

Isolation, Characterization, and Amino Acid Composition of a New Crystalline Protein, Lactollin, from Milk*

MERTON L. GROVES, JAY J. BASCH, AND WILLIAM G. GORDON

From the Eastern Regional Research Laboratory,[†] Philadelphia

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A new crystalline protein, named for convenience "lactollin," has been isolated from bovine milk. It is a simple protein with a unique amino acid composition. Lactollin contains no methionine and very little alanine; it is rich in aromatic amino acids and is a basic protein showing an excess of 19 cationic residues over anionic residues per 43,000 mol wt. Its point of minimum solubility is at pH 8. At pH 9.5 it has an electrophoretic mobility of -1.86 , and at pH 5.0 its sedimentation constant is 3.21 S. A significantly higher amount of lactollin is found in colostrum than in normal milk.

In isolation of the "red protein" (Groves, 1960) from bovine milk, a crystalline protein was found associated with the red fraction, but in very small amounts. We have now prepared enough of this new protein, in yields of 35–90 mg per 57 liters skimmed milk, to describe some of its properties and to determine its amino acid composition. For convenience, the protein has been named "lactollin."

EXPERIMENTAL

Isolation of Lactollin.—The preparation of partially purified lactollin, previously designated "crystalline protein," has been mentioned (Groves, 1960). The initial steps are summarized diagrammatically in Figure 1; in the subsequent chromatographic separation on DEAE-cellulose the lactollin was eluted immediately following the "red protein" peak. Often when a solution of the red fraction was prepared for chromatography, crystals were formed on dialysis against the starting buffer at 2°. These were harvested by centrifugation in the cold prior to chromatography of the supernatant. Spontaneous crystallization of the protein eluted from the column occurred in the collector tubes within a few hours at 25°, but yields were increased by cooling to 2°. After centrifugation the crystalline lactollin was suspended in water and lyophilized.

To determine whether colostrum would be a good source of lactollin, 6.5 liters of skimmed colostrum was fractionated as described. Since colostrum contains larger amounts of protein than normal milk, it was diluted with an equal volume of water before acid-precipitation of the casein. Also, the ammonium sulfate fractionation of the crude red fraction was repeated as an additional purification step. The yield of lactollin from the colostrum was almost four times the best yield obtained from normal milk and amounted to 38 mg (5.9 mg/liter) of crude crystals.

Final Purification.—For recrystallization, the protein was dissolved in dilute acetic acid at pH 5 to give a 1.5% solution, the pH was increased by the slow addition of 0.05 N sodium hydroxide until a slight turbidity developed at a pH of about 6.2, and the solution was stored overnight at 2°. The crystals were harvested by centrifugation. The supernatant was slowly adjusted to pH 8.2, and a second batch of crystals was obtained at 2°. The supernatant contained 0.4–0.5% protein and yielded more crystals on reworking.

Because there was no apparent difference between the first two crops of crystals, they were combined. For the final crystallization a number of samples were combined and dissolved to give a 1.6% solution in 0.05 M phosphate buffer, pH 5.0, to which a few drops of dilute acetic acid were added. The solution was dialyzed at 2° against 0.005 M sodium phosphate buffer, first at pH 6.0, then 7.0, and finally at pH 8.2. After centrifugation, the crystals were suspended in water, dissolved by the addition of dilute acetic acid to pH 5, dialyzed against several changes of distilled water, and recovered by lyophilization. The supernatant from the crystals prepared by this method contained only 0.1% protein. This method of crystallization is to be recommended. The concentration of lactollin may be estimated by use of the experimentally determined absorptivity at pH 5.0 of 16.5 at 280 m μ (1-cm light path, 1% solution).

In dissolving lactollin for recrystallization, it should be noted that dilute acetic acid was added to maintain a pH of about 5. A more acid pH may be harmful, since on recovery of the protein from an electrophoretic experiment at pH 3.5, only about half the protein could be recovered in the form of typical crystals.

Figure 2 shows lactollin crystals under 125 \times magnification. The photograph was taken of an early preparation, and some amorphous material is present.

Typical crystals can also be obtained by dissolving lactollin in glycine buffer at pH 9.5–10, followed by dialysis against water at 2°, although yields have not been determined by this method.

Methods.—Electrophoresis was carried out in a Tiselius-Klett apparatus as well as in starch gel in the presence of urea, as described earlier (Groves *et al.*, 1962).

A Spinco Model E ultracentrifuge was used at a speed of 59,780 rpm for the determination of sedimentation constants.

Nitrogen was determined by the AOAC Kjeldahl method (Ogg, 1960), phosphorus by the method of Sumner (1944), hexose and hexosamine by the methods described by Winzler (1955).

Analyses for amino acids in acid hydrolyzates were by the method of Spackman *et al.* (1958) in a Phoenix amino acid analyzer. Duplicate samples of lactollin were hydrolyzed in 200-fold quantities of 6 N hydrochloric acid in sealed evacuated tubes at 110° for 24, 48, and 96 hours. Cystine was determined in the analyzer as cysteic acid after oxidation of the lactollin by performic acid, as described by Schram *et al.* (1954), destruction of excess oxidant by means of HBr,¹ and acid hydrolysis for 18 hours. Sulfhydryl groups could

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[†] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

¹ We are indebted to Dr. Stanford Moore for details of this procedure prior to its publication (Moore, 1963).

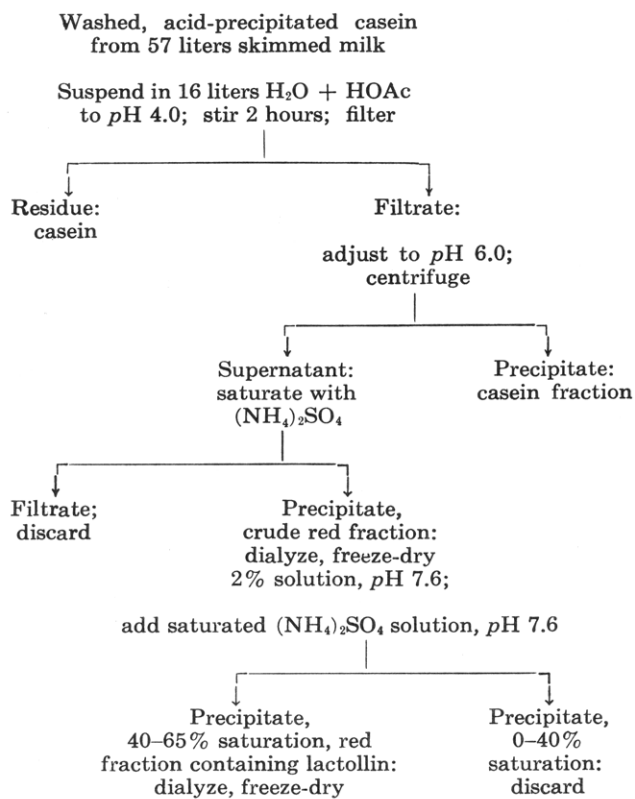


FIG. 1.—Preparation of lactollin.

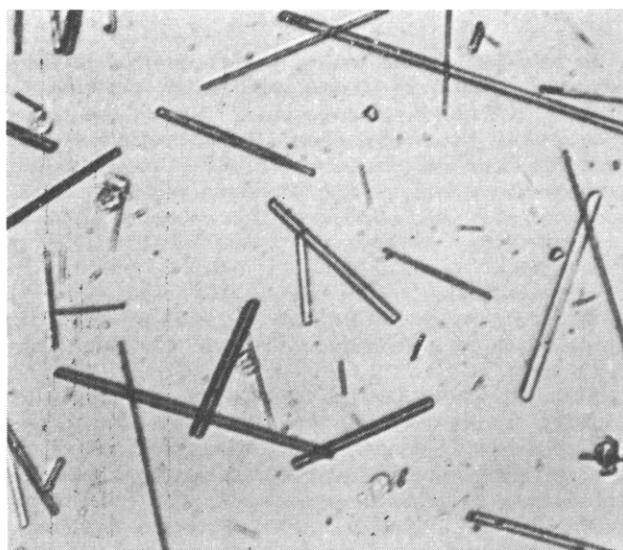


FIG. 2.—Crystalline lactollin (125X).

not be detected in a solution of lactollin in 8 M guanidine HCl when tested with nitroprusside by the procedure of MacDonnell *et al.* (1951). Tryptophan was determined with the solid protein according to procedure N of Spies and Chambers (1949); the time for Reaction I was 6 hours, and for Reaction II, 0.5 hour.

RESULTS

Electrophoretic and Ultracentrifugal Measurements.—Analysis by free boundary electrophoresis was carried out between pH 3.5 and 9.5. On the alkaline side of the isoelectric point, at pH 9.5, only one peak was found (Fig. 3), while at pH 7.0, 4.9, and 3.5 two or more components or asymmetric patterns were observed.

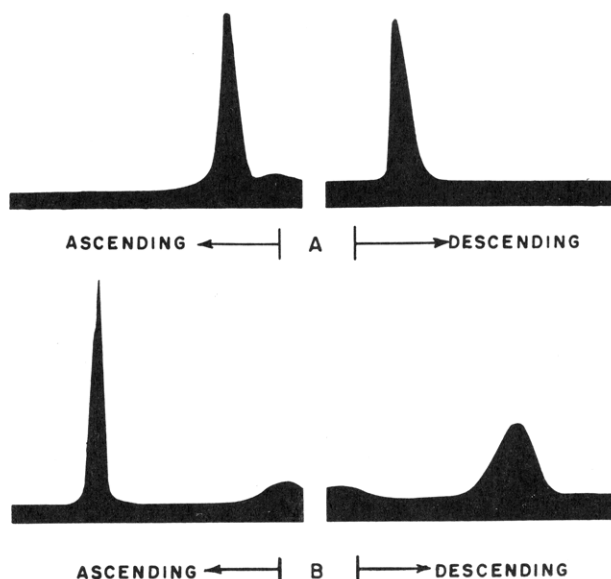


FIG. 3.—Free boundary electrophoresis of lactollin in glycine buffer, 0.1 ionic strength (0.08 M NaCl). (A) pH 9.5, mobility -1.86 ; (B) pH 3.5, mobility $+7.04$.

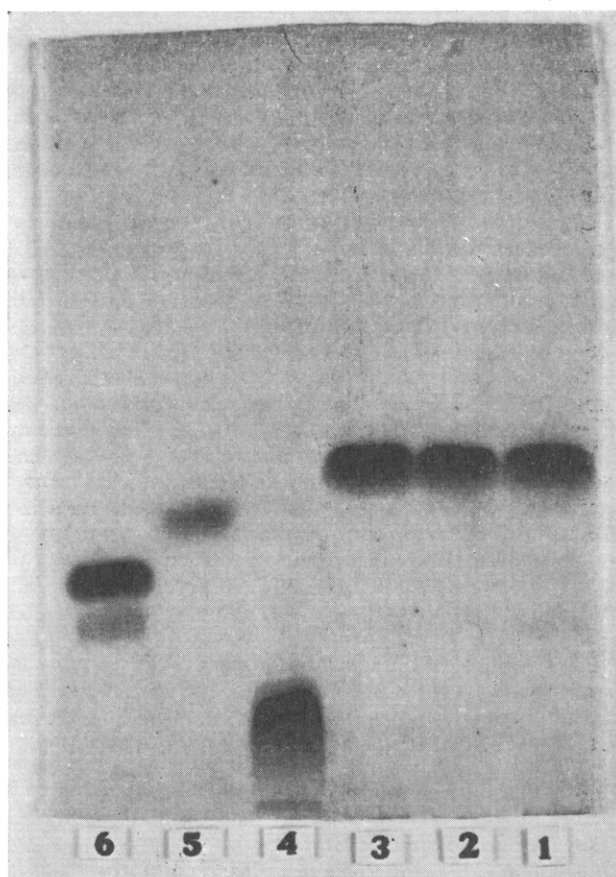


FIG. 4.—Electrophoresis. Starch gel in 5 M urea, pH 3.7. 1, 2, 3: lactollin; 4: red protein (Groves, 1960); 5: α -lactalbumin; 6: β -lactoglobulin.

In contrast to this apparent heterogeneity shown at acid pH, electrophoresis in a starch gel urea medium at pH 3.7 showed only one band (Fig. 4). Numbers 1 and 2 show two different preparations of crystals, while number 3 is protein from the supernatant fraction of lactollin that had been recrystallized once, and shows how efficient one crystallization has been in

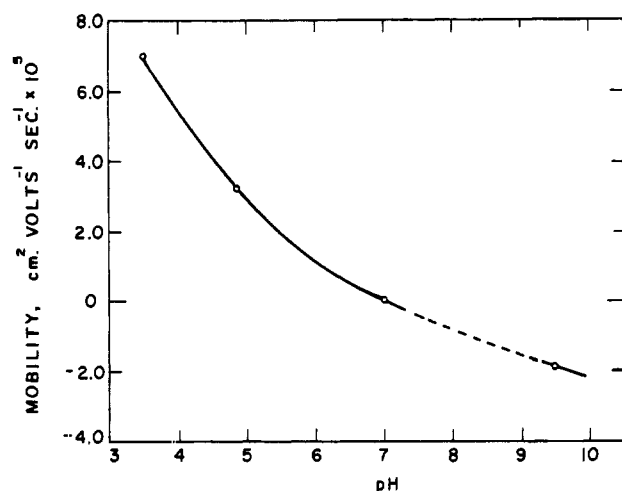


FIG. 5.—Electrophoretic mobility of lactollin as a function of pH at 0.1 ionic strength. The dotted line indicates the insoluble area. pH 3.5 and 9.5: glycine buffer; pH 4.85: sodium acetate; pH 7.0: sodium phosphate.

purifying lactollin. For comparative purposes, the "red protein" (4), α -lactalbumin (5), and β -lactoglobulin (6) are also shown.

The electrophoretic mobility of lactollin at 0.1 ionic strength, plotted against pH, is shown in Figure 5. Although determinations were not made between pH 7.0 and 9.5 because of the insolubility of the protein, the isoelectric point is probably about pH 7.1. Under conditions of lower salt concentration, the minimum solubility of the crystals is near pH 8.

Ultracentrifugation measurements were made on lactollin at pH 5.0 in a sodium acetate buffer of 0.1 ionic strength. Sedimentation coefficients calculated from five determinations gave an average $s_{20,w}$ of 3.21 with a standard deviation of 0.11. Sedimentation coefficients measured at 6° were slightly higher than those at 25°; at both 2.50% and 1.25% protein concentration, and 25°, the $s_{20,w}$ values were the same, while the 6° values showed only a small increase with dilution. There was a very small amount of faster-sedimenting material which was more pronounced in the 25° runs, presumably due to aggregation since the sedimentation of lactollin shows the slow development of a pronounced faster-moving boundary over a period of days. A single determination at pH 10.1 in glycine buffer, 6°, and 1% protein concentration gave a $s_{20,w}$ value of 3.51.

Approximate diffusion coefficients were calculated from the sedimentation diagrams by measuring the chord between the two inflection points on the curves. An average of four determinations gave a $D_{20,w}$ value of 6.83×10^{-7} with a standard deviation of 0.63. The values showed no concentration dependence.

The partial specific volume of lactollin calculated from its amino acid composition (McMeekin *et al.*, 1949) is 0.734. Calculation of the molecular weight using the above sedimentation, diffusion constants, and partial specific volume results in a value of 42,900 \pm 5,000.

Chemical Analyses.—Lactollin contains 16.37% nitrogen. Less than 0.1% phosphorus and 0.14% hexose, 0.15% hexosamine, and 0.3% ash were present, presumably as impurities, in the samples analyzed. Results of the amino acid analyses are summarized in Table I. The figures are averaged results except for the extrapolated values indicated in the table; they have not been corrected for ash or the above impurities.

TABLE I
AMINO ACID COMPOSITION OF LACTOLLIN

	g Amino Acid/ 100 g Dry Protein	g Amino Acid/ Residue/ 100 g Protein	g Amino Acid N/100 g Protein N ^a	Amino Acid Resi- dues ^b / 43,000 g Protein
Aspartic acid	12.24	10.58	7.87	40
Threonine	2.00 ^c	1.70	1.44	7
Serine	7.22 ^c	5.98	5.88	30
Glutamic acid	14.66	12.86	8.53	43
Proline	8.97	7.57	6.67	34
Glycine	1.95	1.48	2.22	11
Alanine	0.82	0.65	0.79	4
Cystine	2.24 ^d	1.90	1.60	4
Valine	4.96 ^e	4.20	3.62	18
Methionine	<0.1			0
Isoleucine	6.63 ^e	5.72	4.33	22
Leucine	8.98	7.75	5.86	29
Tyrosine	9.06	8.16	4.28	22
Phenylalanine	5.65	5.03	2.93	15
Tryptophan	3.77	3.44	3.16	8
Lysine	11.19	9.81	13.10	33
Histidine	5.17	4.57	8.55	14
Amide NH ₂	1.45 ^e		7.29	37
Arginine	7.40	6.63	14.54	18
Total	114.4	98.0	102.7	

^a Based on content of 16.37% N. ^b Rounded off to nearest integer. ^c Extrapolated value from 24, 48, and 96 hr. hydrolyzates, by method of least squares. ^d Determined as cysteic acid. ^e Value from 48 and 96 hour hydrolyzates.

DISCUSSION

In addition to the major, well-recognized proteins which are found in bovine milk, other proteins are present in smaller concentration. Among the minor constituents are enzymes such as lactoperoxidase and xanthine oxidase, trypsin inhibitor, metal-binding proteins, and many other proteins (Whitney, 1958). Lactollin may be included in this category of minor milk proteins. At present, no biological activity or function can be attributed to lactollin, nor has its possible relationship to a blood protein been explored. It is of interest that colostrum is a good source of the protein, but the significance, if any, of this observation is not clear.

Strong evidence for the homogeneity of crystalline lactollin is provided by starch gel electrophoresis. The apparent heterogeneity indicated by some of the Tiselius electrophoretic and ultracentrifugal measurements may perhaps be explained on the basis of aggregates, since a time-dependent aggregation phenomenon has been observed in sedimentation runs. Heterogeneity of the electrophoretic pattern at pH 5 could also be ascribed to interaction of the protein with the un-ionized buffer acid, as shown by Cann and Phelps (1959) with bovine serum albumin and ovalbumin.

As shown by its content of amino acids and the satisfactory totals in both weight and nitrogen summations, lactollin is a simple protein. Its amino acid composition seems to be unique; it is unusual in its lack of methionine, low content of alanine and cystine, and high proportion of aromatic amino acids. Its basic nature in electrophoresis, and as indicated by the pH of minimum solubility, is due to an excess of 19 cationic over anionic side-chain groups per 43,000 g protein: lysine + histidine + arginine = 65; aspartic + glutamic acids - amide = 46.

From the percentages of alanine and cystine in lactollin, minimum molecular weights of 10,870 and

10,730 can be calculated for the protein. It is apparent from the molecular weight as determined by sedimentation that the actual value is about 43,000.

ACKNOWLEDGMENT

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Bovine Milk "Red Protein": Amino Acid Composition and Comparison with Blood Transferrin*

WILLIAM G. GORDON, MERTON L. GROVES, AND JAY J. BASCH

From the Eastern Regional Research Laboratory,† Philadelphia

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The amino acid composition of bovine milk "red protein" has been determined. The basic character of the protein is evidenced by an excess per molecule of 27 cationic residues over anionic residues. Sulfhydryl groups are absent though considerable cystine is present. The protein contains *N*-terminal alanine. The amino acid analyses also prove that the same protein can be isolated from milk in iron-free form. The protein is shown to be different, however, from purified bovine blood transferrin fractions by column chromatography, electrophoretic and ultracentrifugal measurements, and amino acid analysis.

It has been reported that a protein which binds iron ions reversibly can be isolated from cow's milk both as the red, iron-containing compound (Groves, 1960) and as the colorless, iron-free apoprotein (Gordon *et al.*, 1962). In further characterization of this minor milk glycoprotein, its amino acid composition has now been determined and its *N*-terminal group identified. A comparison of "red protein" with bovine blood transferrin has also been made in respect to behavior in column chromatography on DEAE-cellulose, mobility in both paper and free boundary electrophoresis, sedimentation constants, and amino acid composition. Our data, which show the proteins to be different, support the conclusion reached independently by Derechin and Johnson (1962) on the basis of other evidence.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of Proteins.—"Red protein" and apoprotein were isolated by previously published methods (Groves, 1960; Gordon *et al.*, 1962). A sample of bovine blood transferrin prepared from Fraction IV-4, and stated by the supplier to be "about 90% pure by electrophoresis," was purchased from Mann Research Laboratories. It was purified further by column chromatography on DEAE-cellulose as used for the purification of "red protein" except that, with transferrin, stepwise elution was carried out with phosphate buffers of increasing molarity at a uniform pH of 8.3

and at 4°. The elution diagram of the initial purification step is shown in Figure 1. In two duplicate runs a total of 1.1 g of protein applied to the column was separated into three main fractions, T₁, T₂, and T₃, with recoveries (in approximate percentages of the original charge) of 5%, 50%, and 20%, respectively. In its behavior in these runs, the small fraction, T₁, resembled the "red protein." The major fraction, T₂, was rechromatographed under the same conditions but with two additional intermediate steps, with 0.01 and 0.025 M buffers; 25% of the charge was eluted with the 0.01 M buffer, 36% with 0.025 M, and 17% with 0.05 M. Of these three main subfractions, the first was designated T_{2a} and the second, T_{2b}.

Electrophoretic Measurements.—Patterns from paper electrophoresis (in veronal buffer, pH 8.4) of the original transferrin and of the fractions showed that the original protein was heterogeneous, the most heavily stained band corresponding in mobility to fraction T₂, itself somewhat heterogeneous. T₁, also heterogeneous, was the most basic fraction, migrating slowly toward the cathode under these conditions at about the same rate as the homogeneous "red protein." Subfractions T_{2a} and T_{2b} migrated slowly toward the anode as single bands with some trailing. The pattern of T₃ showed only one relatively fast-moving, negatively charged component with considerable trailing. These experiments indicated, as had the chromatographic separation, a resemblance between "red protein" and T₁.

A few patterns were also obtained in a Tiselius electrophoresis apparatus, but not enough T₁ was available for these experiments. The measurements were made at 0.5° in veronal buffer, pH 8.5, ionic strength 0.1, and with protein concentrations of about 1%. Under these conditions, the original transferrin gave two main

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† Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.