference of 10.5 mg. of *DBC*, which is equivalent to about 0.12 percent protein by the amido black 10B test.

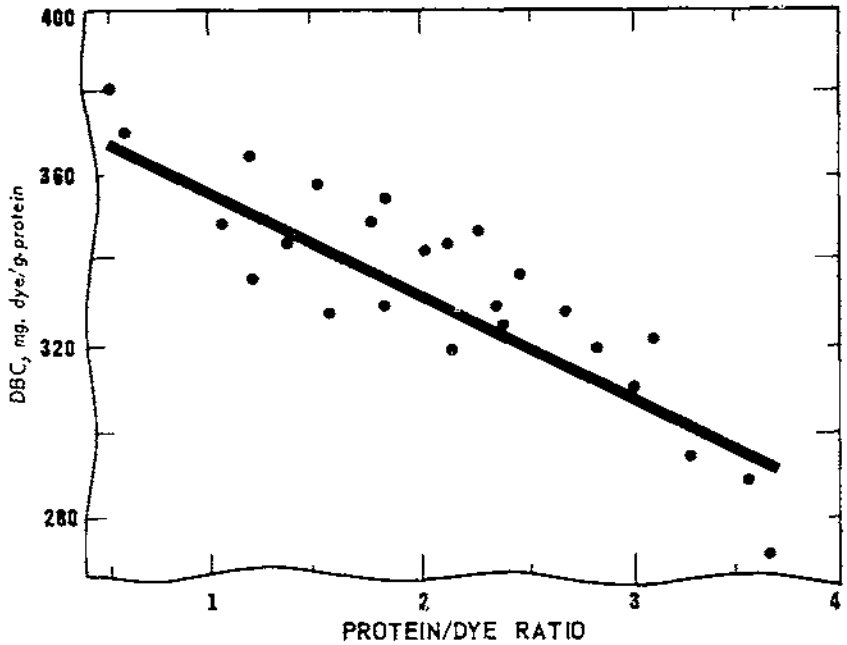


FIGURE 12.—Relation between protein-dye ratio of the reaction mixture and *DBC*; amido black.

The relation between *DBC* and Kjeldahl protein is not linear, but plotting the *DBC* against the protein-dye ratio of the reaction mixture gives a fairly good linear relation, as seen in figure 12.

A study similar to the preceding one was made with orange G dye. *DBC* was determined on seven samples of protein concentration ranging from 2.15 to 5.34 percent and three levels of dye concentration. The relation obtained was similar to that observed with amido black (fig. 13 and 14).

The experiments on the relation between *DBC* and dye concentration were repeated with a single sample of milk of 4.34 percent protein and different dye concentrations. Two separate tests were made in duplicate with each dye. The results are shown in figures 15, 16, 17, and 18. Distinct inverse linear relations were observed between *DBC* and protein-dye ratio for both amido black and orange G though the amount of dye present in the reaction mixture was always in excess.

Multiple equilibria in dye-binding were described by Rosenberg and Klotz (*25*). Working with one protein (serum albumin) rather than a mixture of proteins, they found that when $1/r$ is plotted against $1/(D)$, where $r$ is dye-binding capacity and $(D)$ is a free dye (dye in supernatant), a straight line can be obtained if no interactions other than the protein-dye reaction are involved

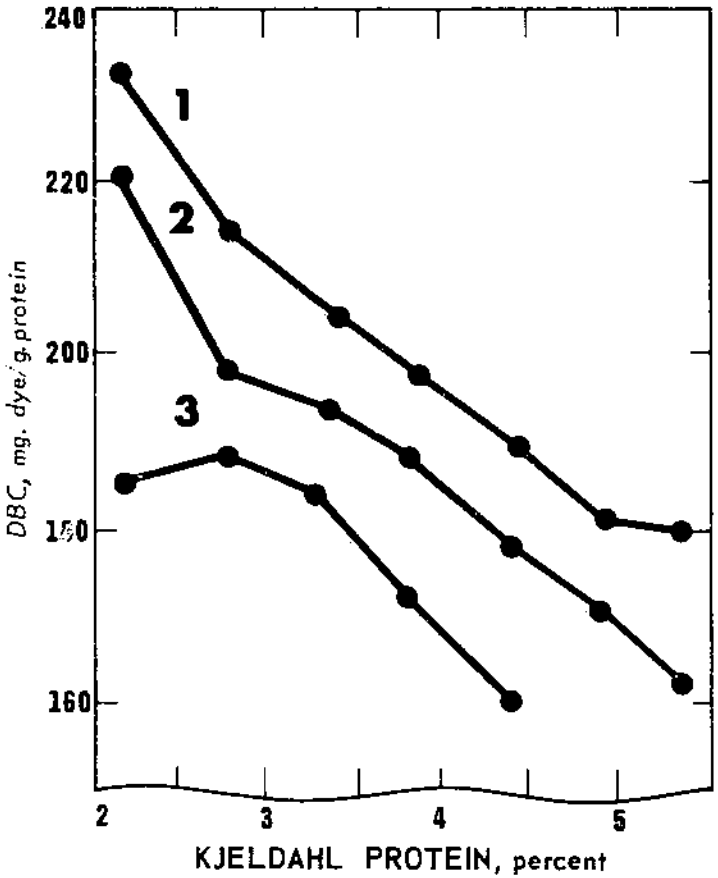


FIGURE 13.—Relation between Kjeldahl protein and various levels of dye concentration (orange G). The dye concentration (in milligrams per liter of buffer solution) is constant for each curve: 1—1,200; 2—1,000; 3—800.

in the dye-binding reaction. The data of the preceding experiments in the present study were accordingly plotted in the same way. Neither dye showed a straightline relationship. One might then conclude that in a complex system such as milk there might be other interactions of dye besides the main reaction of dye with protein. But that was not the case: no fractions of milk other than protein were found to interact with amido black 10B. The interpretation of Rosenberg and Klotz was based on working with a single protein (serum albumin). Milk protein is a mixture of different proteins that vary in dye-binding capacity, so one cannot expect a straight-line relation between the reciprocals of *DBC* and a free dye.

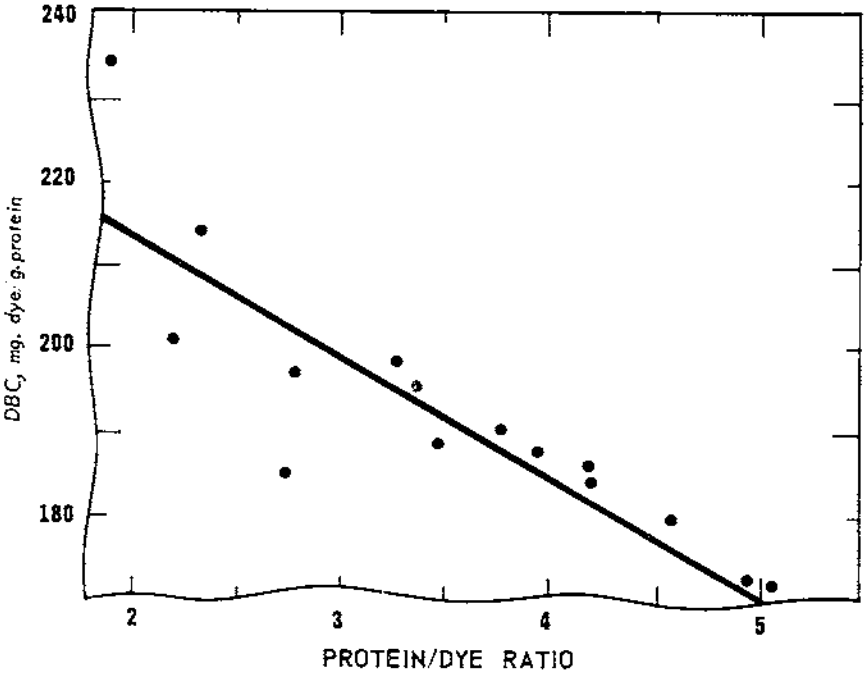


FIGURE 14.—Relation between protein-dye ratio of the reaction mixture and *DBC*; orange G.

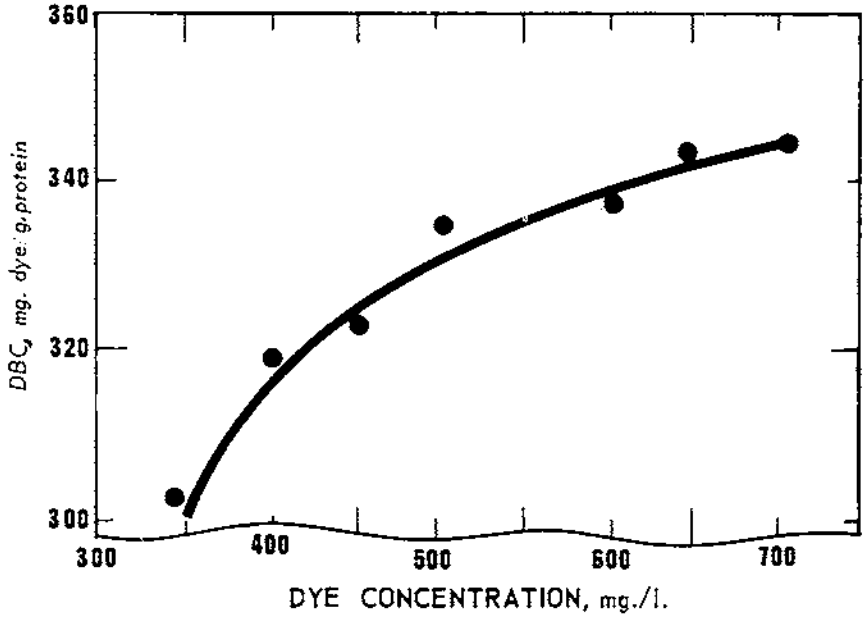


FIGURE 15.—Relation between dye concentration and *DBC*; amido black 10B.

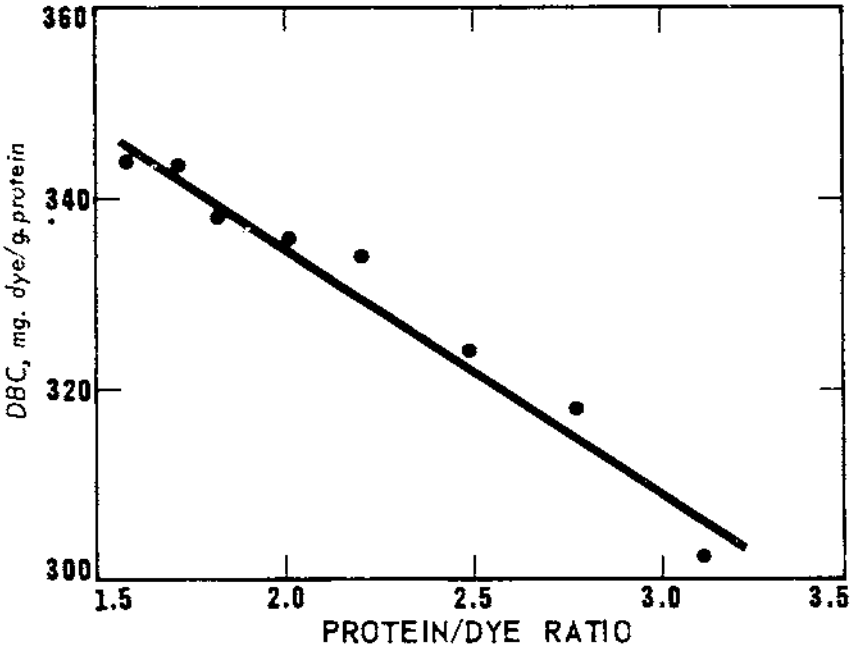


FIGURE 16.—Relation between protein/dye ratio of the reaction mixture and *DBC*; amido black 10B.

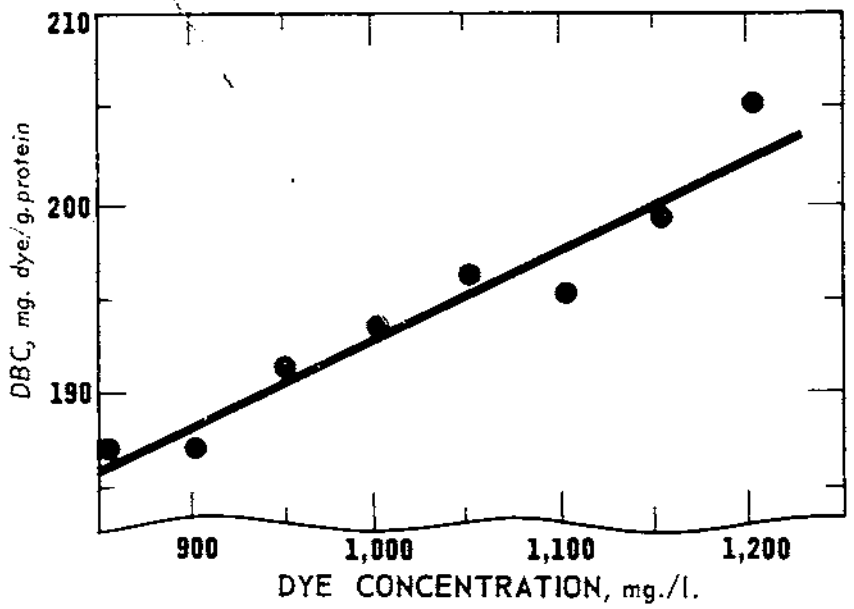


FIGURE 17.—Relation between dye concentration and *DBC*; orange G.

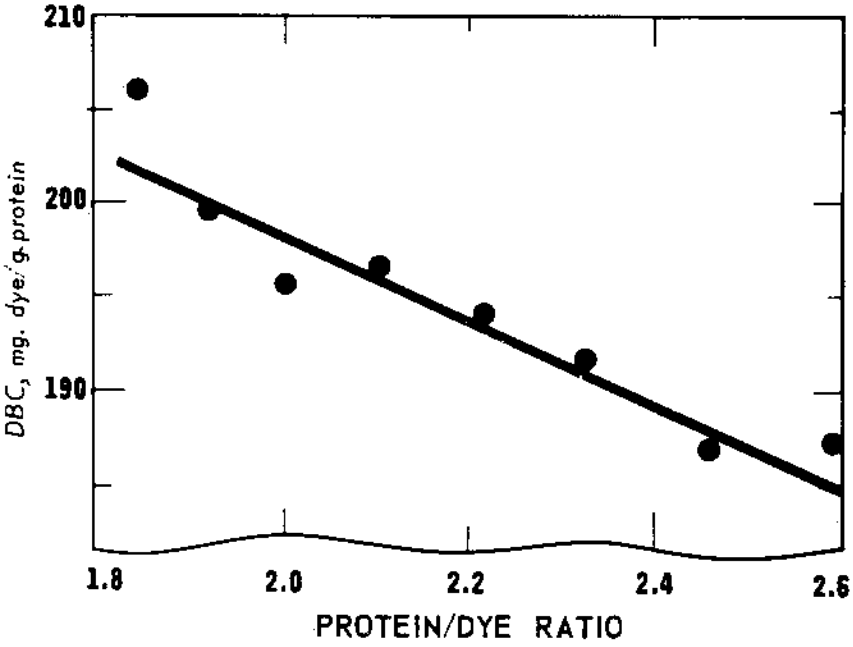


FIGURE 18.—Relation between protein/dye ratio of the reaction mixture and *DBC*; orange G.

## DYE-BINDING CAPACITY OF VARIOUS MILK PROTEIN FRACTIONS

Various milk protein fractions were prepared as described in Experimental Procedures. All preparations except acid casein, Ca-sensitive $\alpha$-casein, Ca-insensitive $\alpha$-casein, and $\kappa$-casein were freeze-dried for use in the experiments. Starch-gel electrophoresis was used as a criterion of purity (see fig. 2 and 3).

About 1 g. of each protein fraction was dissolved in 25 ml. of phosphate buffer of pH 7.0 Total nitrogen of the protein solution was determined by the semimicro-Kjeldahl method. The percentage of protein was calculated by multiplying the total nitrogen value by the conversion factors given in Jenness and Patton (*15, p. 125*). In the absence of the known factor, 6.38 was used. The aliquots of each protein solution were weighed (about 0.5 g.), and *DBC* was determined at eight levels of amido black 10B concentration (350, 400, 450, 500, 550, 600, 650, and 700 mg./l. of buffer solution). The resulting data are presented in figures 19 and 20.

The present data confirm findings of Ashworth and Chaudry (*2*) that the *DBC* of whey protein is considerably greater than that of casein. $\alpha$-lactalbumin has the highest *DBC* of all fractions, followed by lactalbumin and $\beta$-lactoglobulin. The *DBC* of globulin

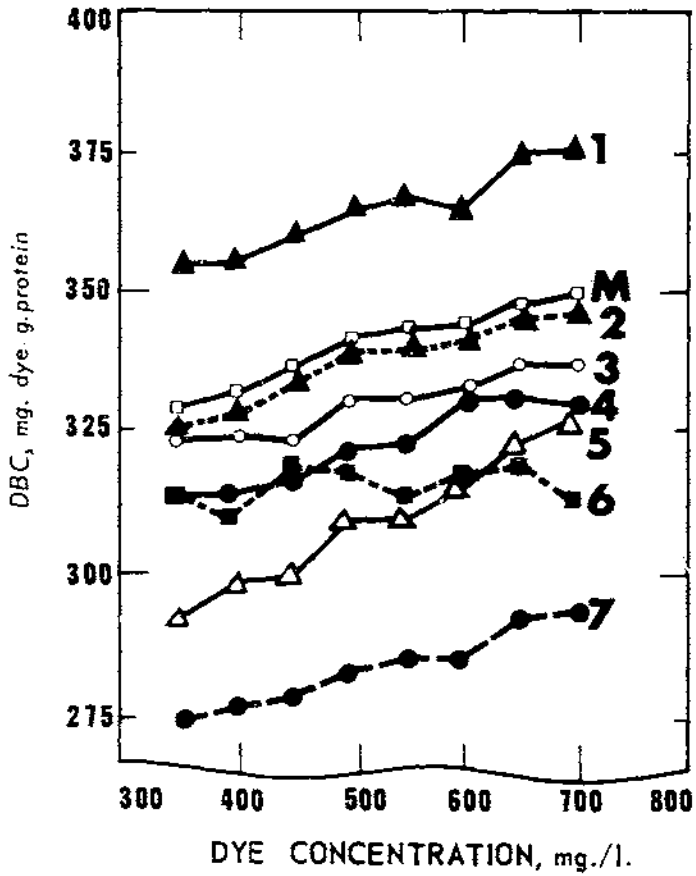


FIGURE 10.—*DBC* of casein fractions (amido black 10B):

| | | |
|---|---|---|
| M | milk protein as a whole | T.N.×6.38=3.23 percent; |
| 1 | Ca-sensitive α-casein | T.N.×6.38=2.83 percent; |
| 2 | α-casein | T.N.×6.44=3.13 percent; |
| 3 | Ca-insensitive α-casein | T.N.×6.38=3.00 percent; |
| 4 | acid casein | T.N.×6.40=2.78 percent; |
| 5 | γ-casein | T.N.×6.49=1.63 percent; |
| 6 | κ-casein | T.N.×6.38=2.67 percent; |
| 7 | β-casein | T.N.×6.53=3.19 percent. |

(pseudoglobulin) is much lower than that of other whey fractions. Ca-sensitive α-casein is the fraction of casein with the highest *DBC*, followed in descending order by α-casein, Ca-insensitive α-casein, κ-casein, γ-casein, and β-casein.

It is of interest to note that the *DBC* of κ-casein and of globulin was essentially the same at all levels of dye concentration, whereas the *DBC* of all other protein fractions increased with dye concentration.

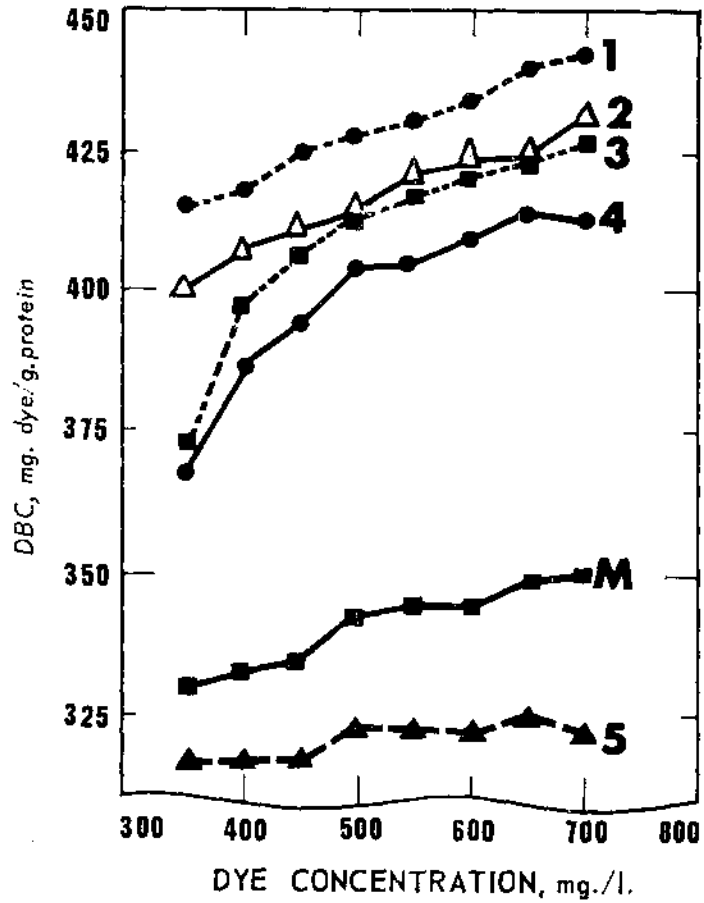


FIGURE 20.—*DBC* of whey protein fractions (amido black 10B):

| | | |
|---|---|---|
| M | milk protein as a whole | T.N.×6.38=3.23 percent; |
| 1 | α-lactalbumin | T.N.×6.30=2.10 percent; |
| 2 | lactalbumin | T.N.×6.38=3.69 percent; |
| 3 | β-lactoglobulin | T.N.×6.41=3.66 percent; |
| 4 | whey protein | T.N.×6.38=3.61 percent; |
| 5 | globulin | T.N.×6.38=2.42 percent. |

## DISCUSSION

The present study investigated the quantitative aspects of dye-binding by milk proteins to determine the limitations of dye-binding methods for rapid, accurate estimation of the proteins in milk. It deals primarily with amido black 10B and, to the lesser extent, orange G, the two dyes used most commonly in dye-binding tests.

Below their isoelectric points, milk proteins bind negatively charged dyes. Since pH governs the number of positively charged

protein groups, the amount of dye bound depends on the pH of the reaction mixture of dye and protein. The relation between pH and the amount of amido black 10B was found to be linear within the range of pH 1.75 to 4.0. A pH of 2.35 was chosen for the standard buffer-dye solution. This is near the optimum pH for a maximum positive charge on the protein molecules without a breakdown of protein by acid hydrolysis.

Different workers have reported different wavelengths at which the optical density of the supernatant (unbound dye) was determined. In our work, spectral analysis of amido black showed the maximum absorption peak between 610 and 620 mμ, and a much smaller peak at 320 mμ. For orange G the maximum absorption was at 480 mμ. Therefore the wavelengths chosen for determination of the optical density of the supernatant from the reaction mixture were 615 mμ for amido black and 480 mμ for orange G. It was confirmed that in the concentrations used in the tests both dyes obey the Beer-Lambert law.

Sensitivity and reproducibility are the two basic criteria of any analytical method. The data of figure 5 show that the amido black method is more sensitive than the orange G method: the difference of 0.01 in optical density corresponds to 0.05 percent protein for amido black and 0.16 percent protein for orange G. These data are in agreement with results of Shiga and coworkers (*28*) and Dolby (*7*). Reproducibility for the methods of both dyes is very good (see table 2). Ten separate analyses of the same sample of milk gave standard deviations of optical densities of 0.0017 and 0.0005 for amido black and orange G, respectively. These values correspond to 0.0095 and 0.008 percent protein. Standard deviation for the Kjeldahl method has been reported to be 0.008 percent protein (*26*).

Other controversial points in the literature have been the time and method of mixing dye and milk. The observation of Richardson (*24*) that the procedure of adding buffer-dye solution to milk is preferable was confirmed. When dye and milk were mixed in this manner, the optical density of the supernatant did not change even after it was stirred for 15 minutes with a mechanical stirrer (see table 3.) The volume proportions of milk to dye solution are 1:40. If, in contrast, milk is added to the dye solution (pH 2.35), a partial acid coagulation of casein takes place and at least 30 seconds of stirring is required to disperse the coagulated proteins.

Of three commonly used preservatives—$HgCl_2$, $K_2Cr_2O_7$, and HCHO—only $HgCl_2$ was found to have no significant effect on the dye-binding test. $K_2Cr_2O_7$ lowered optical density by partially oxidizing the dye and thus increasing the apparent protein content; HCHO reacted with $NH_3^+$ groups of protein in competition with the dye, resulting in an apparent lowering of the protein content.

The effect of common processing treatments on the *DBC* of milk protein was studied. Homogenizing up to 4,000 p.s.i., condensing, and heating for 15 minutes up to 90° C. did not affect the *DBC*

of milk protein. With concentrated milk, it is essential to dilute the sample to a concentration of protein equal to that of normal milk before a sample is taken for the test. It is of interest that although 15 minutes at 90° denatured whey proteins almost completely, it did not affect *DBC*. Denaturation of serum albumin destroys its dye-binding ability (*16*). Denaturation of many other proteins enhances this property (*20*), and *DBC* has been used as a sensitive criterion of denaturation (*12*). It is possible but not likely, that *DBC* is enhanced in some denatured whey proteins and destroyed in others in such a way that the net effect is nil. The probable reason is that whey proteins are always denatured under conditions of the test by acid environment (pH of reaction mixture is 2.35) and/or by breaking of hydrogen bonding by amido black 10B. Kronman and coworkers (*18*) have observed the aggregation of α-lactalbumin at a pH below 4.0 and concluded that this is consequence of a "denaturation-like" conformational change at the molecular surface.

It was shown that heating milk enough to induce browning decreases *DBC*. This would be expected because of the interaction of the amino groups with the aldehyde groups of sugar in the browning reaction. The relations between intensity of browning and *DBC* is linear (see fig. 8).

Extensive proteolysis of milk increases *DBC* (fig. 9). This is interpreted as not the effect of proteolysis per se but rather the effect of the decrease in the protein-dye ratio that results from the test procedure. With a decrease in true protein content by proteolysis the optical density of the supernatant is increased (fig. 9), as would be expected. The increase in optical density would be somewhat greater if it were not for an increase in *DBC* resulting from the increase in protein-dye ratio with a progressive proteolysis.

Mass protein analyses of producers' milks from three different areas of California by dye-binding (amido black 10B) and Kjeldahl methods confirmed the high accuracy of amido black methods. Standard errors of estimate for the regression lines for milk of each area were 0.077, 0.082, and 0.064 percent. The linear regression equations relating Kjeldahl percentage of milk protein to optical density were statistically homogeneous for two areas, but that of the third was statistically significantly different from the other two. Figures 9, 11, 13, 15, and 17 show that the reaction of protein with dye is not entirely stoichiometric and that the *DBC* changes with the protein-dye ratio in the reaction mixture; a straight-line inverse relation is obtained (fig. 12, 14, 16, 18) when *DBC* is plotted against the protein-dye ratio. This limitation of the dye-binding methods is not serious, because the most extreme variation in protein content in normal milk is from 2.8 to 4.5 percent, a difference of 1.7 percent, which in the amido black test would cause an error of approximately ± 0.12 percent protein. The probable explanation for the variation in *DBC* with protein-

dye ratio is that it is possible for more than one dye molecule to be bound by a protein molecule, and hence the interaction between protein and dye is a multiple-equilibria reaction (*25*) dependent upon the concentration of free dye. Furthermore, there may be interaction among bound dye molecules.

The data on the *DBC* of various protein fractions (figs. 19, 20) showed that all proteins of milk differ in *DBC*. The *DBC* of whey proteins except pseudoglobulin is considerably higher than the *DBC* of casein fractions. α-Lactalbumin has the highest *DBC*. Similar to milk protein as a whole, the *DBC* of each protein fraction, except κ-casein and globulin fraction (pseudoglobulin), varies inversely with protein-dye ratio in the reaction mixture.

## SUMMARY

Factors affecting determination of milk proteins by dye-binding procedures were studied. Of the two dyes most commonly used for protein determination, amido black 10B and orange G, amido black 10B was judged to be more suitable because it gives a greater change in optical density per unit of protein. The study is therefore mainly concerned with this dye.

Of three commonly used preservatives—$HgCl_2$, $K_2Cr_2O_7$, and HCHO—only $HgCl_2$ was found to have no significant effect on the dye-binding test. Homogenizing, condensing, and heating to 90° C. for 15 minutes did not affect the dye-binding capacity (*DBC*) of milk protein. Heating sufficient to cause browning did reduce *DBC*. Extensive proteolysis increased *DBC*. The slight hydrolysis that may take place in storage had no effect on *DBC*.

A slight increase in *DBC* was observed with increasing butterfat content through the 0- to 4.1-percent range, with no further effect at higher levels of butterfat. The *DBC* decreased linearly as the protein-dye ratio in the solution increased. The resulting error would, however, be only approximately ± 0.12 percent for samples within the normal variation of protein content of milk (2.8 to 4.5 percent).

Data on the *DBC* of various protein fractions showed considerable variations. These differences in *DBC* exclude the possibility of using the dye-binding protein test on atypical milk such as colostrum, mastitic milk, and milk of very late lactation. Tests with mastitic milk did show poor correlation between protein by Kjeldahl and protein by dye binding.

Mass protein analysis by the amido black method of producers milk from three different areas of California showed standard errors of estimate of 0.077, 0.082, and 0.064 percent protein compared with Kjeldahl protein. Two areas gave regression equations that were statistically homogeneous with one another but that were significantly different from the third area. Reasons for the differences are not clear; however, milk from the third area is mostly from breeds different from those in the first two areas.

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# APPENDIX

TABLE 10.—*Accuracy of automatic pipets* [1]

| Trial | 20 ml. pipet [2] | | | 25 ml. pipet [2] | | |
|---|---|---|---|---|---|---|
| | Weight of water | $d$ | $d^2$ | Weight of water | $d$ | $d^2$ |
| | *Grams* | | | *Grams* | | |
| 1 | 19.8973 | +78 | 6,084 | 24.9188 | +62 | 3,844 |
| 2 | 19.8760 | −135 | 18,225 | 24.9147 | +21 | 441 |
| 3 | 19.8970 | +75 | 5,625 | 24.9167 | +61 | 3,721 |
| 4 | 19.8979 | +84 | 7,056 | 24.9120 | −6 | 36 |
| 5 | 19.8795 | −100 | 10,000 | 24.9095 | −35 | 1,225 |
| 6 | 19.8750 | −145 | 11,025 | 24.9034 | −92 | 8,464 |
| 7 | 19.9000 | +105 | 11,025 | 24.9116 | −10 | 100 |
| 8 | 19.8964 | +69 | 4,761 | 24.9117 | −9 | 81 |
| 9 | 19.8786 | −106 | 11,236 | 24.9107 | −19 | 361 |
| 10 | 19.8969 | +74 | 5,676 | 24.9151 | +25 | 625 |
| Total | 198.8946 | −1 | 100,513 | 249.1262 | −2 | 18,898 |
| Average | 19.8895 | | | 24.9126 | | |
| | $S.D.=0.0104$ | | | $S.D.=0.0046$ | | |

[1] The specified volume was measured ten times with each pipet and the delivered volume was weighed each time.

[2] $d=(X_i-\bar{X})\times 10^4$

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# END