SOME CHARACTERISTICS OF THE LACTOGLOBULIN FRACTION OF BOVINE MILK

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By

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INTRODUCTION

The classical lactoglobulin fraction of colostrum and milk has been obtained by saturating whey with magnesium sulfate or by half-saturation with ammonium sulfate. In general, lactoglobulin refers to the slowest electrophoretic components in colostrum and milk. The American Dairy Science Association Committee on the nomenclature, classification, and methodology of milk proteins (Jenness et al., 1956) tabulated a mobility ranging from -1.8 to -2.2×10^{-5} ($cm.^{2}\ v.^{-1}\ sec.^{-1}$) to this fraction. Upon prolonged dialysis the lactoglobulin separates into a water soluble pseudoglobulin and a water insoluble euglobulin; pseudoglobulin remains in solution and the euglobulin precipitates out. These proteins have often been called the "immune lactoglobulins" because they were associated with antibody activity (Smith, 1946a, b). However, Pierce (1955) has shown that these components were present in milk in the absence of specific immunization. In addition to their immune activity they have been reported to contain carbohydrates.

Advancements in techniques used in the fractionation and purification of proteins have made it possible to demonstrate the presence of new components in protein fractions which once were considered as homogeneous. The general assumption has prevailed, based on ultracentrifugal evidence, that the pseudoglobulins and euglobulins are heterogeneous protein fractions.

The objective of this study was to isolate electrophoretically "homogeneous" lactoglobulin components and to characterize the constituent conjugated carbohydrate moiety.

REVIEW OF LITERATURE

Eugling (1880) was the first to recognize globulin in milk. He reported that following prolonged treatment of diluted colostrum whey with carbonic acid white flakes separated out which were soluble in five per cent salt solution. Therefore he considered these flakes to be globulin. A similar suggestion was made by Hammersten (1883) who observed that after separating casein from milk the filtrate contained albumin and a substance separated by saturation with magnesium sulfate. His experiment led him to suggest that the precipitate was a globulin.

The name "lactoglobulin" was introduced by Sebelien (1885) who obtained a flocculent precipitate by saturating whey with magnesium sulfate. This protein fraction appeared to be identical in characteristics with serum globulin. Further, he found an abundance of lactoglobulin in bovine colostrum. Halliburton (1890) denied the existance of globulin in milk and regarded the isolated substance as lactalbumin. However Sebelien (1891) proved that albumin was not precipitated by saturating whey with magnesium sulfate, but remained in solution from where it could be precipitated by the addition of acetic acid.

Storch (1897) used sodium sulfate at room temperature to isolate the globulins from milk by saturating it with the salt, which, at room temperature, was equivalent to a 14-19 per cent solution.

Schlossmann (1896-1897) separated globulin by saturating milk with magnesium sulfate and observed that the globulin, after prolonged standing, collected on the surface of the liquid. Simon (1901) re-examined the

procedures of Sebelien (1891) and Schlossmann (1896-1897) and obtained globulin fractions which proved to be similar in their composition and solubility.

Bauer and Engel (1911) compared colostrum and milk and found that globulin was more active than albumin in building antibodies. They observed no biological difference between milk and colostrum. The same was found to be valid for the proteins of blood serum compared to those of milk and/or colostrum. Crowther and Raistrick (1916) reported that milk globulin, like blood globulin, could be separated into euglobulin and pseudoglobulin. They precipitated globulin with magnesium sulfate, redissolved it in distilled water, and dialyzed it for six days in running water. The globulin was thus separated into two fractions; euglobulin--insoluble in water and pseudoglobulin--soluble in water.

Dudley and Woodmann (1918) and Woodmann (1921) investigated euglobulin and pseudoglobulin and found that these proteins were structurally identical. Racemization was employed to establish identity or non-identity of related proteins. They investigated the optical rotational properties of euglobulin and pseudoglobulin in alkaline solution and also made a comparative study of the optical properties of amino acids derived from the hydrolysis of racemized euglobulins and pseudoglobulins.

Howe (1922) precipitated globulins from colostrum with sodium sulfate and considered the material precipitated up to 14.2 per cent at 34° C. to be euglobulin; that at 14.2 - 18.4 per cent as pseudoglobulin I and casein (casein was obtained from the filtrate by acidification); that at

18.4 - 21.5 per cent as pseudoglobulin II. This last fraction was recovered in small yields and there was no positive evidence that this was a separate protein. Howe (1921) obtained these fractions by following an isolation scheme he employed to fractionate blood proteins.

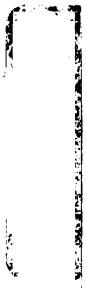
Smith (1946a, b; 1948) made a series of outstanding studies and contributions to our knowledge of the lactoglobulin fraction. He found that the globulin precipitated by magnesium or ammonium sulfate was a mixture of proteins. He devised a scheme for isolating an electrophoretically homogeneous globulin fraction by fractionation with ammonium sulfate. However studies in the ultracentrifuge revealed that all of the isolated lactoglobulins contain more than one fraction. The globulin character of the isolated protein fractions was indicated by their precipitation at low concentrations of ammonium sulfate, low solubility near the isoelectric point, and by marked increase in solubility in the presence of neutral salts. Smith reported that exhaustive dialysis of the lactoglobulin fraction resulted in the separation of euglobulin (water insoluble) and pseudoglobulin (water soluble) fractions and that immune activity was associated with both of these proteins. Further, he showed by electrophoretic analysis that the lactoglobulin of milk and colostrum were identical. An elemental analysis of lactoglobulins by the same investigator revealed an absence of phosphorus, but showed the presence of sulfur and carbohydrates. Smith and Greene (1947) reported that lactoglobulins had a high threonine content, and that cystine and methionine accounted for the sulfur of these proteins. The total carbohydrate content of

lactoglobulins in milk and colostrum was contributed from the protein bound hexose and hexosamine (Smith, Greene and Bartner, 1946; Smith, 1946b). Smith (1946b) reported the values of pH 5.6 and 6.05 for the isoelectric points of pseudoglobulin and euglobulin, respectively.

Smith (1946a, b) was the first to use electrophoresis and ultracentrifugation as well as chemical analysis in comparing the properties of lactoglobulin fractions. He records electrophoretic mobilities for pseudoglobulin and euglobulin of milk and colostrum of -2.5 and -1.7, and -2.2 and -1.9, respectively. Murthy and Whitney (1958) reported values of -2.04 and -1.76 for similar protein fractions.

The sedimentation coefficient of the principal component (about 84 - 89%) of colostrum and milk was reported to be approximately $S_{20,w} = 7$ (Smith, 1946a, b). The second most concentrated component had a value of 10 Svedberg units. A third component, with a sedimentation coefficient of about 20 Svedberg units, was found only in the euglobulin fraction. He also reported a component of 2 to 3 Svedberg units in the pseudoglobulin fraction of colostrum. Deutch (1947) reported somewhat lower values and Murthy et al. (1958) higher values for similar protein fractions.

A new method for isolating lactoglobulins from milk and colostrum was introduced by Kenyon, Anderson and Jenness (1959), as an adaptation of a method developed by Horejsi and Smetana (1956) for the isolation of gamma-globulin from blood serum. In this method Rivanol (2-ethoxy-6,9-diamino-acridine lactate) was used to form a metal-combining globulin complex which remained in solution. The precipitated proteins were



filtered off. Rivanol was removed from the supernatant by adsorption on activated charcoal, leaving a crude lactoglobulin fraction in solution.

EXPERIMENTAL PROCEDURE

Procedures for the Isolation of Lactoglobulins

Electrophoretically homogeneous euglobulin and pseudoglobulin fractions were separated from the lactoglobulin of milk and/or colostrum by previously reported methods and include:

a. fractionation of bovine milk and colostrum with ammonium sulfate (Smith, 1946a, b);

b. fractionation with sodium sulfate (Howe, 1922);

c. fractionation by the use of Rivanol (Kenyon et al., 1958).

The fractionation of bovine milk with ammonium sulfate. The scheme for the isolation and fractionation of the lactoglobulin is shown in Figure 1. Fresh raw milk was separated three times at 40° C. in a laboratory-size cream separator. The skimmilk was acidified with 0.1 N HCl to pH 4.6 and the casein removed by filtration. The acid whey was adjusted to pH 6.5 with 0.1 N NaOH and ammonium sulfate added to 0.5 saturation to precipitate the crude globulin (Fraction A). Fraction A was redissolved to about three per cent protein concentration, the pH adjusted to 4.6 and ammonium sulfate added to 0.25 saturation. The ensuing precipitate (Fraction C) was removed by centrifuging in a Servall centrifuge for 15 minutes at 25,000 xG. The supernatant was filtered through a thick layer of glass wool. The lactoglobulins (Fraction D) were precipitated from the supernatant at 0.4 saturation with ammonium sulfate at pH 6.0. This fraction was reworked by dissolving in distilled water to about three per

cent protein concentration, adjusting pH to 4.5 and filtered. Ammonium sulfate was added to 0.3 saturation and Fraction E was precipitated. The supernatant was brought to pH 6.0 and ammonium sulfate added to 0.4 saturation to obtain the precipitate (Fraction F). Precipitates were redissolved and dialyzed free of salt against distilled water. The lactoglobulin fractions were then dried by lyophilization.

The fractionation of bovine colostrum with ammonium sulfate. The scheme for fractionation and isolation of lactoglobulins from colostrum is shown in Figure 2. After separating the fat from the colostrum the skimmilk was diluted fivefold with distilled water and slowly adjusted to pH 4.5 with 0.5 N HCl. The casein precipitate (Fraction A) was removed by filtration. The lactoglobulins were precipitated at pH 6.0 by adjusting the whey to 0.3 saturation with ammonium sulfate (Fraction B). Ammonium sulfate was added to the supernatant to 0.5 saturation and Fraction C was precipitated. Fractions B and C were redissolved in distilled water to about five per cent protein concentration, cleared of residual undissolved material by filtration, and reprecipitated at the same limits of salt concentration. The lactoglobulins were redissolved and dialyzed against distilled water. The pseudoglobulin and euglobulin fractions were lyophilized.

The fractionation of bovine milk and colostrum with sodium sulfate. The scheme for the isolation and fractionation of lactoglobulins is shown in Figure 3. Fresh raw milk was separated three times at 40° C. The skimmilk was acidified with 0.1 N HCl to pH 4.6 and the casein removed

by filtration. The acid whey was adjusted to pH 6.5 and 14.2 per cent with respect to sodium sulfate, added at 34° C. to precipitate the euglobulin fraction. The precipitated fraction was collected by centrifugation, redissolved and dialyzed free of salt prior to lyophilization. Sodium sulfate was added to the supernatant to increase the salt concentration to 18.4 per cent. The precipitated pseudoglobulin was collected by centrifugation, redissolved, dialyzed salt free and lyophilized.

The fractionation of bovine milk and colostrum with Rivanol. The scheme of isolation and fractionation of lactoglobulins is outlined in Figure 4. Fresh raw milk was separated three times at 40° C. The skimmilk was acidified with 0.1 N HCl to pH 4.6 and the casein removed by filtration. To one volume of the acid whey to be fractionated, 3.5 volumes of 0.4 per cent aqueous Rivanol solution was added. The solution was adjusted to pH 8.5 to precipitate the lactoglobulins which were filtered off. Activated charcoal was added to remove the Rivanol by adsorption and filtered off. The clear filtrate, containing lactoglobulins, was pervaporated, dialyzed against distilled water. The euglobulins and pseudoglobulins were recovered and dried by lyophilization.

Analytical Methods

Electrophoresis. All electrophoretic data were obtained with a Perkin-Elmer Model 38-A Electrophoresis apparatus. Protein solutions were made up in veronal buffer, pH 8.6, $\mu = 0.1$ and dialyzed for six hours using a magnetic stirrer or for seventy-two hours without a stirrer in

two veronal buffer changes of 500 milliliters at 2° C. The completion of dialysis was determined by measuring the specific conductivity of the protein solution. The specific conductivity was calculated from the following equation:

$$K = \frac{cc}{R}$$

where

K - specific conductivity

cc - cell constant

R - resistance of solution in ohms observed at 1° C.

Protein concentrations were determined after dialysis by determining the difference between the dry weight of the buffer and the dialyzed protein solution. The electrophoretic mobilities were calculated using the following equation:

$$\mu(\text{cm.}^2\,\text{v.}^{-1}\,\text{sec.}^{-1}) = \frac{d\,a\,K}{i\,t\,R\,m}$$

where

d - distance migrated (cm.)

a - cross sectional area of the cell (cm.^2)

K - specific conductivity cell constant (ohms) = 0.8491

i - current (amps)

t - time (sec.)

R - resistance of buffer (ohms)

m - magnification factor = 1.1

The field strength was calculated from the following equation:

$$F = \frac{i}{a\,K}$$

where

F - field strength or potential gradient

i - current (amps)

a - cross sectional area of the cell ($cm.^2$)

K - specific conductivity of the buffer-protein solution

Ultracentrifugal analysis. Sedimentation coefficients were determined by the sedimentation-velocity technique, employing a Spinco Model E Centrifuge equipped with analytical accessories. The determinations were made at 20° C., and veronal buffer at pH 8.6 and ionic strength of 0.1 was used as a carrier for the protein solutions. Sedimentation coefficients were calculated from the following equation:

$$S = \frac{\Delta d/m}{4\pi^2 (x + \frac{r}{m}) rps^2 t}$$

where

S - sedimentation coefficient (sec.)

Δd - distance migrated in unit time (cm.)

m - magnification factor = 2.1

x - distance of refractive gradient from axis of rotation (cm.) = 5.72

$4\pi^2$ - 39.5

r - distance half-way between the peaks used for calculation (cm.)

rps - revolutions per second

t - time corresponding to observed position of sedimenting boundary indicated by subscripts (sec.)

Nitrogen determination. The official A. O. A. C. (1950) micro-Kjeldahl method was used to measure the nitrogen content of the various protein fractions.

Carbohydrate determination. Protein bound carbohydrates were determined according to procedures outlined by Glick (1955). Hexose was determined by means of an orcinol method for which galactose served as a standard. Hexosamine was determined on the basis of the reaction observed with galactosamine when used as a standard. Authentic fucose was employed as a standard for the fucose determination. Diphenylamine was employed in the determination of protein bound neuraminic acid. Orosomucoid, containing 11. 2 per cent neuraminic acid, was employed as a secondary standard for neuraminic acid.

Fractionation of Proteins on Diethylaminoethyl-Cellulose (DEAE) Columns

In general the fractionation procedure developed by Rackis, Sasame, Anderson, and Smith (1959) for chromatography of soybean proteins on a DEAE-cellulosic column was followed.

Preparation of the adsorbent column. The cellulosic anion-exchange adsorbent (DEAE) was suspended in water, stirred and titrated to pH 7. 6 with a concentrated solution of dihydrogen phosphate. The adsorbent was washed several times on a Buchner funnel with 0. 01 M sodium phosphate buffer at pH 7. 6 and re-suspended in the same buffer. The fines were decanted from the suspension after settling for 30 minutes. Following equilibration for at least 12 hours with the buffer, a slurry of the adsorbent

was poured into the exchange column, equipped with a fritted disc at the bottom, and allowed to settle until a constant column height was achieved. The packed column was connected to a fraction collector and washed with several volumes of the phosphate buffer. The column assembly used was similar to that described by Hirsh and Ahrens (1958). The packed column had dimensions of 16 - 17 x 1. 5 cm.

Following the completion of a chromatographic separation, the adsorbent was removed from the column and washed with 1 N NaOH until free from protein. This was checked by addition of 10 per cent TCA solution to equal volume of wash water following the regeneration of the adsorbent. The regenerated adsorbent was packed as described above.

Preparation of the sample. Samples containing 300 milligrams of lyophilized protein in 10 milliliters of 0. 01 M sodium phosphate buffer at pH 7. 6 were poured onto the column and allowed to enter the adsorbent under flow conditions induced by gravity. The walls of the column were washed down with 5 milliliters of the buffer, followed by pouring 5 milliliters of buffer on top of the column before elution was started.

Development of the chromatogram. Gradient elution was carried out at room temperature and NaCl (0 --- 0. 345 M) in 0. 01 M sodium phosphate buffer at pH 7. 6 was used to develop the chromatograms. Ten milliliter fractions were collected at a rate of 60 to 70 milliliters per hour with an automatic collector equipped with a 10 milliliter syphon. The fractions which compriseddiscrete peaks were combined, dialyzed, lyophilized and subsequently analyzed.

Examination of the effluent. The absorbance of each effluent fraction was determined in a Beckman DK-2 Spectrophotometer at 278 mμ. The fractions comprising discrete peaks were combined, dialyzed free of salt against distilled water, lyophilized and subsequently analyzed.

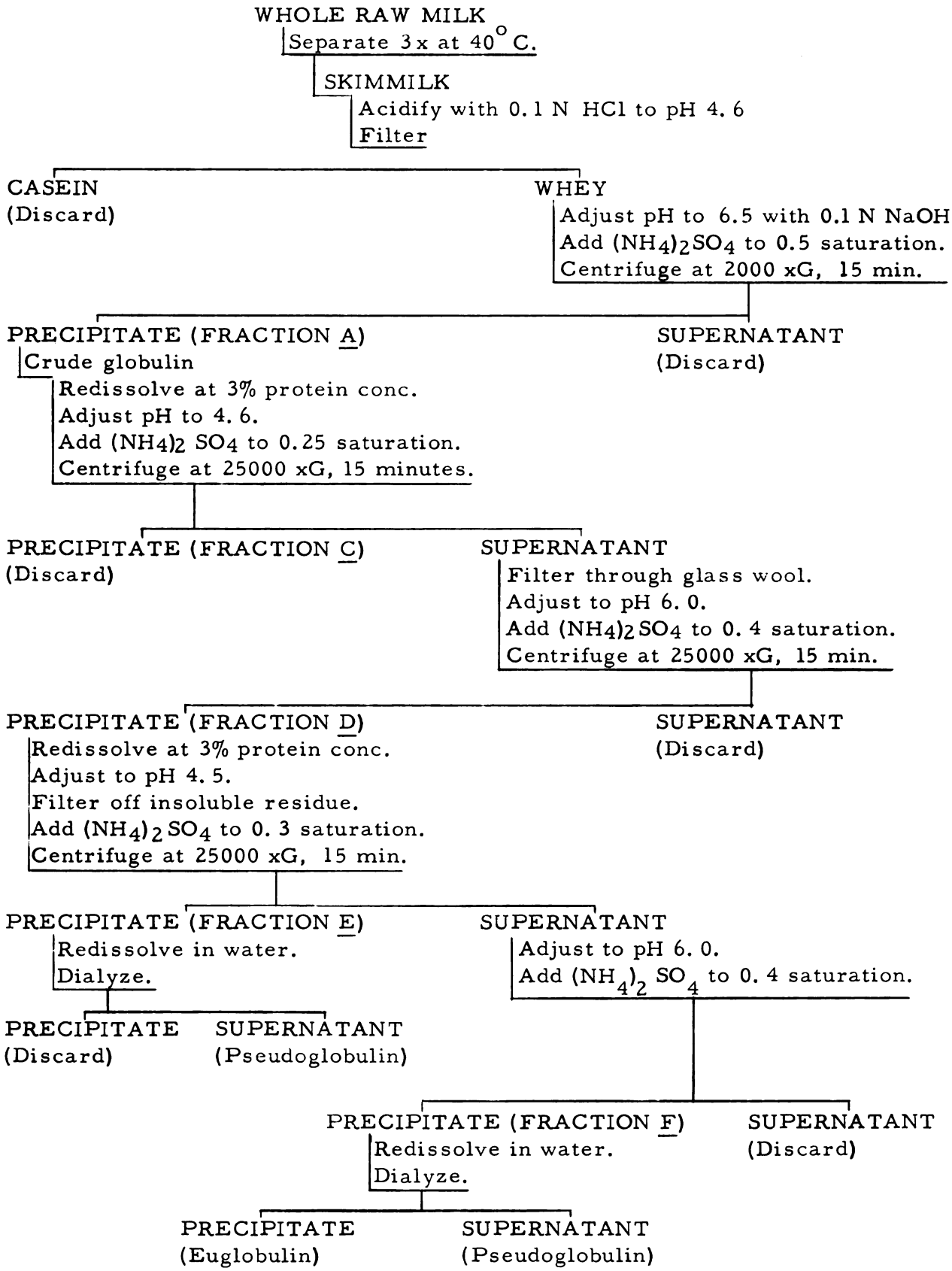


Figure 1. A schematic diagram showing the fractionation of bovine milk with ammonium sulfate.

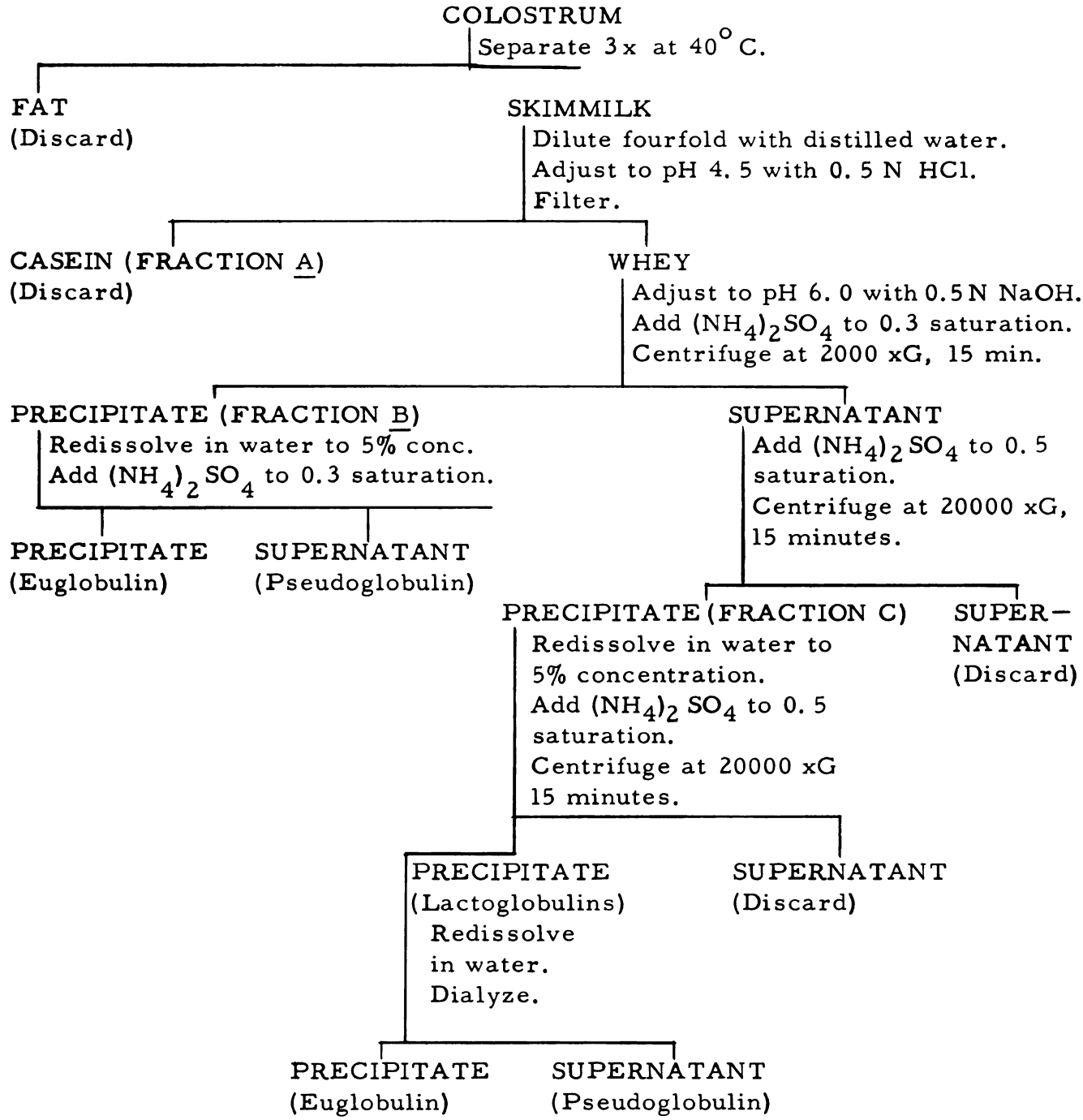


Figure 2. A schematic diagram showing the fractionation of bovine colostrum with ammonium sulfate.

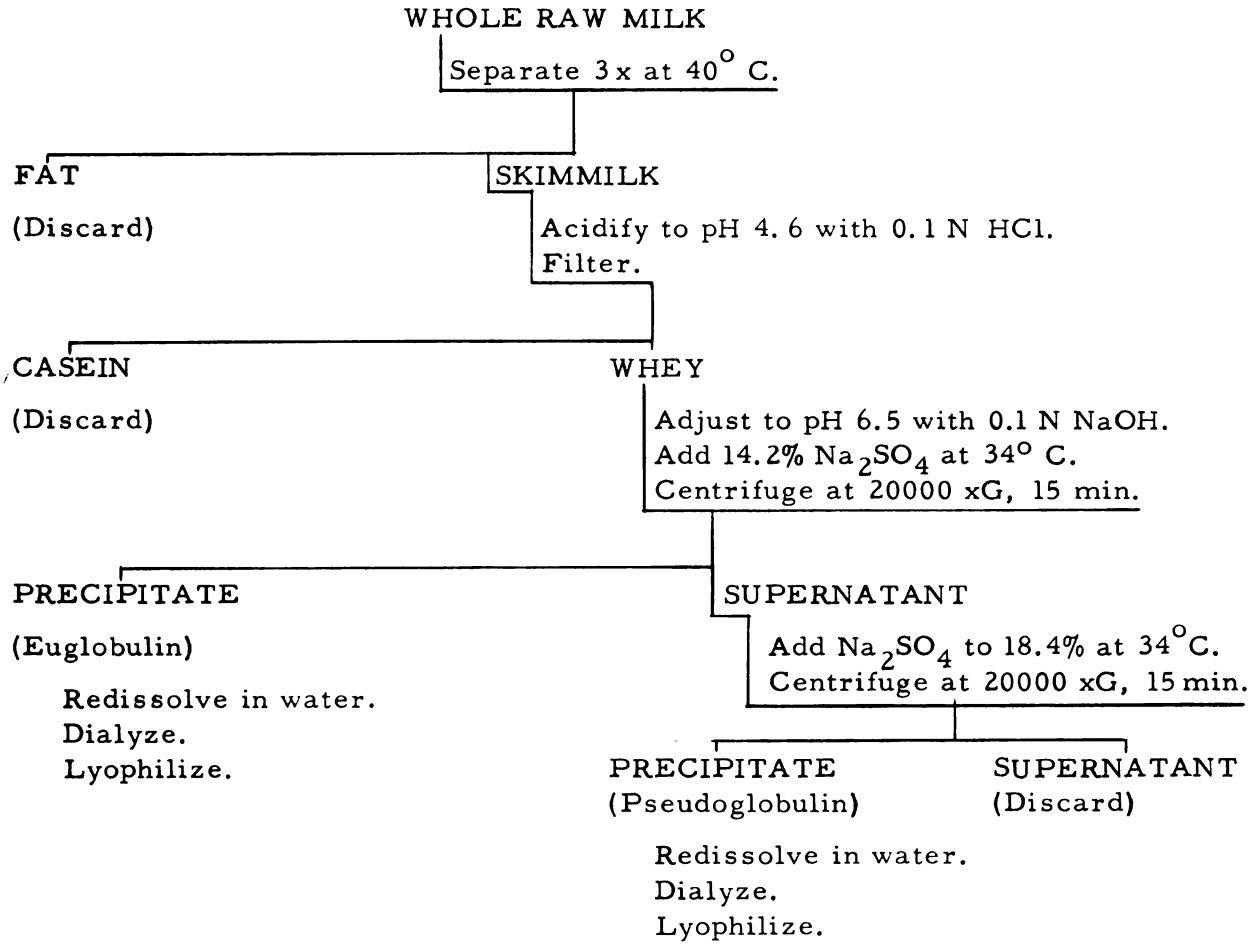


Figure 3. A schematic diagram showing the fractionation of bovine milk and colostrum with sodium sulfate.

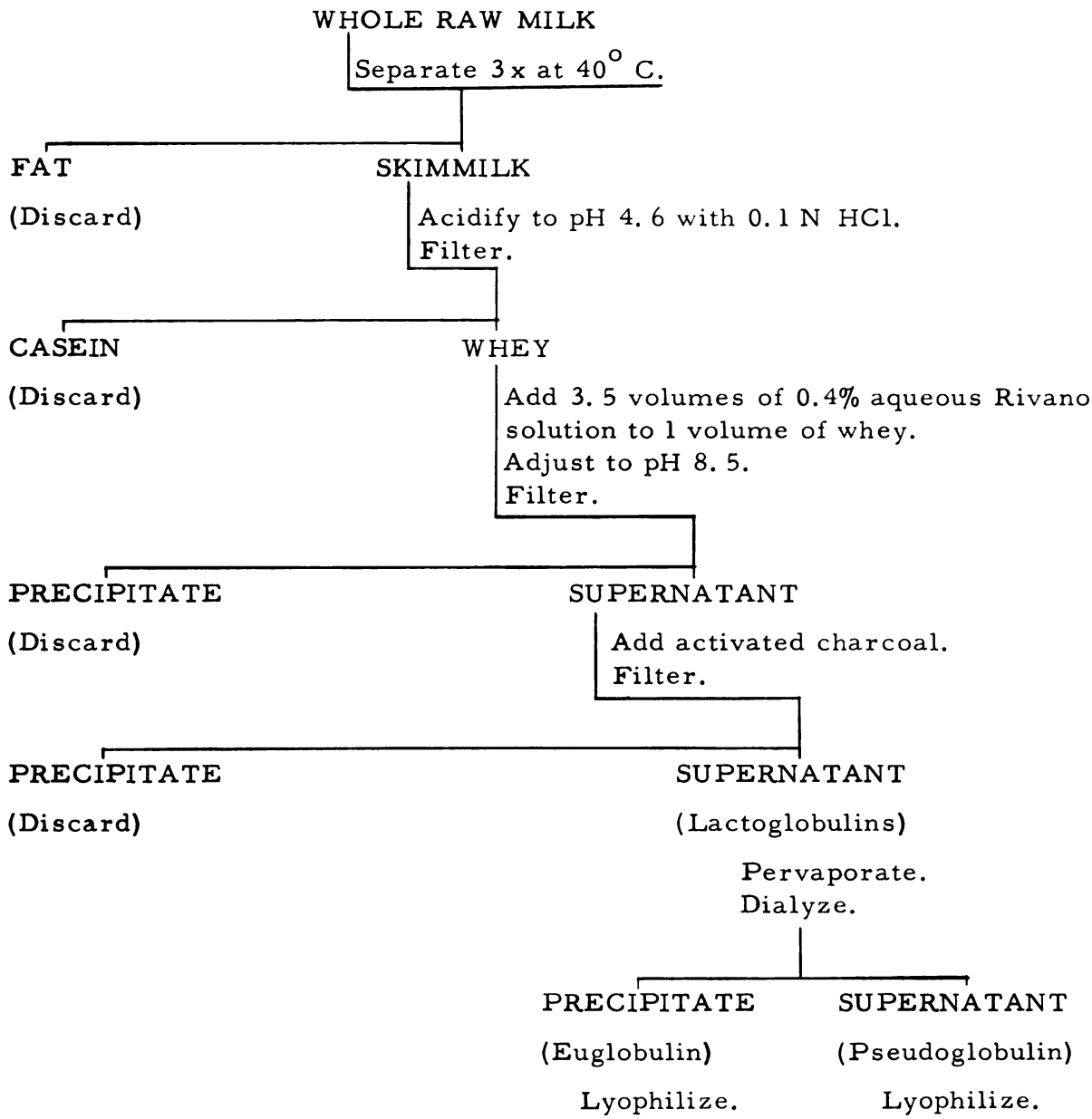


Figure 4. A schematic diagram showing the fractionation of bovine milk and colostrum with Rivanol.

EXPERIMENTAL RESULTS

Isolation of Lactoglobulins

The best isolations of lactoglobulins based on electrophoretic homogeneity were obtained by following the procedure of Smith (Figure 1) in which ammonium sulfate was used as the salting-out agent. To obtain workable amounts of electrophoretically homogeneous lactoglobulins, this fractionation procedure as shown in Figure 1 was adapted and used for the fractionation of colostrum. The free-boundary electrophoretic patterns of the fractions isolated are shown in Figure 9; their electrophoretic mobilities are listed in Table II.

Fractionation of Lactoglobulins on DEAE-Cellulose Columns

The elution diagram of acid whey proteins from the DEAE cellulosic column is shown in Figure 5. Figures 6, 7 and 8 show the elution diagrams of electrophoretically homogeneous lactoglobulin, pseudoglobulin and euglobulin fractions respectively (Figure 9). The reproductability of the position and height of the chromatographic peaks were good. Identical elution patterns were obtained in all cases.

In Figure 6, area under peak 1 consists of proteins collected in tubes 3 to 9; area under peak 2 of effluent collected in tubes 12 to 22; area under peak 3 of effluent in tubes 23 to 50. In Figure 7, area under peak 1 consists of effluent collected in tubes 4 to 12, and area under peak 2 of effluent in tubes 14 to 53. In Figure 8, area under peak 1 consists of effluent in tubes 3 to 14; area under peak 2 of effluent in tubes 15 to 57.

Analysis of Fractions

Nitrogen determination. The results of the Kjeldahl nitrogen determinations for lactoglobulin fractions of milk and colostrum are listed in Table I. The concentration of proteins was calculated on a dry weight basis.

Ultracentrifugal characteristics. Sedimentation-velocity studies were made on lactoglobulin, pseudoglobulin, euglobulin, and pseudoglobulin Fraction 2 from the DEAE-cellulose column. The sedimentation-velocity diagrams for these fractions are shown in Figure 10. Table III lists the sedimentation-velocity coefficients for all components in the above listed fractions.

Carbohydrate content. Protein bound hexose, hexosamine, fucose and neuraminic acid concentrations of the lactoglobulin fractions and their components are recorded in Table IV. The results were calculated on a dry weight basis.

Electrophoresis. The electrophoretic patterns of the lactoglobulin fractions are shown in Figure 9 and their mobilities are listed in Table II.

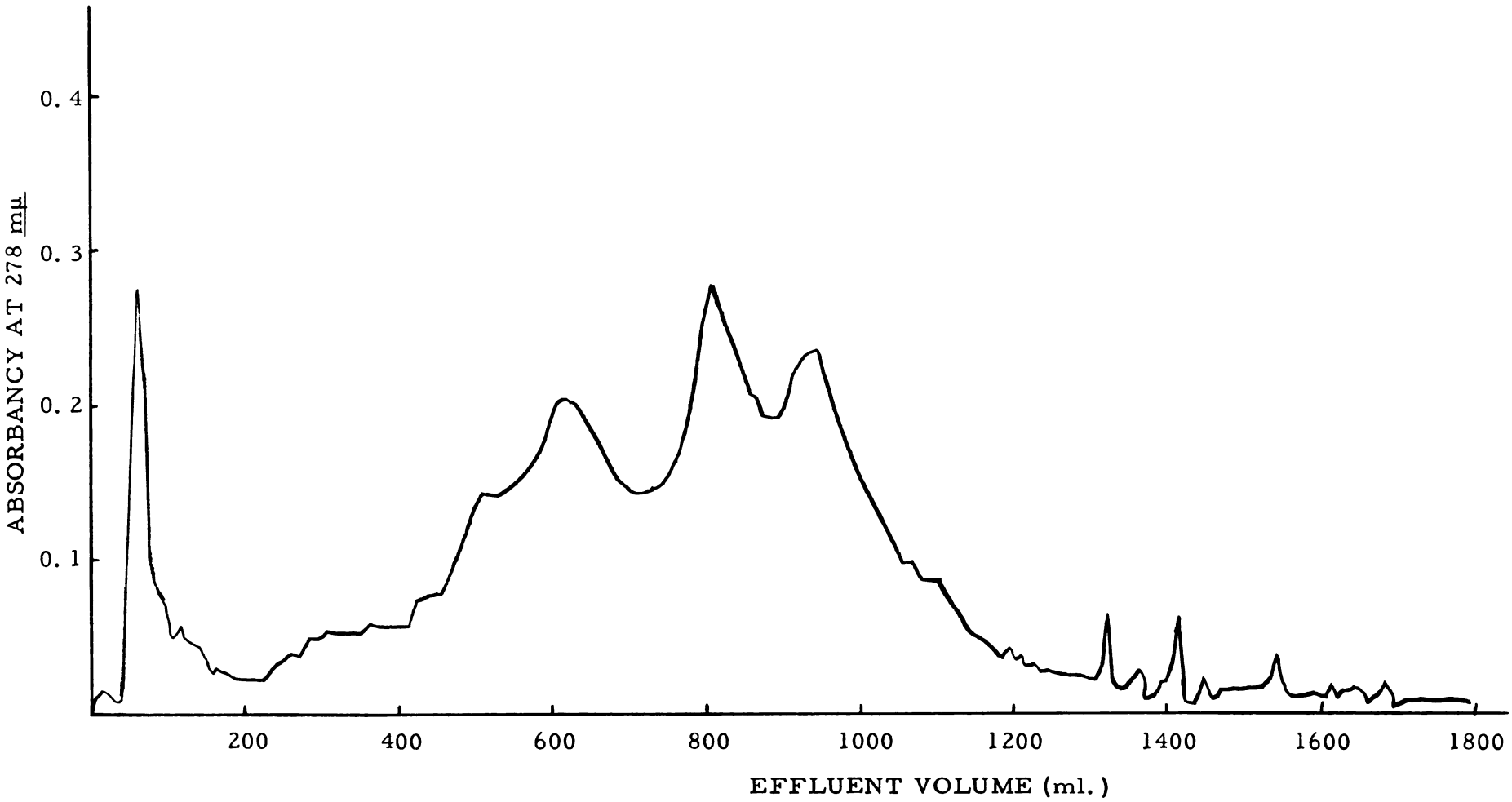


Figure 5. Gradient elution diagram of whey proteins from a DEAE-cellulose column.

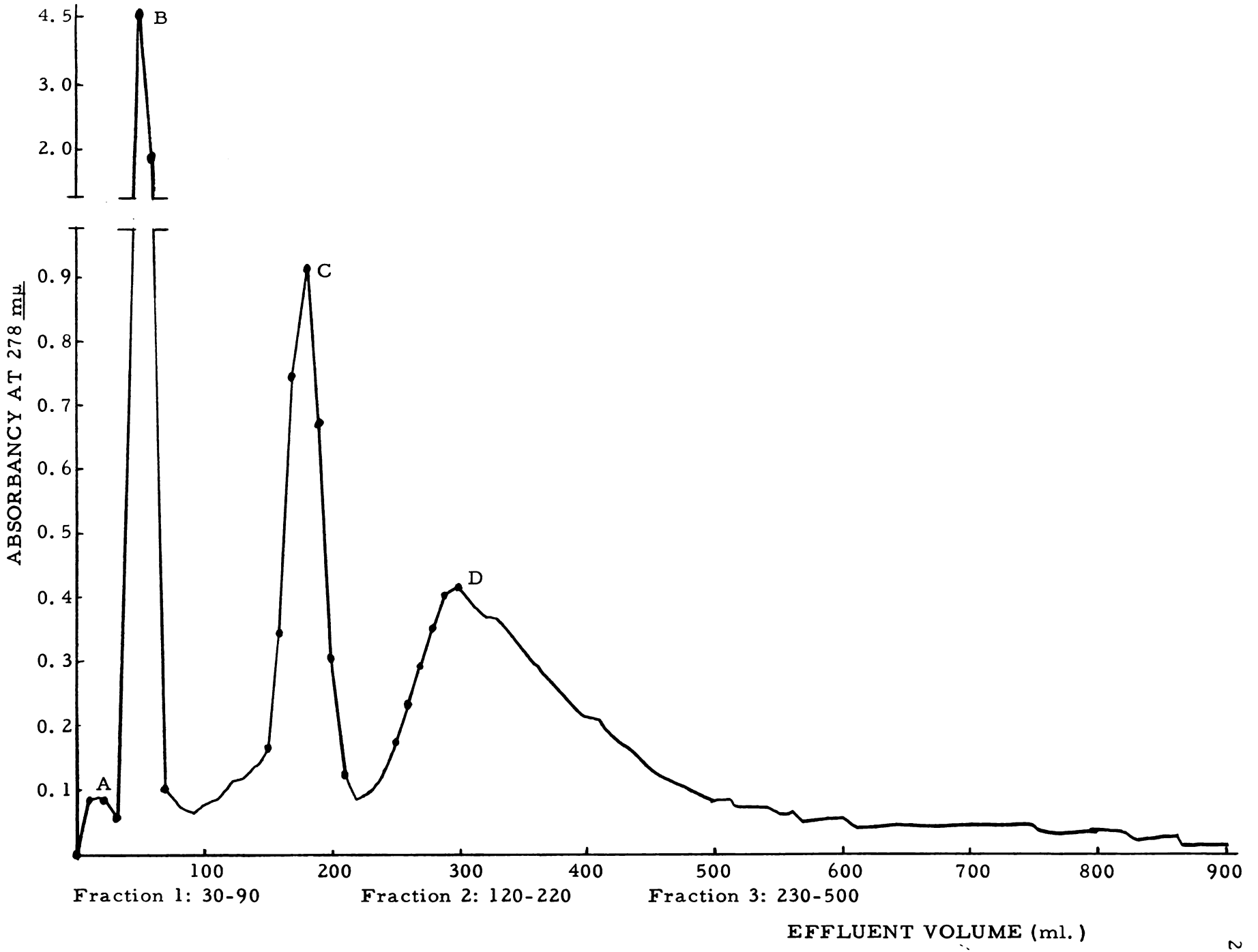


Figure 6. Gradient elution diagram of lactoglobulins from a DEAE-cellulose column.

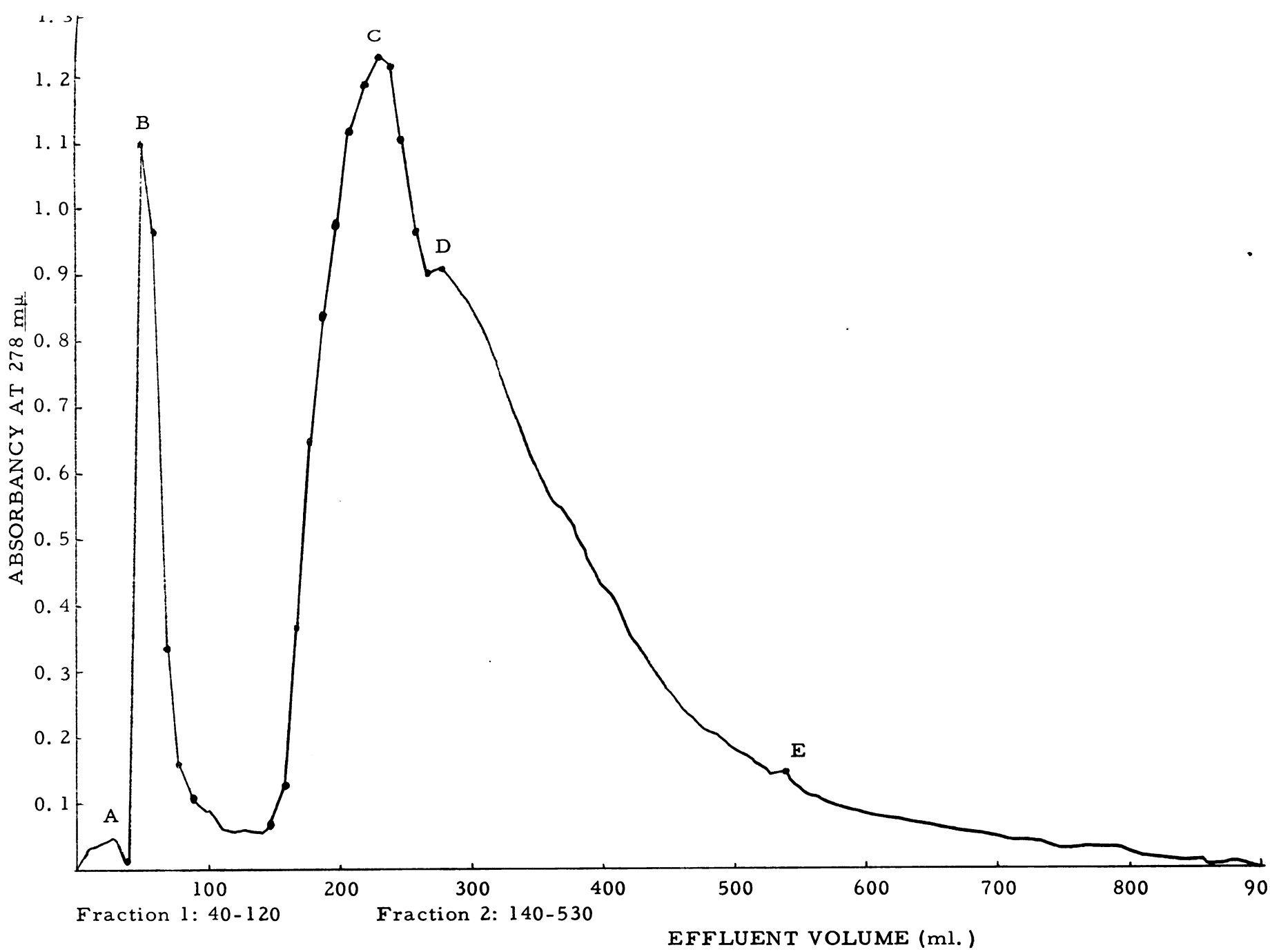


Figure 7. Gradient elution diagram of pseudoglobulin from a DEAE-cellulose column.

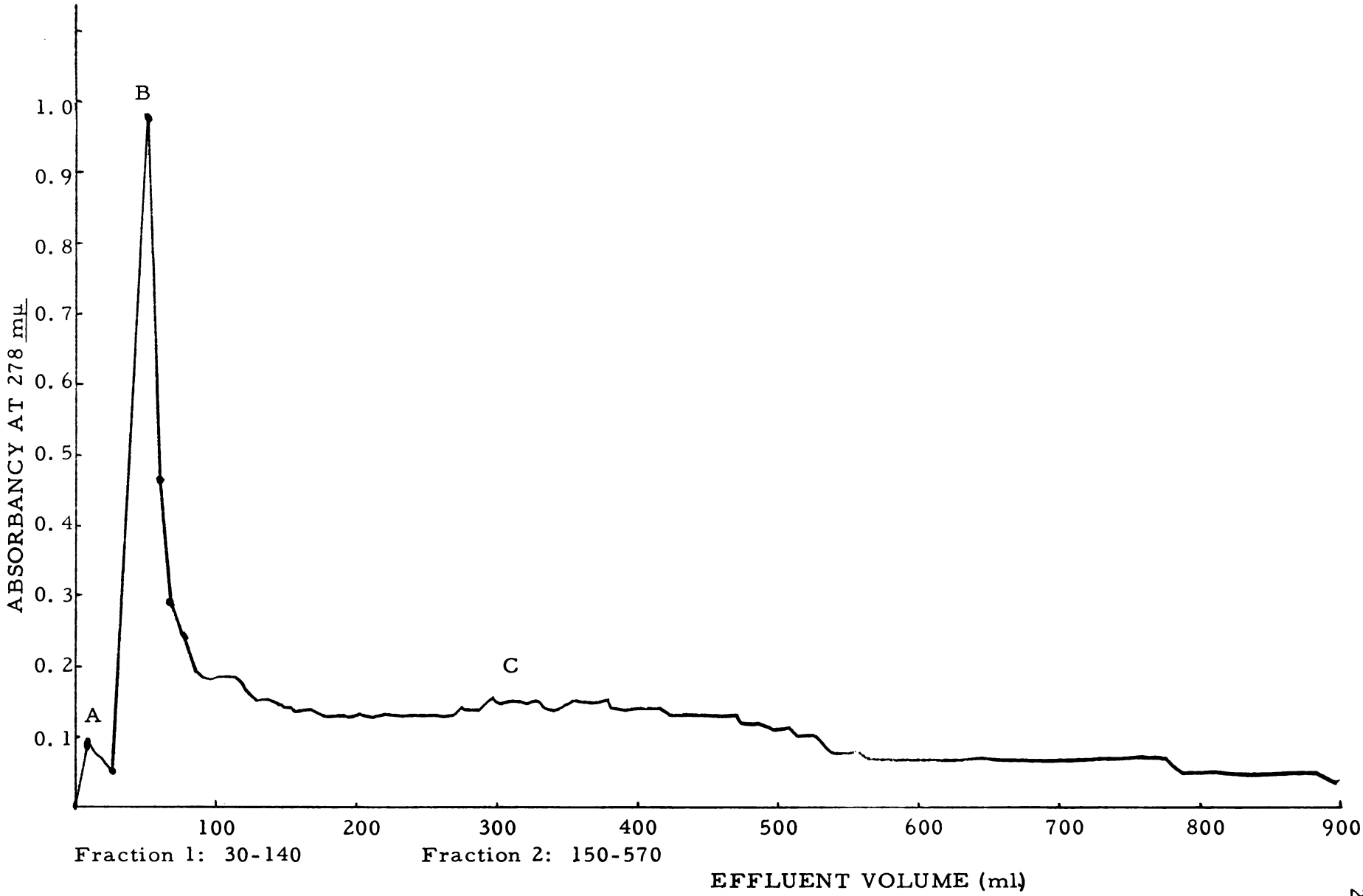


Figure 8. Gradient elution diagram of euglobulin from a DEAE-cellulose column.

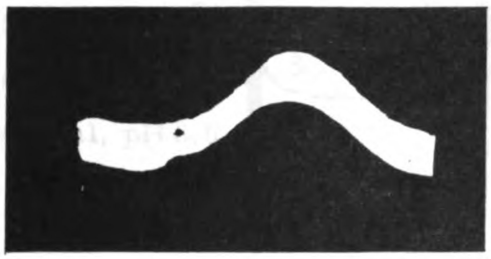
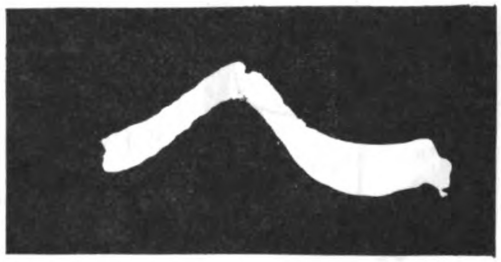
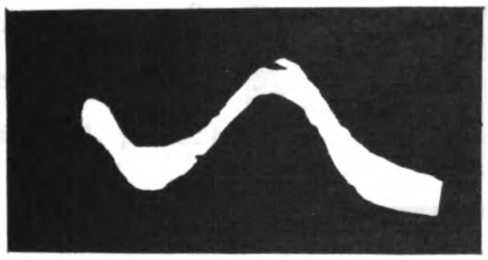
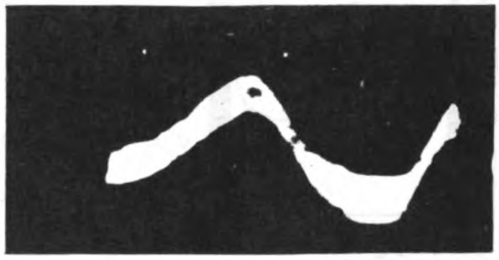
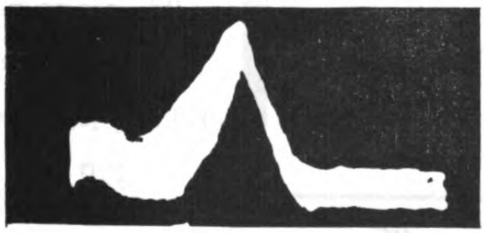
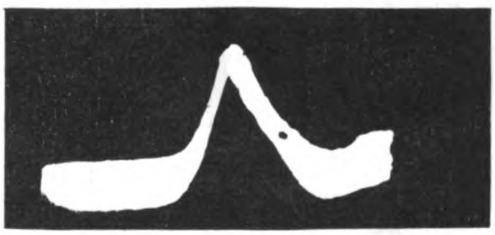
Ascending	Descending
Lactoglobulin Fraction:	
	
8800 sec.	12.1 volt cm.$^{-1}$
Pseudoglobulin Fraction:	
	
8100 sec.	12.1 volt cm.$^{-1}$
Euglobulin Fraction:	
	
8700 sec.	12.1 volt cm.$^{-1}$

Figure 9. Electrophoretic patterns of lactoglobulin fractions in veronal buffer, pH, $\Gamma/2 = 0.1$.

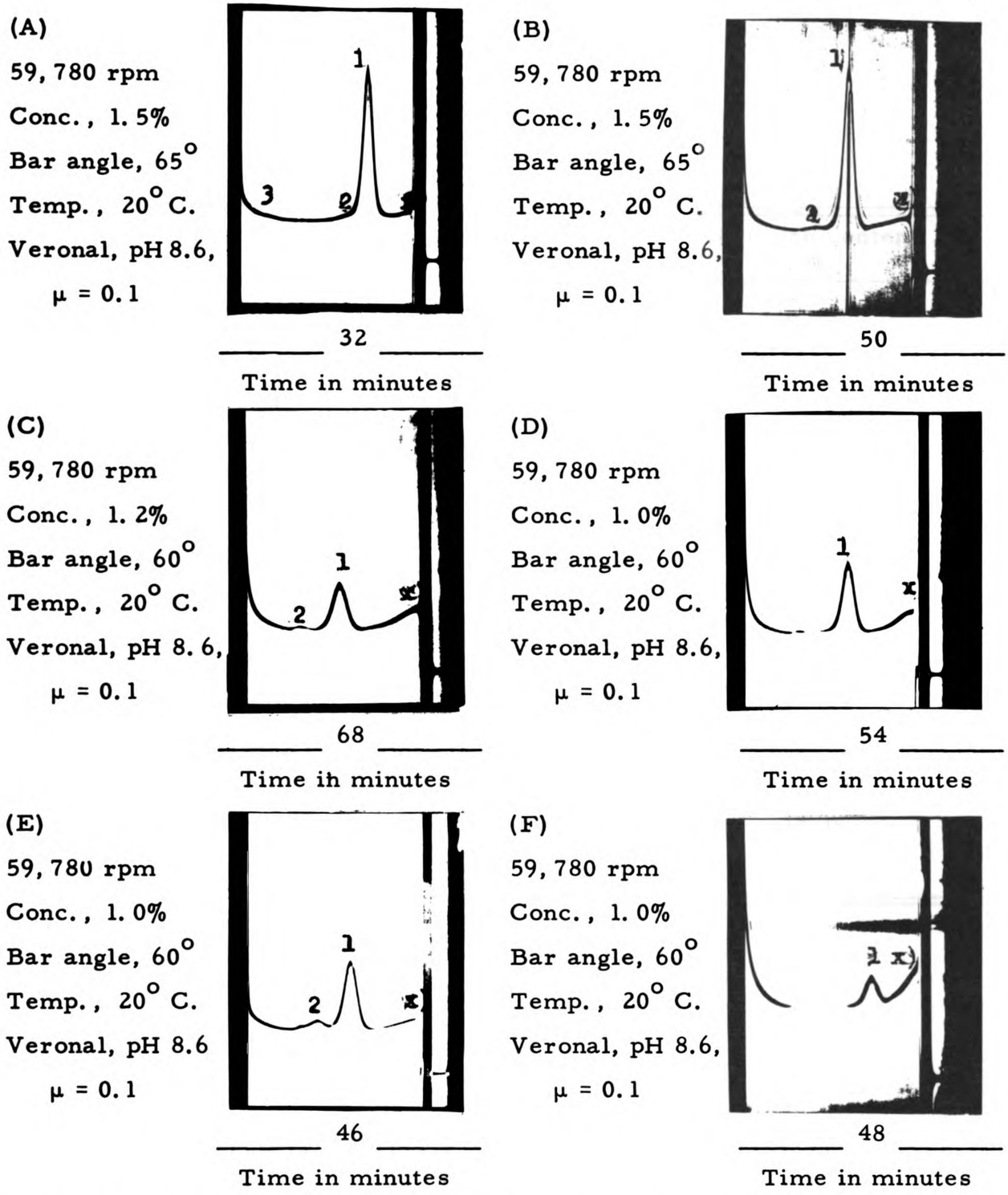


Figure 10. The ultracentrifugal diagrams of: (A) lactoglobulin, (B) pseudoglobulin, (C) pseudoglobulin fraction 2 from DEAE-cellulose column, (D) supernatant of dialyzed pseudoglobulin fraction 2 from DEAE-cellulose column, (E) precipitate of dialyzed pseudoglobulin fraction 2 from DEAE-cellulose column, (F) pseudoglobulin from DEAE-cellulose column minus fraction 2. x denotes protein of a slow sedimenting component which could not be measured.

TABLE I

Nitrogen content of lactoglobulin fractions of bovine milk and colostrum

Protein fraction[a]	Nitrogen content (%)
Milk	
Lactoglobulin	15.72
Pseudoglobulin	15.58
Euglobulin	15.83
Colostrum	
Lactoglobulin	15.68
Pseudoglobulin	15.16
Euglobulin	16.07

[a]Obtained by salting out with ammonium sulfate.

TABLE II

Electrophoretic mobilities of lactoglobulin fractions of bovine colostrum in veronal buffer, pH 8.6, $\Gamma/2 = 0.1$, at 1° C.

Protein fraction[a]	Concentration (%)	Electrophoretic mobility (μ)[b] Descending	Ascending
Lactoglobulin	1.5	-1.96	-2.13
Pseudoglobulin	1.5	-1.93	-2.09
Euglobulin	0.8	-1.68	-1.75

[a]Obtained by salting out with ammonium sulfate.

[b]μ = $cm.^2\ v.^{-1}\ sec.^{-1} \times 10^{-5}$

TABLE III

Sedimentation-velocity coefficients (S_{20}) for lactoglobulins of bovine colostrum in veronal buffer, pH 8.6 $\Gamma/2 = 0$.

Protein fraction	Concentration (%)	Sedimentation-velocity coefficients ($S_{20} = s_{20} \times 10^{-13}$ sec.) Peaks		
		1	2	3
Lactoglobulin[a, b]	1.5	6.10	9.50	20.63
Pseudoglobulin[a, b]	1.5	6.83	9.68	-
Pseudoglobulin fraction 2 from DEAE-cellulose column[b]	1.2	6.15	9.34	-
Supernatant of the dialyzed pseudoglobulin fraction 2 from DEAE-cellulose column[b]	1.0	6.43	-	-
Precipitate of the dialyzed pseudoglobulin fraction 2 from DEAE-cellulose column[b]	1.0	6.72	10.06	-
Pseudoglobulin from DEAE-cellulose column without fraction 2[b]	1.0	6.81	-	-
Euglobulin[a, b]	0.7	7.11	9.85	20.86

[a] Obtained by salting out with ammonium sulfate.

[b] These fractions contain one very slow sedimenting component which could not be measured.

TABLE IV

Carbohydrate content of the lactoglobulins of bovine colostrum

Protein fraction	Carbohydrate (mg./100 mg. protein)				
	Hexose	Hexosamine	Fucose	Neuraminic acid	Total
Lactoglobulin[a]	0.71	1.50	0.54	1.66	4.41
Lactoglobulin from DEAE-cellulose column (Figure 6)					
Fraction 1	1.42	1.93	0.61	1.81	5.77
Fraction 2	1.96	2.28	0.75	2.27	7.26
Fraction 3	1.50	2.43	0.84	2.57	7.34
Pseudoglobulin[a]	0.84	2.15	0.73	2.12	5.84
Pseudoglobulin from DEAE-cellulose column (Figure 7)					
Figure 1	1.20	3.15	0.98	3.18	8.51
Figure 2	1.44	1.15	0.49	1.51	4.59
Euglobulin[a]	3.24	2.56	0.86	2.80	9.46
Euglobulin from DEAE-cellulose column (Figure 8)					
Fraction 1	3.90	3.00	0.89	3.03	10.82
Fraction 2	2.90	2.40	0.77	2.34	8.41

[a]Obtained by salting out with ammonium sulfate.

DISCUSSION

In this study the term "lactoglobulins" was applied to the components of colostrum with an electrophoretic mobility ranging from -1.8 to -2.2.

Isolation of Lactoglobulins

In the comparison of the four different methods of fractionation and isolation of lactoglobulins, the best results were obtained by the procedure of Smith (1946b), as outlined in Figure 1. Fraction F which represents the lactoglobulin appeared to be electrophoretically homogeneous. Pseudoglobulin and euglobulin fractions obtained from Fraction F upon dialysis showed electrophoretically homogeneous characteristics. The pseudoglobulin, Fraction E, was also electrophoretically homogeneous. However, the yields of these proteins from bovine milk were too small, and large quantities of milk had to be used to obtain sufficient amounts for further studies.

In the isolation of lactoglobulins from colostrum by salting-out with ammonium sulfate (Figure 2), only the pseudoglobulin fraction showed electrophoretically homogeneous characteristics. The lactoglobulin and the euglobulin fractions showed several peaks in their electrophoretic patterns. The euglobulin fraction was pinkish-gray in its appearance suggesting the presence of other proteins.

The sodium sulfate fractionation procedure as outlined in Figure 3 did not produce homogeneous preparations.

The Rivanol fractionation procedure (Figure 4) was simple to perform

and the fractions obtained were electrophoretically homogeneous. However, it was not possible to remove completely the clarifying charcoal which was carried in the lyophilized fractions and therefore obviating further analytical analysis. This method offers a simple procedure for obtaining lactoglobulins if a substance other than charcoal could be employed for the removal of the Rivanol.

To obtain sufficient quantities of electrophoretically homogeneous components, the lactoglobulins were prepared from colostrum by the method outlined in Figure 1. The lactoglobulin preparation designated Fraction F was used throughout this study. Figure 9 presents the electrophoretic patterns of this fraction and its components.

Fractionation of Lactoglobulin on DEAE-Cellulose Columns

Studies in the analytical ultracentrifuge have revealed that the lactoglobulin preparations contained more than one component. In this phase of the study an attempt was made to separate as many components of the lactoglobulin fraction as possible.

The chromatograms presented here (Figures 6-8) demonstrate the separation of lactoglobulins into constituent components by following their elution from a DEAE-cellulose column. The chromatogram for acid whey proteins (Figure 5) is in close agreement with the elution diagram presented by Yaguchi, Jennings, and Tarassuk (1959). Figure 6 shows the resolution of electrophoretically homogeneous lactoglobulin fraction into four distinct peaks, designated A to D in the order of their elution.

Similarly, Figure 7 shows the resolution of an electrophoretically homogeneous pseudoglobulin fraction into five distinct peaks, designated A to E. Data from this diagram suggest that areas under peaks C and D were the main components of the pseudoglobulin fraction. Counterparts of these same peaks appear in the chromatogram for lactoglobulin (Figure 6). However, in this instance they appear as separate peaks, an observation attributed to the smaller protein concentration. The small peak E in Figure 7 was reproducible in every fractionation and therefore evidenced to be a distinct component. In Figure 8 the electrophoretically homogeneous euglobulin fraction was resolved into two distinct peaks (A and B) and a relatively flat region (C). This region seems to cover many proteins or various states of aggregation of constituent proteins having similar affinities for this adsorbent. This flat region is masked by the two distinct peaks C and D of pseudoglobulin in the lactoglobulin chromatogram (Figure 6). Area under peak A in all chromatograms appears to be proteins not adsorbed on the column. A very sharp peak B is present on all chromatograms.

An interesting observation was made upon prolonged dialysis of pseudoglobulin Fraction 2 from the DEAE-cellulose column. The fraction separated into a water insoluble (white precipitate) and a water soluble component. This suggested that one of the components plays the role of a stabilizer in the pseudoglobulin complex. It is also possible that the column partially denatured the pseudoglobulin complex. A similar observation was made with the lactoglobulin Fraction 2 from DEAE-cellulose column.

Analysis of Fractions

Nitrogen content. The results in Table I are in close agreement with previously reported nitrogen values (Smith, 1946a, b).

Electrophoretic mobilities. The mobility of lactoglobulin was approximately the same as observed by Smith (1946a), -2.1; whereas that of pseudoglobulin was different from his value of -2.2 (1946b). Murthy et al. (1958) report a value of -2.04. The mobility of euglobulin conforms with the data reported by Murthy et al. (1958), -1.76; and that reported by Smith (1946b), -1.80.

Ultracentrifugal characteristics. The data listed in Table III show that all of the protein preparations contained several ultracentrifugal sedimentation boundaries. The values observed for lactoglobulin were approximately the same as reported by Deutch (1947), namely, 6, 10 and 20 Svedberg units. Pseudoglobulin yielded two values which were in close agreement with Smith's (1946a) values of 7 and 10 Svedberg units. Murthy et al. (1958) reported 7.69 and 10.7 Svedberg units. The differences can be accounted for by the different protein concentrations used in this study as well as the manner in which the experimental data were corrected. Euglobulin yielded three boundaries with sedimentation-velocity coefficients in close agreement with Smith's (1946b) values of 7, 10 and 20 Svedberg units but lower than those observed by Murthy et al. (1958), namely, 7.93, 11.85 and 22.98 Svedberg units. The lactoglobulin fractions contained one slow sedimenting boundary which could not be measured, because it did not move far enough to make a

measurement. Smith (1946b) reported this particular component only in the pseudoglobulin fraction.

The purified pseudoglobulin, Fraction 2 from the DEAE-cellulose column showed sedimenting boundaries which were a little slower than those observed in the salted out pseudoglobulin.

Sedimentation-velocity coefficients for the components of the pseudoglobulin Fraction 2, DEAE-cellulose column, indicated that the supernatant of dialyzed pseudoglobulin has only one principal component instead of the two values reported for the entire pseudoglobulin fraction. However, the precipitate of the same dialyzed pseudoglobulin Fraction 2 yielded two values. This can be interpreted as an indication that the column plays a role in the dissociation of the pseudoglobulin complex.

Carbohydrate content. Smith (1946b) and Smith, Greene and Bartner (1946) presented analytical data for hexose and hexosamine. They reported higher values for hexose in pseudoglobulin, 2.52, and lower values for hexosamine in euglobulin and pseudoglobulin; 1.58 and 1.52, respectively.

SUMMARY

This study was initiated for the purpose of isolating lactoglobulin fractions, separated into as many components as possible, and to determine the nature and location of the chemically bound carbohydrates. Lactoglobulins (whole lactoglobulins, i.e. pseudoglobulins and euglobulins) were fractionated from colostrum employing the method described by Smith (1946b) for bovine milk. These fractions were further separated into their constituent components on a DEAE-cellulose column. The lactoglobulin fraction was separated into four components, the pseudoglobulin into five, and the euglobulin into two distinct peaks and one relatively flat region consisting of many proteins having similar affinities for the adsorbent. The area measured under peak A seemed to represent proteins not adsorbed on the column. A sharp peak B is also characteristic for all chromatograms. Proteins comprising the areas of peaks C and D were the main components of the pseudoglobulin fraction. These two peaks in the lactoglobulin diagram seem to cover up the flat region of the euglobulin. Component E of the pseudoglobulin fraction did not appear distinctly in the lactoglobulin chromatogram probably because of the relatively lower concentration.

When pseudoglobulin Fraction 2 from the DEAE-cellulose column was dialyzed against distilled water, it separated into water insoluble and water soluble components. This suggested that one of the components plays the role of a stabilizer in the pseudoglobulin complex. It is also

possible that the column contributed to the denaturation of the pseudoglobulin. The water soluble component was found to be the principal component and had but one sedimenting boundary at 6.4 Svedberg units.

The analytical ultracentrifugal analysis showed that lactoglobulins contained a principal component of about 7 Svedberg units, and a second component of about 10 Svedberg units. The lactoglobulin and euglobulin fractions both showed a component of 10 Svedberg units. The lactoglobulin and euglobulin fractions both showed a component of about 20.5 Svedberg units. The pseudoglobulin fraction showed components with values of 6.8 and 9.7 Svedberg units. Further, all three fractions possessed one very slow sedimenting component.

Carbohydrates were found to be present in all lactoglobulin fractions and their components. The highest carbohydrate content was in the euglobulin fraction, 9.46 per cent. Pseudoglobulins had a total carbohydrate content of 5.84 per cent. The concentrations of hexose, hexosamine, fucose and neuraminic acid were found highest in the euglobulin fraction. The fractions obtained from the fractionation on the DEAE-cellulose column showed a different distribution of carbohydrates. The concentration of hexose was the highest in the euglobulin in Fraction 1--3.90 per cent. The pseudoglobulin Fraction 1 possessed the highest values for hexosamine, fucose and neuraminic acid; 3.15, 0.98, and 3.18 per cent, respectively.

CONCLUSIONS

The fractionation of bovine milk and colostrum showed that the best method for isolating electrophoretically homogeneous lactoglobulin components was achieved through the salting out procedure with ammonium sulfate (Figure 1).

Fractionation of the electrophoretically homogeneous lactoglobulin fractions on the DEAE-cellulose column resulted in the resolution of these fractions into several components. Thus, the lactoglobulin fraction was separated into four components; the pseudoglobulin into five components; the euglobulin into two distinct components and a fraction consisting of several proteins having similar affinities for the adsorbent.

The main component of pseudoglobulin fraction from the DEAE-cellulose column, when dialyzed against distilled water, separated into water insoluble and water soluble components. This suggested that one of the pseudoglobulin components emerging from the DEAE column plays the role of a stabilizer for the pseudoglobulin complex, or, that the column partially denatured the pseudoglobulin complex.

Studies in the analytical ultracentrifuge revealed that all lactoglobulin fractions contained several sedimenting boundaries. The pseudoglobulin fraction, when separated on the DEAE-cellulose column, showed a component, which, after dialysis, had only one sedimenting boundary.

Carbohydrates were found in all lactoglobulin fractions and their components. Euglobulins had the highest carbohydrate content.

Pseudoglobulin Fraction 1 from the DEAE-cellulose column showed the highest concentration of hexosamine, fucose and neuraminic acid.

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