

PREPARATION OF SOME IRON-BINDING PROTEINS AND α -LACTALBUMIN FROM BOVINE MILK

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SUMMARY

The preparation of the iron-binding milk proteins—"red protein," blood transferrin, and lactoperoxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7)—by chromatography on DEAE-cellulose and phosphocellulose is described. The "red protein" is distributed in the casein and whey fractions of milk. In some fractions it forms complexes with other proteins which drastically change its chromatographic behavior. The transferrins found in the milk and blood of an individual cow are shown to be the same by gel electrophoresis. The preparation of an electrophoretically homogeneous α -lactalbumin by chromatography on DEAE-cellulose is also described.

INTRODUCTION

The "red protein" (also called lactotransferrin¹), blood transferrin, and lactoperoxidase (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) are iron-binding proteins found in milk. These proteins have recently attracted considerable attention, as indicated by the variety of procedures developed for their purification. For example, the "red protein" was prepared from acid-precipitated casein by GROVES² while GORDON *et al.*³ isolated a similar protein from rennet whey. DERECHIN AND JOHNSON⁴ fractionated the "red protein" and blood transferrin by chromatography of the albumin fraction of whey on DEAE-cellulose, and MORRISON AND HULTQUIST⁵ prepared lactoperoxidase and the "red protein" by adding to milk a resin that preferentially combines with these two basic proteins.

The present study is an extension of previous work on the separation of these iron-binding proteins by the use of ion exchange resins. Their distribution in the casein and whey fractions of milk will be described. By means of these procedures, it is also possible to isolate α -lactalbumin purer by the criterion of gel electrophoresis than that prepared by extensively used methods^{6,7} involving salt fractionation and recrystallization.

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EXPERIMENTAL PROCEDURE AND RESULTS

Fractionation of the milk

Milk from individual cows* was passed through a cream separator and the casein in the skim milk was then precipitated at pH 4.6. The whey was separated from the casein by filtration through a cloth bag, and the whey proteins were precipitated by saturation of the whey with ammonium sulfate. The precipitate was then dialyzed against distilled water at 3° until free of ammonia, and a precipitate was removed by centrifugation at 25°. The precipitate and supernatant, hereafter referred to as Whey P and Whey F, were then lyophilized and stored at 3°. The ratio of the Whey F to Whey P proteins was generally higher for milk containing BB β -lactoglobulin than the ratio 3-1 for milks with AA and AB β -lactoglobulin.

Electrophoresis

Disc electrophoretic determinations were made in the standard 7.5 % acrylamide gel⁸ (pH 9.5) and also in 7.5 % gel⁹ at pH 4.3. In all the pH-9.5 gels the marker dye, a sharp band that migrates ahead of the proteins, is found near the bottom of the gels.

Chromatography

The use of DEAE-cellulose has been described². Phosphocellulose with a capacity of 0.96 mequiv/g was obtained from the Gallard and Schlesinger Chemical Corp., Garden City, N.J.** (U.S.A.). The resin was prepared for chromatography by washing on a Buchner funnel with 0.1 N sodium hydroxide, followed by water, then suspended in water and decanted to remove fines. It was finally washed on a funnel with 0.1 N HCl followed by water. After use it was also regenerated in the same manner. For chromatography, the resin was suspended in a 0.05 M sodium phosphate buffer (pH 5.0) and adjusted to pH 5.0. Chromatography was carried out at 3° on columns 2 × 28 cm packed at 8 lbs./in² pressure and equilibrated with the starting buffer. In a few instances, a larger column, 3.5 × 25 cm, was used with DEAE-cellulose to accommodate larger samples. For chromatography, the protein was first dissolved in water, adjusted to the desired pH with dilute alkali, and lyophilized. A concentrated solution was then prepared by dissolving the lyophilized protein in the starting buffer and dialyzing at 3° for 16 h. After elution from the column, the various fractions were combined, dialyzed free of salt, and lyophilized.

Whey-F fraction

Stepwise elution of the Whey-F fraction on DEAE-cellulose is shown in Fig. 1. Most of the protein, presumably pH denatured β -lactoglobulin, remained on the column. Fraction 1F was greenish red in color and contained among other proteins the "red protein" and lactoperoxidase. Fraction 2F moved down the column as a light red band and contained only the "red protein"; gel electrophoresis at alkaline pH showed its pattern to be identical to the purified "red protein". Blood transferrin in small amounts was found in tubes 60-90 of Fraction 3F. In some chromatograms the 0.01 M

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** It is not implied the U.S.D.A. recommends the above company or its product to the exclusion of others in the same business.

phosphate buffer step was eliminated; the transferrin and some "red protein" (2F) were then eluted in the first few tubes of the 0.025 M phosphate buffer peak. The major component in Fraction 4F was α -lactalbumin.

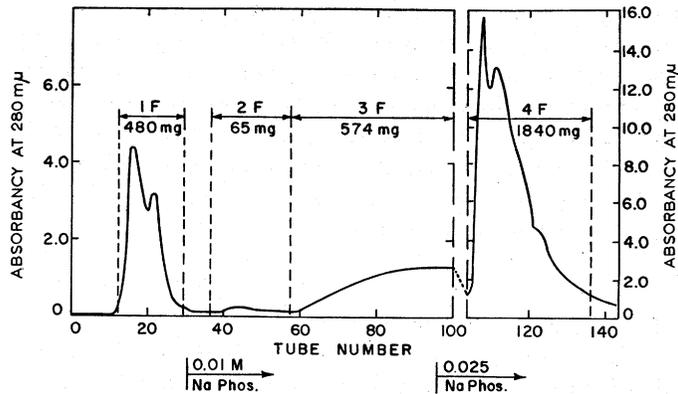


Fig. 1. Typical chromatograph of the Whey-F fraction on DEAE-cellulose at pH 8.2. Starting buffer 0.005 M sodium phosphate (pH 8.2). 12 g protein in 80 ml were applied to a 3.5×25 cm column; effluent collected in 18-ml fractions at a rate of 36 ml/h. Combined fractions are numbered as indicated between dashed lines.

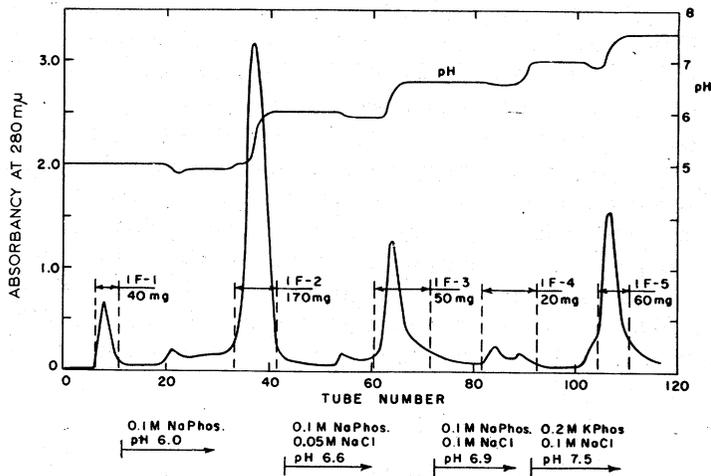


Fig. 2. Stepwise elution diagram of Fraction 1F on phosphocellulose with a starting buffer of 0.05 M sodium phosphate (pH 5.0). 0.48 g in 6 ml was applied to a 2×31 cm column; effluent collected in 12-ml fractions at a rate of 24 ml/h. Combined fractions are numbered as indicated between dashed lines.

Fraction 1F. Chromatography of the 1-F protein fraction (Fig. 1) on phosphocellulose is shown in Fig. 2 and disc electrophoretic patterns of the fractions in Fig. 3. The protein corresponding to Peak 1F-1 contained several proteins. The largest fraction was found in Peak 1F-2. Green fractions of lactoperoxidase were present in 1F-3 and 1F-4 although with one cow the protein in 1F-3 was colorless. The purified "red protein" was found in the 1F-5 fraction.

Good resolution with the pH 9.5 gel is shown for Fraction 1F-1 and 1F-2; the latter shows four bands indicated by numbers in Fig. 3. Lactoperoxidase (1F-3) and the "red protein" (1F-5) are poorly resolved in this system.

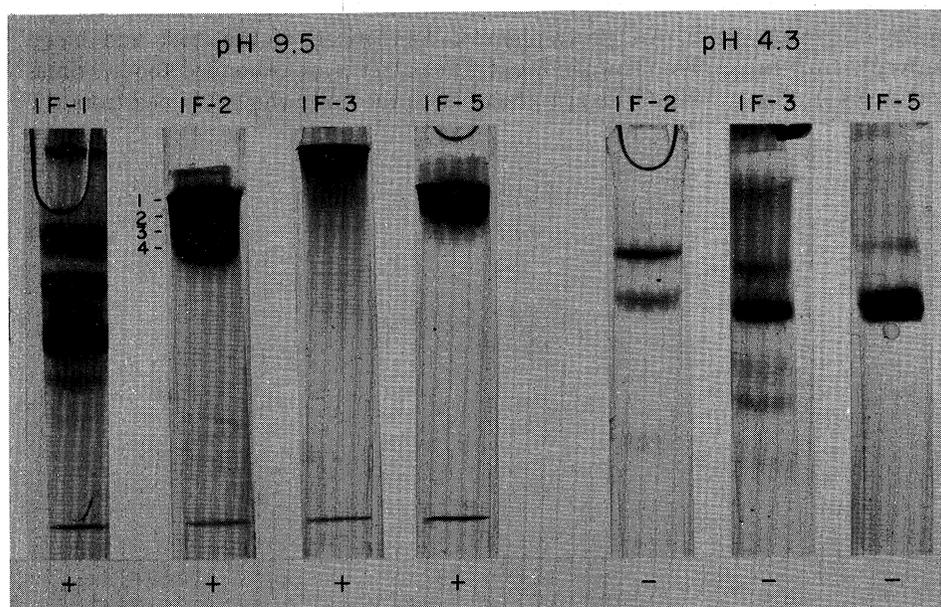


Fig. 3. Disc electrophoretic patterns of fractions shown in Fig. 2. 1F-3, lactoperoxidase; 1F-5, "red protein". The bands are indicated by numbers for 1F-2.

Disc electrophoresis at acid pH revealed two bands for the 1F-2 fraction, while the "red protein" (1F-5) had three closely related bands together with a minor slow moving zone. Electrophoresis of the apoprotein, prepared by removal of the iron by means of acid, gave a similar pattern, indicating that iron does not contribute to differences in mobility under these conditions. The lactoperoxidase fraction (1F-3) revealed several zones together with some material that did not move into the lower gel. Peroxidase measurements on these gels showed activity in the protein corresponding to the major band and also in the smaller slower moving bands.

Fraction 2F. Since Fraction 2F contained only the "red protein," as determined by gel electrophoresis, it was not rechromatographed.

Fraction 3F. The fraction containing transferrin, 3F (Fig. 1), 0.57 g, was chromatographed on a 2×30 cm phosphocellulose column with a starting buffer of 0.05 M sodium phosphate (pH 6.0). This was followed by buffer changes of 0.1 M sodium phosphate (pH 6.0) and 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride. The bulk of the protein (0.46 g) was eluted with the starting buffer while the transferrin was eluted with the 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride and amounted to 5 mg. Disc electrophoretic patterns of the two fractions are shown in Fig. 4 together with the original material put on the column. Although the transferrin fraction contained a few minor contaminants, as shown by disc electrophoresis, the preparation was quite good consid-

ering the fact that only about 1% of the protein was in the starting material. The transferrin shown contains three bands migrating close together.

In order to compare the electrophoretic pattern of the transferrins found in milk with those in the blood serum of an individual cow, Dr. KIDDY kindly furnished some samples of bovine blood. An electrophoretic comparison of the milk and blood transferrins from two cows is shown in Fig. 5. The patterns are magnified about 7 times those shown in Fig. 4. Transferrin rich fractions of the milk were obtained from the

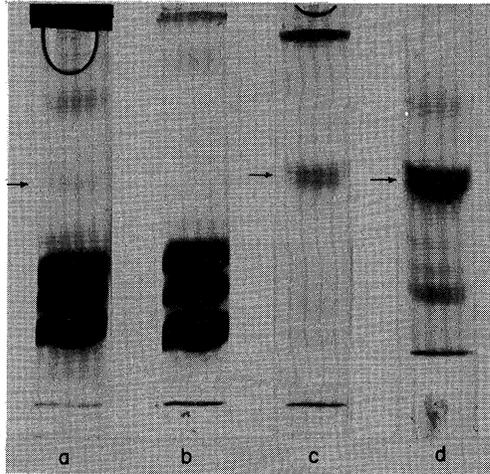


Fig. 4. Disc electrophoretic gels (pH 9.5) of the transferrin fraction 3F (Fig. 1) before and after chromatography on phosphocellulose. a, 3F before phosphocellulose; b, first fraction from phosphocellulose; c, transferrin fraction; d, same as c except higher protein concentration and shorter electrophoretic time. The transferrin is indicated by the arrow.

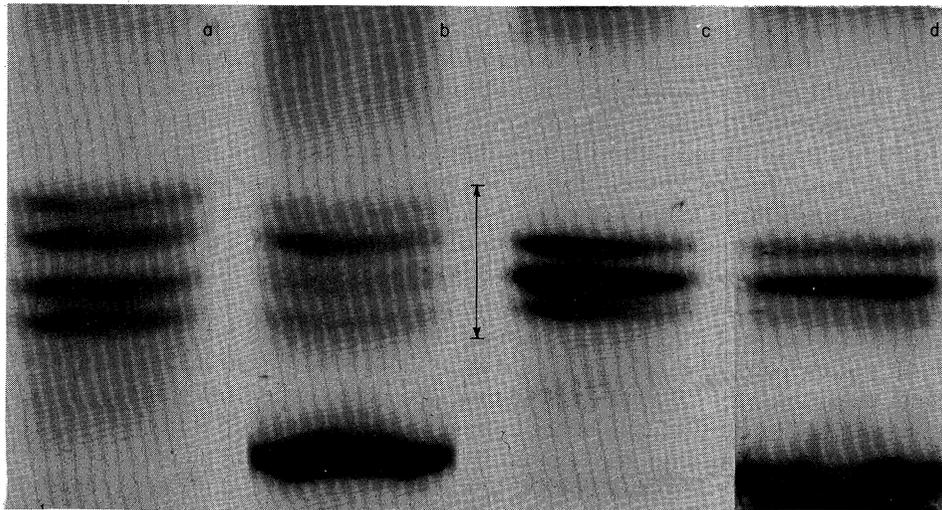


Fig. 5. Gel electrophoretic comparisons (pH 9.5) of transferrins (indicated by the vertical arrow) found in the milk and blood of individual cows. The transferrin fraction from milk was obtained by chromatography of Whey F on DEAE-cellulose. a, Cow A, blood serum; b, Cow A, whey fraction; c, Cow B, blood serum; d, Cow B, whey fraction.

DEAE-cellulose column were used. With Cow A, 4 similar bands are present in both the milk and serum; however, there appears to be a larger space between the two center bands for the blood transferrin than for the corresponding protein in milk. With Cow B, the 3 transferrin bands found in the serum are identical to those in the milk. The transferrins for 3 other cows were also examined and found to be identical for both the blood and milk of the same cow.

Fraction 4F. Rechromatography on DEAE-cellulose of the 4-F fraction is shown in Fig. 6 together with disc electrophoretic patterns of the fractions (Fig. 7). The Whey-F fraction and α -lactalbumin (C), prepared by salt fractionation and recrystallization, are also included for comparison. The photographic reproduction of C in Fig. 7

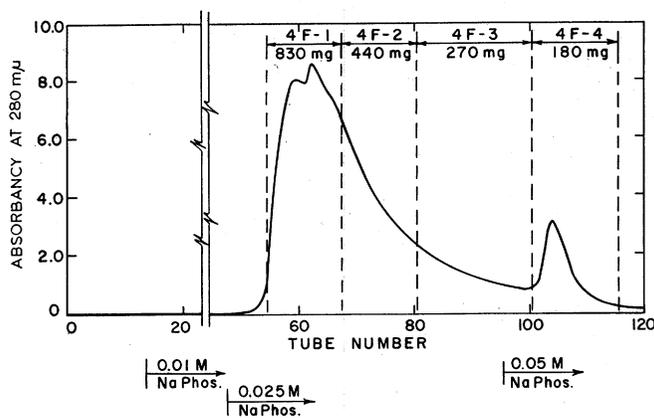


Fig. 6. Elution diagram of the α -lactalbumin-rich Fraction 4F (Fig. 1) when rechromatographed on DEAE-cellulose at pH 8.2. Starting buffer 0.005 M sodium phosphate (pH 8.2). 1.84 g protein in 27 ml was applied to a column 2×34 cm; effluent collected in 16-ml fractions at a rate of 32 ml/h. Combined fractions are numbered as indicated between dashed lines.

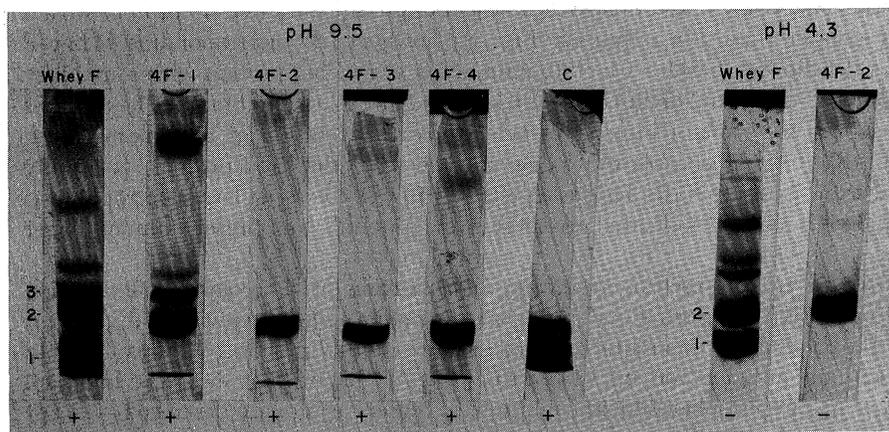


Fig. 7. Disc electrophoretic patterns of α -lactalbumin fractions shown in Fig. 6. α -Lactalbumin (C) prepared by salt fractionation and recrystallization and Whey F are also shown for comparison. The numbers identify the following proteins: 1, β -lactoglobulin A, B; 2, α -lactalbumin; 3, serum albumin.

exaggerates the amount of faster moving material actually seen in the gel. As shown by disc electrophoresis at both acid and alkaline pH, the best preparation of α -lactalbumin was fraction 4F-2 (0.44 g), representing 25 % of the recovered material. Two fractions (4F-3 and 4F-4) amounted to another 25 % of the material, and their electrophoretic patterns showed only a small amount of slower moving impurity.

Whey-P fraction

The Whey-P fraction was chromatographed on a 2×30 cm DEAE-cellulose column equilibrated with 0.005 M sodium phosphate buffer (pH 8.2). A concentrated opaque solution, 5.1 g in 55 ml, was applied to the column after centrifugation to remove a small amount of insoluble material (0.10 g dry). Aliquots of 12 ml of the eluate were taken at a rate of 24 ml/h. The distribution of the protein in the various fractions is shown in Table I. The first and largest fraction (1P) was opaque on elution but was not colored, as was found with the corresponding Whey-F fraction. With the

TABLE I

DISTRIBUTION OF PROTEINS FROM THE WHEY-P FRACTION AFTER CHROMATOGRAPHY ON DEAE-CELLULOSE

<i>Elution buffer</i>	<i>Fraction number</i>	<i>Protein weight (g)</i>
0.005 M Sodium phosphate (pH 8.2)	1P	1.32
0.01 M Sodium phosphate (pH 8.2)	2P	0.11
0.025 M Sodium phosphate (pH 8.2)	3P	0.80
0.05 M Sodium phosphate (pH 8.2)	4P	0.94
0.10 M Sodium phosphate (pH 8.2)	5P	0.81
0.10 M Sodium phosphate (pH 6.0) 0.3 M NaCl	6P	0.54

Whey-P fraction, the 0.01 M phosphate eluate (2P) contained a small amount of transferrin. The Whey-F fraction, rich in α -lactalbumin, contained no protein with a mobility greater than α -lactalbumin (Fig. 7); however, the corresponding Whey-P fraction (3P) showed, besides α -lactalbumin, a faster moving protein with a mobility corresponding to β -lactoglobulin and similar in amount to α -lactalbumin. The proteins present in 4P were not of interest in this study. The elution of a salmon-colored fraction (5P) at 0.1 M phosphate was unexpected since the "red protein" is usually eluted at a low ionic strength. The last fraction (6P) was colorless. Disc electrophoresis of Fraction 5P and 6P indicated that the "red protein" was present among other proteins.

Fraction 5P. On rechromatography of a portion of the colored 5-P fraction (0.50 g) on DEAE-cellulose at pH 6.0 with a combination of stepwise and gradient elution, the colored fraction required a higher ionic strength than 0.1 M phosphate for elution and amounted to 100 mg. Chromatography of this fraction dissolved in 2.5 ml on a 0.9×49 cm phosphocellulose column with buffers as already described—except with a 0.05 M phosphate starting buffer (pH 6.0)—gave essentially two fractions. A colorless fraction amounting to 51 mg (5P-5), identical to the purified "red protein" by disc electrophoresis was obtained where the "red protein" was normally eluted.

Fraction 6P. The eluate of the 6-P fraction from DEAE-cellulose, as mentioned earlier, was not colored; however, chromatography of this fraction on phosphocellulose gave results similar to that of Fraction 5P. From an opaque solution of 460 mg protein in 16 ml applied to a 2×31 cm column, 0.314 mg (6P-1) was eluted with the starting buffer and 41 mg (6P-5) of the colored "red protein" were obtained.

Fractions 5P-1 and 6P-1 evidently complex with the "red protein" and drastically change its chromatographic behavior. Disc electrophoresis of the two revealed a large number of similar bands and phosphorus determination on the 6P-1 fraction gave a value of 1.5 percent.

DISCUSSION

The "red protein" apparently can form complexes or become associated with other proteins. It is found in significant amounts in both the acid-precipitated casein fraction and in the whey fraction of milk. When the "red protein" from the casein or the Whey-F fraction is chromatographed on DEAE-cellulose, it is eluted from the column at a low ionic strength, as would be expected for a basic protein. On chromatography of the Whey-P fraction, however, the "red protein" requires a high ionic strength for elution. Evidently in this fraction the basic "red protein" is complexed with an acidic fraction due to the high phosphorus content and consequently requires high ionic strength for elution. The "red protein" in the complex can be resolved with a cation exchanger.

Since a colorless protein closely resembling the "red protein"^{3,10} and capable of binding iron has been isolated from milk, it was not surprising to find this protein in some of the fractions herein described. On chromatography of the Whey-P fraction with DEAE-cellulose, the eluate of Fraction 5P (Table I) is salmon-colored while Fraction 6P is essentially colorless. After chromatography on phosphocellulose, about the same amount of colored (red) protein is obtained (40 mg) from each fraction. This suggests that the iron-binding protein in Fraction 6P eluted from DEAE-cellulose does not bind iron, perhaps because of the complex. When the complex is broken up with phosphocellulose, the protein is then able to bind iron. Since the complexing fractions in both 5P and 6P are similar, as determined by disc electrophoresis, it is not clear why one and not the other would bind iron. It is interesting to note that on fractionation by the phosphocellulose method, only the colored (red) protein is obtained and not the apoprotein. This was found when iron-free protein was prepared and chromatographed. Presumably, there is enough iron impurity in the buffer to combine with the protein under these conditions.

The "red proteins" isolated from the various protein fractions of milk obtained from an individual cow reveal no differences in gel electrophoretic behavior. However, there are small but significant differences in electrophoretic patterns of the "red protein" obtained from different cows, and this will be the subject of a further communication.

The lactoperoxidase fractions are not pure. Based on the ratio of the absorbancy at 412 to 280 $m\mu$ of 0.9 for the pure protein, they contain 30-40 % lactoperoxidase. Further purification of these fractions will be required. Most of the lactoperoxidase in skim milk is found in the Whey-F fraction, while only a small amount is associated with the "red protein" in the casein fraction.

The genetic studies on β -globulin (transferrin) polymorphism in cattle by starch gel electrophoresis of blood serum show six phenotypes^{11,12}. Although transferrins in the blood serum from the cows in this report differ in phenotypes, the transferrins in the milk and blood serum from the same cow are identical, or closely related, as determined by disc electrophoresis. This is consistent with the finding of DERECHIN AND JOHNSON⁴ that transferrin isolated from pooled milk and from the blood serum of cows gives similar starch-gel electrophoretic patterns. Since the amount of transferrin found in milk is small and its genetic type is similar to that found in the blood, it is probable that the proteins have a common origin. Immunoelectrophoretic comparisons⁴ show that the transferrin prepared from pooled milk and blood are closely related.

Transferrin in milk is found in the whey where it is distributed between the Whey-F and Whey-P fractions.

The major impurity in the α -lactalbumin fraction obtained from the DEAE-cellulose column is serum albumin. On rechromatography of this fraction, most of the impurities are concentrated in the first part of the α -lactalbumin elution peak, the latter part containing electrophoretically pure α -lactalbumin. When paper electrophoretic determinations were used extensively to identify proteins, α -lactalbumins prepared by the usual methods always showed a minor protein moving just ahead of the main protein band which could not be removed by recrystallization or other fractionation procedures⁷. WETLAUFER¹³ also noted a "fast" lactalbumin by paper electrophoresis and found that the "fast" component isolated by electrophoresis had the same sedimentation coefficient and ultraviolet spectrum as the original material. With α -lactalbumin prepared by the ion exchange cellulose method, no "fast" component was found and attempts to produce it by exposing the protein to conditions similar to those used in regular fractionation procedures failed. This suggests that the minor fraction is an impurity and not a transformation product of the fractionation procedure.

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