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Quantitative Ionophoretic Determination of Some Whey Proteins in Skim Milk

The quantitative determination of whey proteins is a matter of considerable interest in dairy technology. Studies with α-lactalbumin and β-lactoglobulin, singly and in mixtures, have shown that recovery is quantitative if a precoat of β-lactoglobulin is applied ionophoretically. This technique, applied to whey, yields quantitative values for the chief protein components. Streaking of milk samples 1 inch from the apex toward the cathode permits the easy resolution of β-lactoglobulin in milk, and its subsequent quantitative determination.

WHEY PROTEINS, particularly β-lactoglobulin, are a rich source of the essential amino acids and contribute to such diverse properties as flavor, color, body, and keeping quality of milk and milk products. Differentiation between these proteins in their native and denatured state furnishes a basis for the determination of the degree of high heat treatment which milk has undergone, information which is important in baking technology.

In applying paper electrophoretic methods to the quantitative study of the proteins of milk and whey, questions of technique arose in connection with the specific properties belonging to these proteins. Conditions defining optimum resolution and the translation of the patterns in terms of quantitative relative and absolute concentration values posed a series of problems.

Limitations were met similar to those encountered with other protein systems. However, with several innovations in technique, some measure of success was achieved and the analysis of whey and skim milk yielded satisfactory results. These innovations and the results obtained with their application, as well as the magnitude of losses due to adsorption and the influence of variations in staining techniques, are reported and discussed.

Apparatus and Technique

An instrument was constructed which was patterned after a model used by Durrum (*7*) and differed from it only in dimensional details. Paper strips, 10 × ¾ inch, cut from Whatman's 3-mm. filter paper were hung over a glass rod on a rack. The strips were wet with a quantity of buffer solution and allowed to equilibrate overnight. Electrical contact between the buffer and the strips was effected by means of 11 × 2 inch wicks. The buffer solution (pH 8.6, ionic strength 0.05) contained 0.01 and 0.05 mole per liter of barbital and sodium barbital, respectively. Manually regulated power was furnished by an improvised unit delivering rectified alternating current between 130 and 500 volts over a current range from 0 to 100 ma. Manual regulation at approximately 15-minute intervals sufficed to keep the current constant to within 2%. For lower voltages, a 110-volt house line was available, acting through a variable resistance.

Preliminary experiments showed that the use of voltages intermediate between 110 and 500 volts, yielding, for nine strips, current at 18 ma., provided optimum resolution in approximately 2 hours. The use of lower voltages extended the time required to obtain the same resolution, without apparent advantage. The use of higher voltages and currents led to undesirable heating effects, and the loss of resolution. Extending the migration path by prolonging the time of migration resulted in blurring and loss of resolution.

Application of Sample. The samples were applied as streaks at a point 1 inch from the apex toward the cathode. A Breed pipet containing 0.01 ml. of sample was held in a near horizontal position and its contents were blown gently onto the wet strip as the pipet was moved to and fro across the strip. Application of the sample away from the apex by means of pipet was less demanding on operator skill than application at the apex.

Streaking of the sample 1 inch from the apex, at the point of dynamic equilibrium with respect to fluid flow, resulted in better resolution of β-lactoglobulin in whey and skim milk. Graph 7 of Figure 1 represents a pattern belonging to a sample of skim milk which had been applied at the apex. Figure 3,*A*, on the other hand, represents a sample of the same skim milk which had been applied at a point 1 inch toward the cathode. Only with off-center streaking is sufficient resolution achieved to permit the easy identification of β-lactoglobulin—the most advanced zone in Figure 3,*A*.

Although the reason for this effect is not clear, the existence of conductivity gradients along the paper due to evaporation may be a determining factor.

There is also an increase in the resolution between the various whey components, an effect which is significant with respect to the resolution of the zone belonging to the immunoglobulin fraction of whey. The patterns showing this distinction have not been included. A zone belonging to lactose appeared in strips which had been dried at a relatively high temperature—110° C. or higher. This zone is found 1 inch from the apex toward the cathode irrespective of the point of application of the sample. At the point, the flow due to endosmosis is balanced by the flow caused by evaporation. The band is brown in color and becomes visible as a result of a browning reaction which occurs

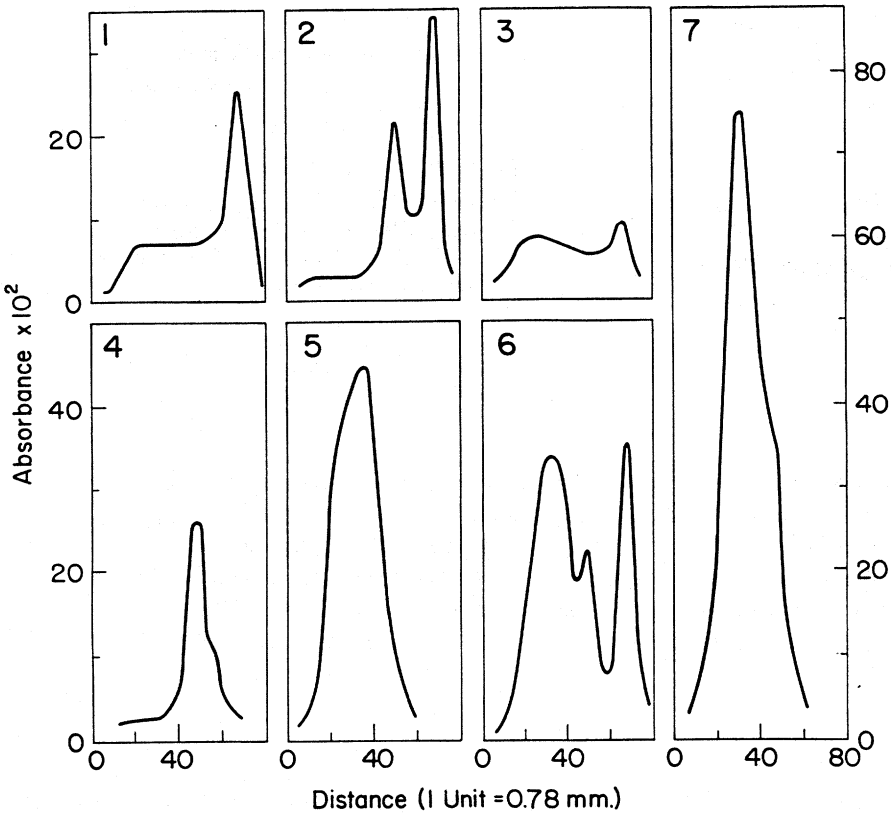

Figure 1. Effect of heating milk and solutions containing α-lactalbumin and β-lactoglobulin at 85° C. for 30 minutes. Loss in resolution in streaking at apex (see also Figure 3,*A*)

The patterns represent:
1. **0.25% α-lactalbumin, and 0.5% β-lactoglobulin, heated**
2. **0.25% α-lactalbumin, and 0.5% β-lactoglobulin, unheated**
3. **0.5% β-lactoglobulin, heated**
4. **0.5% β-lactalbumin, heated**
5. **Supernatant from centrifuged heated skim**
6. **Supernatant from raw skim**
7. **Raw skim—0.01 ml. applied at apex (see Figure 3,*A* for corresponding sample streaked 1 inch from apex)**

Supernatant centrifuged 4 hours at 4000 r.p.m. in refrigerated International Centrifuge. Approximately 50% of calcium caseinate–phosphate complex removed

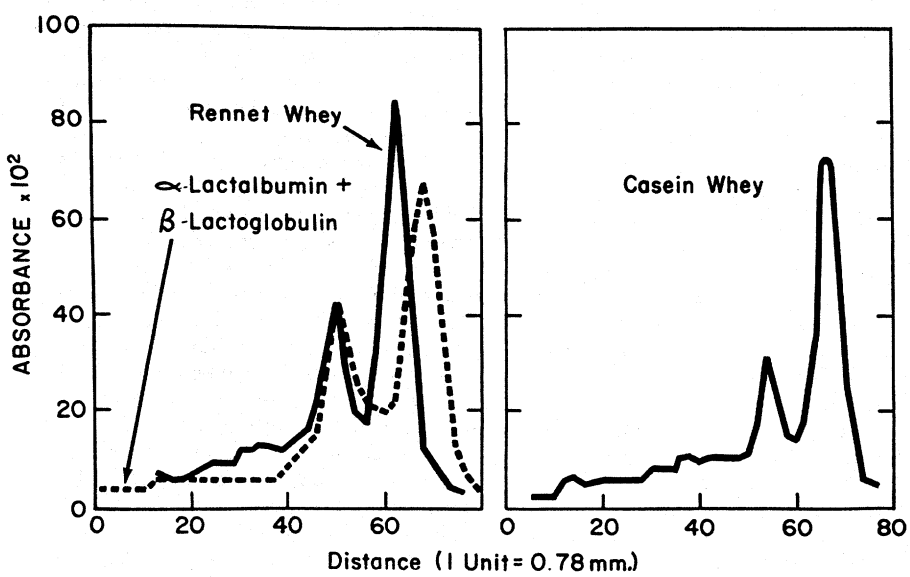

Figure 2. Ionophoretic patterns.
Left. **Rennet whey and mixture of α-lactalbumin and β-lactoglobulin**
Right. **Casein whey**

Rennet whey, 0.01 ml.; α-lactalbumin, 0.025 mg.; and β-lactoglobulin, 0.05 mg. Casein whey, neutralized to pH 6.8 and filtered, 0.01 ml. Ionophoresis in veronal buffer, pH 8.6; ionic strength 0.05, current 18 ma. per nine 10 × ¾ inch strips

in the alkaline pH range at high drying temperature.

Drying and Staining. Strips, tautly held in a horizontal position, were dried at 50° to 60° C. by means of an improvised unit based on a fan heater.

The staining solution, essentially that recommended by Block and Durrum (*1*), contained 0.1 gram per liter of bromophenol blue, 50 grams of zinc sulfate, and 50 ml. of glacial acetic acid. The first rinse solution contained 2% acetic acid and was applied successively over 5-, 5-, and 10-minute periods. A final rinse applied for 2 minutes contained 0.5% sodium acetate and 10% acetic acid. Porcelain 12 × 8 × 2 inch trays, thoroughly coated with paraffin, were employed. The strips rested flatwise on a series of ridges (waxed glass rods), and their ends were anchored down by means of stainless steel strips inclined at a small angle. Solutions (approximately 70 ml. per strip) were poured onto these anchor strips, which acted as baffles for the gentle distribution of the solution. Used solutions were removed by means of a siphon. The quantity of dye which is taken up depends not only on the molecular properties of the protein but also on the character of the protein film which forms in drying. Thick films take up dye at a slow rate, thin films at a more rapid rate. The net quantity absorbed is the resultant of two opposing factors, the rate of absorption and the rate of leaching. The extent of leaching depends upon the character of the film and the degree of mixing to which the strips and solutions have been exposed. Whether a long staining interval is employed, or a short one, a linear relation is not achieved except over a limited range of protein concentration. Long staining periods favor stoichiometric net dye absorption, but linearity is achieved only in the higher protein range; short staining periods are applicable in a comparatively lower range. Unless otherwise noted, a 10-minute staining period was employed. The blotting step was omitted as dye is transferred to the blotter.

Elution. The strips were dried at approximately 60° C. for 1 hour prior to elution, which was used to measure protein concentration. As an eluant, 3 ml. of 0.06*M* sodium barbital (barbital buffer at pH 8.6) was employed, instead of 0.1*N* sodium hydroxide and sodium carbonate in dilute methanol, as used by Block and Durrum (*1*) and Cremer and Tiselius (*2*), respectively. The color of bromophenol blue in the buffer eluant is stable over long periods of time, an important characteristic in lengthy serial determinations. Fifteen minutes with intermittent shaking were allowed for complete elution.

Bands ranging in width from 1.45 mm. to multiples thereof were ruled on the

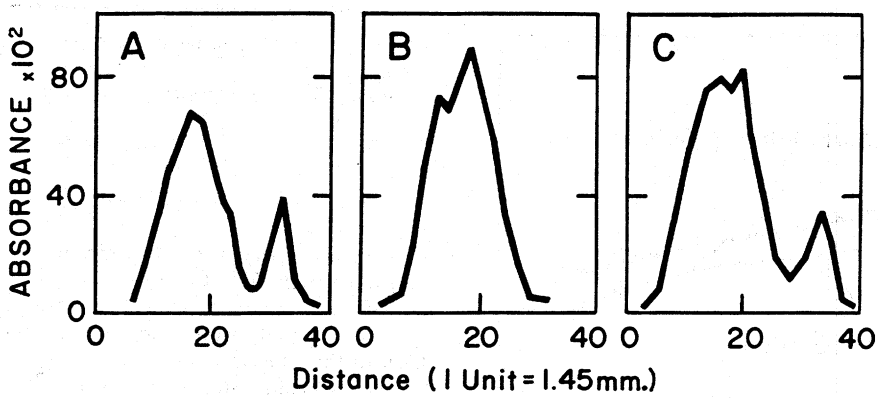

Figure 3. Ionophoretic patterns showing influence of heat treatment on β-lactoglobulin in milk and recovery of β-lactoglobulin added to heated milk

A, 0.01 ml. skim milk; *B*, 0.01 ml. skim milk heated at 85° C. for 30 minutes; *C*, same as *B*; 0.05 mg. of β-lactoglobulin added after cooling

dried dyed strips; the bands were then cut and transferred to culture tubes, and the dye was eluted. Rulings were made to conform to the outlines of zones, and in this manner the occurrence of spurious plateaus and maxima was minimized in those instances in which the outlines were curved. Absorbance measurements of the eluates were made at 590 mμ with a 1-cm. cell. As an alternative to the elution technique, densitometry was tried directly on the strips, but measured dye absorption values were significantly lower than actual values in the range of protein concentration exceeding 0.05 mg. per sq. cm. of filter paper. This failure to follow Beer's law has been observed by other workers (*3*), and although faulty instrumentation may be involved (failure to employ monochromatic band filters for example), the magnitude of the deviation was large enough to preclude such an interpretation.

Experimental Results

Figures 2 and 3 illustrate patterns obtained with whey and skim milk. The graph on the left in Figure 2 shows clearly two zones, the more mobile zone belonging to the β-lactoglobulin present in rennet and whey, and the zone adjacent to it belonging to α-lactalbumin. The least mobile zone is flat, irregular, and indistinct, and belongs to the immunoglobulin fraction. Superimposed on the graph, showing the ionophoretic pattern of rennet whey, is a curve representing the pattern of a solution containing 0.025 mg. of α-lactalbumin and 0.05 mg. of β-lactoglobulin. The superimposition brings out the indistinct zone belonging to the immunoglobulin fraction. The pattern on the right belongs to casein whey and does not differ essentially from that of rennet whey.

Figure 3 shows three patterns, one belonging to skim milk, a second belonging to skim milk heated at 85° C. for 30 minutes, and a third belonging to heated skim milk to which 5 mg. of β-lactoglobulin had been added per ml. The clear-cut separation of the lactoglobulin zone made possible by off-center application of sample and its incorporation in the zone belonging to calcium caseinate–phosphate complex in the heated milk pattern furnish a basis for the direct quantitative determination of β-lactoglobulin in skim milk.

The identity of bands was established by comparison of patterns belonging to whey and milk with those belonging to mixtures of these with known proteins.

Factors Influencing Dye Absorption. Failure to obtain reproducible results from day to day led to an investigation of possible contributing factors. The temperature of drying, the mode of drying, the pH of buffer occluded in the strip prior to drying, the time of staining, and the temperature of the drying were factors requiring control. Tables I and II and Figure 4 illustrate the influence of these factors on the absorption of bromophenol blue. Figure 4 refers to variations in dye absorption corresponding to variations in drying temperature. Streaks of solutions, 0.01 ml., of α-lactalbumin and β-lactoglobulin containing 5 mg. per ml. were made on strips wet with veronal buffer, pH 8.6. The strips were held taut on a rack and dried at the temperatures indicated. Variations are obtained which point to the desirability of temperature control during drying. In this instance, the use of constant temperatures in the range 50° to 60° C. is preferable to the use of the near-scorching temperatures recommended for the drying of other proteins. Drying at room temperature leads to inconsistent results with respect to dye absorption by β-lactoglobulin. The staining of moist strips yields, for lactalbumin, results comparable in magnitude to those obtained with strips dried at relatively low temperatures, while a marked reduction occurs in dye uptake by β-lactoglobulin. This may be related to the inconsistencies observed in drying at room temperature under conditions of varying relative humidity.

Table I refers to results obtained with strips dried at 55° and 120° C., and exposed to the stain for 10-minute and 18-hour intervals. The uptake of dye is not maximal in a 10-minute staining period, nor is linearity achieved under any conditions over the entire range of concentration under study. Extending the staining period brings about increased absorption over the entire concentration range, yet the marked increase in linearity in the high concentration range is effected at the expense of a loss in linearity in the lower range. The extended period leads to maximal absorption; however, the requirement of linearity seemed more important, and as no particular advantage accrued, in this respect, to the use of an 18-hour staining period, the shorter period was used. Linearity with maximal absorption over a wide range of concentration should be achievable through further study.

Dye absorption variations due to different staining bath temperatures were studied and, as a result, a constant temperature of 37° C. was used for staining.

Table I. Influence of Drying Conditions and Staining Time on Dye Absorption

Protein	Wt., Mg.	Absorbance per Mg. A[a]	B[b]	C[c]	D[d]
	0.025	7.16	8.52	5.40	7.28
	0.033	7.38	9.15	5.40	7.77
β-Lactoglobulin	0.050	7.52	10.00	6.50	8.64
	0.060	7.18	...	...	...
	0.100	4.68	8.05	5.60	9.51
	0.025	6.88	7.52	4.28	5.24
	0.033	6.90	...	...	...
α-Lactalbumin	0.050	6.42	8.54	5.92	7.68
	0.060	7.00	...	...	...
	0.100	5.29	8.72	5.49	8.97

[a] Dried at 55° C. for 45 minutes, stained at 37° C. for 10 minutes.
[b] Dried at 55° C. for 45 minutes, stained at 28° C. for 18 hours.
[c] Dried at 120° C. for 30 minutes, stained at 37° C. for 10 minutes.
[d] Dried at 120° C. for 30 minutes, stained at 28° C. for 18 hours.

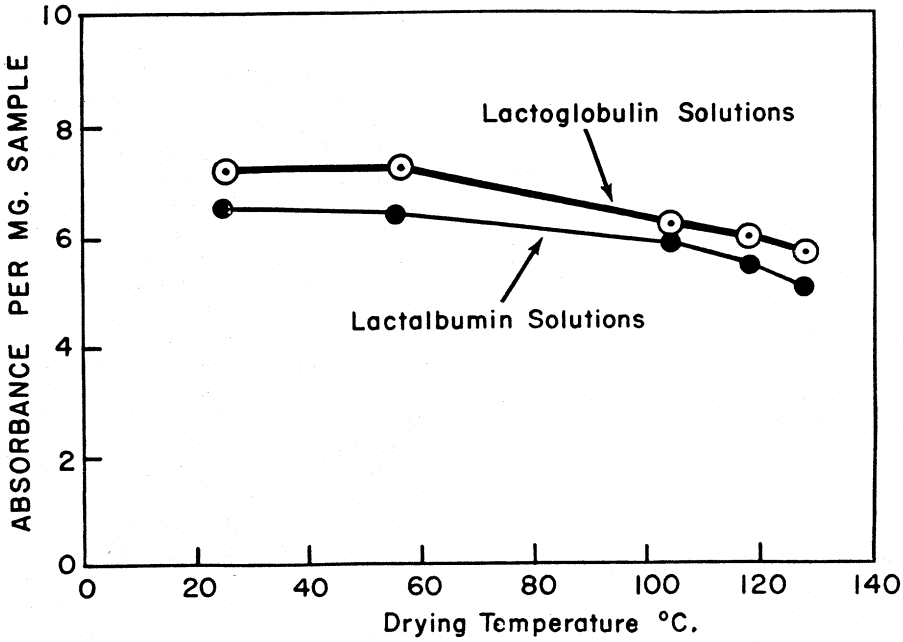

Figure 4. Influence of drying temperature on dye absorption

Holding times for temperatures above 25° C., 30 minutes; room temperature, 45 minutes

Staining Bath Temp., ° C.	Absorbance Value/Mg. Lactalbumin	Absorbance Value/Mg. B-Lactoglobulin
4.4	4.7	4.6
35	6.7	6.9
54.5	6.5	6.8

A drying period of 45 minutes at approximately 55° C. and a staining period of 10 minutes at 37° C., unless otherwise noted, were adopted on the basis of the experimental results.

The dye-combining power of β-lactoglobulin was influenced by the composition of the buffer adhering to the strip prior to drying, while that of α-lactalbumin and the protein of the classical globulin fraction of whey, was independent of pH and buffer type (Table II). The larger β-lactoglobulin value obtained with acetate buffer as compared with the minimum value obtained with phosphate buffer probably reflects the volatility of acetic acid which contributes to an increase in pH during drying. The dye-combining power of this protein should therefore be evaluated whenever a change in buffer and reaction is contemplated. Column 5 (Table II) shows that within experimental error, 5%, the calcium caseinate complex and each of the chief whey proteins has the same dye-combining power when the strips, prior to drying, are wet with veronal buffer at pH 8.6, and the staining operation is standardized.

There was a relatively large change in the dye-combining power of β-lactoglobulin when solutions containing it were heated at 85° C. for 30 minutes. Bromophenol blue derived from 1 mg. of heat-denatured and undenatured protein yielded solutions, the absorbance of which were 11.0 and 7.2, respectively. The corresponding change observed with lactalbumin was not significant.

Adsorption on Filter Paper during Electrophoresis. Table III illustrates the extent to which protein losses due

Table II. Influence of Buffer Used in Electrophoresis on Dye Absorption Capacity of Paper Strips on Staining with Bromophenol Blue

Protein[b]	Wt., Mg.	Absorbance per Mg.[a] Buffer I[c]	Buffer II[d]	Buffer III[e]
α-Lactalbumin	0.028	6.75	7.00	6.98
	0.075	6.56	6.40	6.80
β-Lactoglobulin	0.028	4.98	3.93	6.98
	0.055	4.18	3.80	6.82
Immunoglobulin fraction	0.027	6.50	6.55	6.60
	0.053	6.10	6.65	6.53
Calcium caseinate complex	0.028	...	...	7.18
	0.056	...	...	7.24

[a] Per 3 ml. of solution.
[b] Drying temperature, 50° to 60° C., staining temperature, 37° C. for 10 minutes.
[c] I 0.1*M* Acetate, pH 4.8.
[d] II 0.1*M* Phosphate, pH 6.7.
[e] III 0.06*M* Barbital, pH 8.6.

Table III. Adsorption of α-Lactalbumin and β-Lactoglobulin

(Reproducibility of results in terms of average deviation)

Run[a]	Solution[b]	Wt. of Protein, Mg.	Mean Absorbance[c]	Mean Protein Adsorption, Mg.[c]	Mean Absorbance per Mg.[c]	Mean Distance Migrated, Cm.[c]	Mean Adsorption per Cm., Mg.
1	Standard lactalbumin	0.024	0.120 ± 0.008(4)		5.00 ± 0.33(4)	...	...
	Standard lactoglobulin	0.024	0.117 ± 0.003		4.88 ± 0.12	...	...
	Migrating lactalbumin	0.024	0.033 ± 0.006	0.017	1.37 ± 0.24	2.80 ± 0.10(4)	0.0061
	Migrating lactoglobulin	0.024	0.017 ± 0.005	0.021	0.71 ± 0.10	4.30 ± 0.08	0.0049
2	Standard lactalbumin	0.048	0.297 ± 0.010		6.19 ± 0.19	...	...
	Standard lactoglobulin	0.048	0.294 ± 0.014		6.13 ± 0.29	...	...
	Migrating lactalbumin	0.048	0.152 ± 0.030	0.018	3.16 ± 0.23	3.35 ± 0.05	0.0054
	Migrating lactoglobulin	0.048	0.114 ± 0.003	0.025	2.37 ± 0.06	4.30 ± 0.08	0.0058

[a] Run 1. Nine 10 × 3/4 inch strips; current, 18 ma.; 440 to 307 volts; time, 2 hours.
Run 2. Nine 10 × 3/4 inch strips; current, 18 ma.; 396 to 280 volts; time, 2 hours.
[b] Standard solutions streaked on paper after run, prior to drying. Strips oven dried at 105° C. for 30 minutes.
[c] Albumin solutions were run five times; globulin solutions, four times. All replicates were acceptable except where a number appears in parentheses, giving the number of acceptable replicates. Rejection of observations was based on the Pierce Chauvenet criterion.

Table IV. Effect of Application of β-Lactoglobulin Coating on Recovery of Added Lactalbumin and Lactoglobulin

Run	Precoat	Solution	Wt., Mg.	Distance to Zone, Cm.	Width of Zone, Cm.	Corrected Absorbance, Mobile Zone	Corrected Absorbance Standards	Recovery, %
1[a]	None	Lactalbumin	0.025	2.3	0.9	0.090	0.147	61
		Lactoglobulin	0.025	3.0	0.9	0.062	0.136	46
	Lactoglobulin 0.1 mg.	Lactalbumin	0.025	2.4	1.2	0.139	0.147	95
		Lactalbumin	0.050	2.2	1.3	0.318	0.294	108
		Lactoglobulin	0.025	3.0	1.2	0.132	0.136	97
2[b]	Lactoglobulin 0.05 mg.	Lactoglobulin	0.025	3.1	1.1	0.173	0.168	102
		Lactoglobulin	0.050	2.9	1.1	0.367	0.337	109
	Lactoglobulin 0.1 mg.	Lactoglobulin	0.025	2.7	1.3	0.155	0.168	92
		Lactoglobulin	0.050	3.1	1.4	0.347	0.337	103

[a] 0.01 ml. of precoat applied; current, 18 ma. at 455–320 volts passed through nine 10 × 3/4 inch strips for 1 hour. Samples were applied, electrophoresis continued for 2 added hours with same current.
[b] 0.01 ml. of precoat applied; current, 18 ma. at 350 to 264 volts passed through nine 10 × 3/4 inch strips for 65 minutes. Samples then applied and electrophoresis continued for 2 hours.

to adsorption affect quantitative relations and that, in a single run, reproducibility of results is sufficiently good to lend a quantitative character to the results; the percentage deviation is the same which would normally be encountered in colorimetric micro and semimicroanalysis.

Protein adsorption was calculated from the difference between the absorbance of solutions containing eluted dye derived from the stationary and migrating protein zones, on the assumption that a linear relation exists in the range from 0 to 0.024 mg. of protein. The mean adsorption per cm. migrated appears to be independent of the quantity of protein applied to the strip and to be the same for both β-lactoglobulin and α-lactalbumin, irreversible and of fixed magnitude.

Because of several adsorption variables, the use of correction factors to compensate for adsorption losses is not feasible. However, the invariability of the loss with respect to both the kind and concentration of protein suggested that if the paper could be exactly saturated with irreversibly adsorbed β-lactoglobulin or α-lactalbumin, quantitative analysis of the proteins would be possible.

Effect of Precoating with β-Lactoglobulin on Recovery of β-Lactoglobulin and α-Lactalbumin. With the application in a prescribed manner of β-lactoglobulin as an adsorbed saturated precoat prior to the application of the sample, quantitative recovery of both lactalbumin and lactoglobulin becomes feasible (Tables IV to VI). Precoating was accomplished by streaking 0.01 ml. of a solution containing 5 to 10 mg. per ml. on the strip, allowing electrophoresis to proceed for 1 to 2 hours, applying the samples on the strips, and continuing electrophoresis for an additional 2 hours. Per cent recovery values given in the last column of Table IV for solutions containing single proteins show that in the absence of a precoat, there were losses of 39 and 54% in lactalbumin and lactoglobulin, respectively. In the presence of a precoat, recoveries were quantitative within experimental error. Average recoveries of lactalbumin at the two levels, 0.025 and 0.050 mg., were 95 and 108%; average recovery of lactoglobulin, 97 and 106%. Equally good results were obtained with protein precoat derived from streaks containing 0.05 or 0.1 mg. of protein, and this signifies that saturation of the migrating path is obtained at the lower, 0.05-mg., level.

Table VI shows recovery of lactalbumin and lactoglobulin from mixtures of these proteins either in the presence or absence of a precoat. From a mixture containing 0.025 mg. of each of the two proteins, 0.017 mg. of lactalbumin and 0.008 mg. of lactoglobulin were recovered in an experiment in which no precoat was applied. From strips containing precoat, the corresponding recoveries were 0.025 and 0.025 mg. Results were equally favorable for mixtures containing varying proportions of proteins, and were quantitative not only in relative but also in absolute magnitude. Whether a precoat is applied or not it is necessary to correct for background color contained in the zone. The weak background color due to the precoat is uniform and does not weaken contrast significantly.

Table VI shows also the results of analysis of rennet and casein whey. Rennet whey was prepared by the addition of 0.1 ml. of rennet extract to 100 ml. of skim milk at 30° C. A slightly opalescent filtrate was obtained, which on standing became turbid.

Casein whey was prepared by the addition of 1*N* hydrochloric acid to skim milk with stirring at 30° C. until a pH of 4.6 was reached. The mixture was filtered and the filtrate was brought to pH 8.4 by means of sodium hydroxide. The precipitate which formed was removed by filtration and a clear filtrate was obtained, each 100 ml. of which represented the whey present in 105 ml. of skim milk. Rennet whey was diluted to the same degree.

The composition of whey is variable and depends to a large extent on the

Table V. Effect of Application of β-Lactoglobulin Coating on Recovery of Added Lactalbumin and Lactoglobulin[a]

Solution	Wt., Mg.	Corrected Absorbance, Mobile	Corrected Absorbance Standards	Recovery, Mg.	Recovery, %	Recovery Relative Concn., Concn. Alb. / Concn. Glob.
Lactalbumin	0.025	0.181	0.173	0.026	104	0.52
Lactoglobulin	0.050	0.376	0.340	0.055	110	
Lactalbumin	0.050	0.351	0.173[b]	0.050	100	0.91
Lactoglobulin	0.050	0.379	0.340	0.055	110	
Lactalbumin	0.050	0.345	0.173[b]	0.050	100	2.0
Lactoglobulin	0.025	0.170	0.170	0.025	100	
Lactalbumin	0.025	0.165	0.173	0.024	96	1.0
Lactoglobulin	0.025	0.160	0.170	0.024	96	

[a] 0.01-ml. of precoat (0.05 mg. of lactoglobulin) applied; current, 18 ma. at 297 to 279 volts passed through nine 10 × 3/4 inch strips for 1 hour; samples were applied, electrophoresis continued for 2 added hours with same current.
[b] Value for 0.025-mg. samples.

method of preparation used to define it. This has been pointed out by Sørensen (7). Coprecipitation in precipitation of a fraction may alter relative concentrations in the remaining fraction.

Within experimental error the same values, 4.1 to 4.2 mg. per ml., were obtained for the concentration of β-lactoglobulin in rennet and casein whey.

Values of 1.3 and 1.5 mg. per ml. were obtained for α-lactalbumin in casein and rennet whey, respectively, and corresponding values of 0.8 and 1.4 mg. per ml. were obtained for the proteins of the immunoglobulin fraction. Because the zone belonging to the protein of the immunoglobulin fraction is poorly defined, the error attending its determination is considerable, and the values cited show corresponding variability. Percentage-wise, the relative concentrations in casein and rennet whey were:

	Casein Concn., %	Rennet Concn., %
β-Lactoglobulin	67	60
α-Lactalbumin	20	21
Immunoglobulin fraction proteins	13	19
Protein/ml., mg.	6.3	7.0

The concentration of β-lactoglobulin in one sample of skim milk was also determined. β-Lactoglobulin moves ahead of the main body of milk proteins in ionophoretic experiments with veronal buffer, at pH 8.6, and with sample applied 1 inch from the apex toward the cathode. A clearly defined zone is obtained and the quantity of dye absorbed by β-lactoglobulin is easily determined. The zones belonging to β-lactoglobulin and α-lactalbumin do not appear in ionophoretic patterns of skim milk heated at 85° C. for 30 minutes. These become involved in the band belonging to the main body of milk proteins as a result of interaction in a complex manner of β-lactoglobulin with the constituents of milk (see Figure 1, graphs 1 to 6, inclusive). If known concentrations of β-lactoglobulin are added to heated skim milk, this added protein appears as a zone at the expected site. From a standard curve obtained in this manner, relating the concentration of added β-lactoglobulin to the concentration recovered, the concentration of β-lactoglobulin in skim milk may be calculated. This calculation is based on the assumption that losses of β-lactoglobulin due to adsorption are of equal magnitude in patterns belonging both to unheated milk, and to heated milk containing added β-lactoglobulin. On this basis, the concentration of β-lactoglobulin in a sample of skim milk, the pattern of which is shown in Figure 2, was found to be 0.5 gram per 100 ml., an unexpectedly high value compared with values for whey which may be calculated from boundary electrophoretic data (6).

Table VI. Influence of Lactoglobulin Coating on Recoveries from Mixed Solutions of Lactoglobulin and Lactalbumin, and on Recoveries from Casein and Rennet Whey

Sample No.[a]	Solution: Lactalbumin, mg.	Solution: Lactoglobulin, mg.	Solution: Casein whey, ml.	Solution: Rennet whey, ml.	Recoveries: Lactalbumin, mg.	Recoveries: Lactoglobulin, mg.	Recoveries: Immunoglobulin fraction	Recoveries: Total protein
1	0.025	0.025	...	...	0.025	0.025	....	0.050
2	0.050	0.025	...	...	0.053	0.024	....	0.077
3	0.050	0.050	...	...	0.053	0.051	....	0.104
4	....	....	0.01	...	0.013	0.042	0.008	0.063
5	....	....	...	0.01	0.015	0.041	0.014	0.070
6	0.025	0.025	...	...	0.017	0.008	....	0.025
7	0.050	0.050	...	...	0.046	0.012	....	0.058
8	....	....	0.01	...	0.011	0.024	0.001	0.036
9	....	....	...	0.01	0.015	0.029	0.006	0.051

[a] 0.01 ml. of lactoglobulin solution containing 5.0 mg. per ml. applied as a precoat. Current, 18 ma. and 297 to 279 volts passed through nine 10 × 3/4 inch strips for 43 minutes; samples were applied, and electrophoresis continued for 2 hours. Samples 1 through 5 are coated; samples 6 through 9 are not coated.

Discussion

In the present work on paper electrophoresis of whey, only three zones are clearly defined. There is a suggestion of a fourth—a very small plateau on the leading edge of the β-lactoglobulin zone (Figure 2) which may belong to serum albumin (5). As artifacts are much more apt to appear in ionophoresis than in boundary electrophoresis, in calculations this zone was assigned to β-lactoglobulin. Therefore, the value assigned to β-lactoglobulin, 0.41 to 0.42 gram per 100 ml. whey, may be in error to the extent of approximately 0.03 gram per 100 ml. The zone ascribed to α-lactalbumin appears to correspond to component *c* rather than to component *d* in the designation of Smith (6). Experiments with pure solutions of α-lactalbumin and with whey filtrates, from which the immunoglobulin fraction had been removed with ammonium sulfate at 58% saturation and pH 6.0, support this conclusion. Solutions containing the immunoglobulin fraction, precipitated three times with ammonium sulfate at 58% saturation, shows two zones on ionophoresis, one of which corresponds to the zone belonging to α-lactalbumin. Smith found a corresponding protein in his impure immunoglobulin fractions which he ascribed, however, to the presence of component *c*.

Recently Larsen and Jenness (4) have compared their boundary electrophoretic patterns with those of Smith. They have questioned the conclusions based on the work of Smith, and have proposed that the component *c* in Smith's patterns belongs to α-lactalbumin, and that component *d* represents a small and hardly discernible boundary between those belonging to α-lactalbumin and β-lactoglobulin. They cite relative concentrations of 62, 21, and 15% for the β-lactoglobulin fraction (β-lactoglobulin plus serum albumin plus component *d*), α-lactalbumin, and the immunoglobulin fraction. These values compare favorably with the corresponding values obtained ionophoretically in this laboratory—67, 20, and 13%.

Protein values are given in absolute quantities and the total protein found in casein and rennet whey—0.63 and 0.70 gram per 100 ml., respectively—agrees with the average of values given in the literature and which refer to the difference between total whey nitrogen calculated as protein, and the sum of proteose and nonprotein nitrogen.

With pure solution of α-lactalbumin, it has been observed that the leading edge of the lactalbumin zone has a tendency to spread during ionophoresis. This tendency was intensified with a solution of α-lactalbumin which had been heated at 85° C. for 30 minutes, showing that during ionophoresis of lactalbumin, changes occurred similar to those which take place on heating lactalbumin solutions. However, Larsen and Jenness (4) report the same kind of asymmetry in their boundary electrophoretic patterns belonging to recrystallized α-lactalbumin, and the observed pattern is peculiar to α-lactalbumin.

The high value obtained for the β-lactoglobulin content of skim milk, 0.5%, belongs to a single sample and does not represent an average value. Possibly in processing skim milk for either rennet or casein whey, some β-lactoglobulin is lost as a coprecipitate.

Samples were applied to the strip with a minimum of processing. No fractionation, dialysis against buffer, or concentration of the proteins was attempted. In the interest of greater accuracy with respect to the minor protein constituents of whey, the employment of concentrated solution of whey solids or of fractions rich in the immunoglobulin fraction may prove desirable. This would

require the use of separate strips, or a modified technique for the analysis of protein in the strips which would cover a wide range of protein concentration.

A number of papers on ionophoresis of milk and whey proteins by Schulte and Müller (*8*) which appeared recently, lthough not bearing directly on the quantitative determination of these proteins, are of interest in connection with the results reported in this paper.

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