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*

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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 *p*H 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, T_1, T_2, and T_3, with recoveries (in approximate percentages of the original charge) of 5%, 50%, and 20%, respectively. In its behavior in these runs, the small fraction, T_1, resembled the "red protein." The major fraction, T_2, 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, *p*H 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 T_2, itself somewhat heterogeneous. T_1, 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 T_3 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 T_1.

A few patterns were also obtained in a Tiselius electrophoresis apparatus, but not enough T_1 was available for these experiments. The measurements were made at 0.5° in veronal buffer, *p*H 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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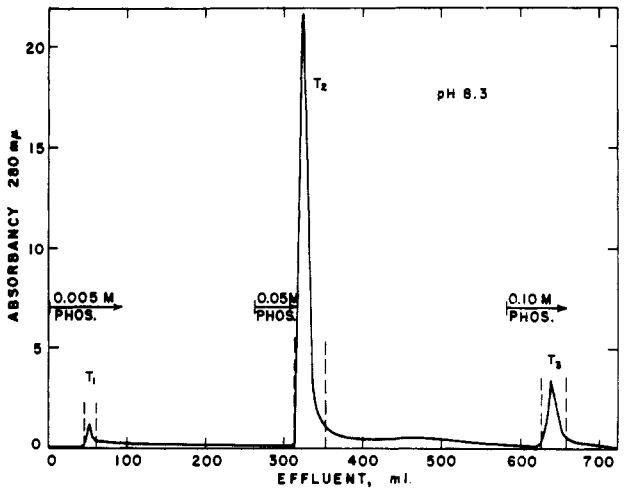

FIG. 1.—Stepwise elution of transferrin: 0.5 g transferrin in 4 ml 0.005 M buffer applied to a 2 × 30 cm column of DEAE-cellulose, broken vertical lines indicate volumes of effluent combined to yield fractions T_1, T_2, and T_3.

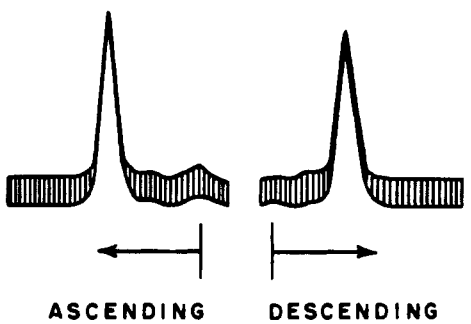

FIG. 2.—Electrophoretic pattern of transferrin fraction T_{2a} in Veronal buffer, *p*H 8.5, after 2 hours at a field strength of 7.8 v/cm.

peaks, the major one with a mobility of −2.9 and the minor with a mobility of −6.8. Fraction T_{2a} gave a single symmetrical peak, mobility −2.9, with only a trace of slower-moving impurity (Fig. 2). The major component in fraction T_{2b} had a mobility of −3.0, but more slower-moving material was present than in T_{2a}. The pattern of the comparatively homogeneous T_3 showed a symmetrical major peak, mobility −6.8, with small amounts of slower-moving impurities. At this *p*H the mobility of the "red protein" was −0.2 (Groves, 1960).

Ultracentrifuge Experiments.—Sedimentation constants were determined in the Spinco Model E ultracentrifuge. One per cent solutions of the proteins, prepared in sodium phosphate–sodium chloride buffer, ionic strength 0.1, *p*H 7.0, were used. Values for $s_{20,w}$ calculated for the major sedimenting peak in each fraction, were as follows: original transferrin, 4.65; T_1, 3.4; T_{2b}, 4.7; T_3, 3.9. Fraction T_{2a} gave a monodisperse pattern with $s_{20,w}$ = 4.7. The comparable value for "red protein" was 5.25 (Groves, 1960).

Iron-binding Properties of Transferrin.—Both subfractions T_{2a} and T_{2b} were considered to be reasonably pure transferrins because of their reddish brown color, their ability to bind iron reversibly, and the resemblance of their spectral characteristics and other physical properties to those of human transferrin (Surgenor *et al.*, 1949) and of swine transferrin (Laurell, 1953). The following results of experiments with T_{2a}, the purer fraction as judged by its electrophoretic and ultracentrifugal patterns, were essentially the same as those with T_{2b}.

Iron-free T_{2a} was prepared by dialyzing a 0.5% solution in 0.1 M acetate buffer, *p*H 5.0, against 0.15 M disodium versenate at room temperature for 8 hours, then at 4° against acetate buffer overnight, and finally against water. To ensure removal of iron versenate complex, the protein solution was then passed through a 0.9 × 9.5-cm column of Dowex-1-X10, 200–400 mesh, in the chloride form, under which was a 0.3-cm layer of the same resin in the hydroxide form. The protein was recovered by lyophilization, with an estimated loss in the chromatography of 20%.

The iron-binding capacity of the almost colorless product, determined by the method of Warner and Weber (1953) in the manner employed previously (Gordon *et al.*, 1962), was 0.10% Fe. By comparing the absorption spectra of the fully saturated Fe-transferrin and the iron-free form, a difference spectrum was obtained which showed the characteristic maximum at 465 mμ and minimum at 410 mμ. From the difference spectrum, the absorptivity of the Fe-transferrin at 465 mμ was found to be 0.42 (1-cm light path, 1% solution, *p*H 7.6).

Amino Acid Analysis.—Analyses were done by the method of Spackman *et al.* (1958), with automatic recording equipment. Duplicate samples of the "red protein" were hydrolyzed in 200-fold quantities of 6 N hydrochloric acid in sealed, evacuated tubes at 110° for 24, 48, 96, and 120 hours. The results for serine and threonine are extrapolated values by the method of least squares, since progressive decomposition occurred; similarly, from the ammonia analyses, which increased with time, an approximate value for amide ammonia was obtained by extrapolation to zero time. Determinations of valine and isoleucine in 24-hour hydrolysates were omitted in the calculation of final averages because of incomplete liberation of these amino acids. Total cystine was estimated as cysteic acid after performic acid oxidation according to Schram *et al.* (1954) and subsequent hydrolysis for 24 hours. By means of the nitroprusside test, as used by MacDonnell *et al.* (1951), sulfhydryl groups could not be detected in a solution of the "red protein" in 8 M

TABLE I
AMINO ACID COMPOSITION OF "RED PROTEIN"

	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 Residues[b]/mw 86,100[c]
Aspartic acid	9.95	8.60	6.87	64
Threonine	4.73[d]	4.01	3.65	34
Serine	4.89[d]	4.05	4.28	40
Glutamic acid	11.24	9.86	7.02	66
Proline	4.23	3.57	3.38	32
Glycine	4.20	3.19	5.14	48
Alanine	6.61	5.27	6.82	64
Cystine	5.15	4.38	3.94	18
Valine	6.02	5.09	4.72	44
Methionine	0.84	0.74	0.52	5
Isoleucine	2.37	2.04	1.66	16
Leucine	9.46	8.16	6.63	62
Tyrosine	4.25	3.83	2.16	20
Phenylalanine	4.98	4.44	2.77	26
Tryptophan	3.44	3.14	3.10	15
Lysine	8.24	7.22	10.36	49
Histidine	1.83	1.62	3.25	10
Amide NH_3	1.22[d]		6.59	(62)[e]
Arginine	7.19	6.45	15.18	36
Total		85.7[f]	98.0	668

[a] Based on nitrogen content of 15.24%. [b] Rounded off to nearest integer. [c] Groves (1960). [d] Extrapolated value. [e] Omitted from total. [f] See text.

TABLE II
COMPARISON OF AMINO ACID COMPOSITION OF "RED PROTEIN," ITS APOPROTEIN, AND TRANSFERRIN FRACTIONS[a]
(micromoles per 100 micromoles analyzed)

	Red Protein	Apo-protein	Original Transferrin	T_1	T_{2a}	T_3
Aspartic acid	9.2	9.0	10.1	9.4	11.8	9.1
Threonine	4.8	4.8	5.7	6.3	5.1	5.4
Serine	5.5	5.4	6.7	8.0	6.2	5.2
Glutamic acid	9.3	9.4	9.6	8.0	8.2	11.4
Proline	4.5	4.4	5.0	5.5	4.6	5.2
Glycine	6.9	6.8	5.9	6.7	6.8	3.9
Alanine	9.2	9.3	7.0	5.7	7.2	7.1
Half-cystine	3.8	4.2	3.6	3.0	3.6	4.1
Valine	5.8	5.7	5.8	6.1	5.4	5.7
Methionine	0.63	0.64	1.1	1.2	1.4	0.89
Isoleucine	2.1	2.0	2.4	2.8	2.6	2.1
Leucine	9.0	9.0	8.0	6.6	7.2	9.1
Tyrosine	3.0	2.9	3.3	3.6	3.5	3.2
Phenylalanine	3.7	3.7	4.2	4.1	4.1	4.4
Lysine	7.0	7.1	7.7	7.1	8.2	9.0
Histidine	1.5	1.4	2.4	2.1	2.2	2.7
Ammonia	9.0	9.0	8.0	9.6	8.9	7.7
Arginine	5.1	5.1	3.5	4.0	3.3	3.8

[a] 24-Hour hydrolysates; molar percentages calculated by adding all amino acids and ammonia (in micromoles found) and equating the total to 100.

guanidine hydrochloride. Tryptophan analyses by the method of Spies and Chambers were reported previously (Gordon and Basch, 1961). The amino acid composition of the "red protein" is shown in Table I. The figures are averaged results or extrapolated values from all hydrolysates with the aforementioned exceptions.

Because of limited amounts of the various transferrin fractions and of apoprotein, only 24-hour hydrolysates of these were prepared, and in several instances only one complete amino acid analysis was made. For the same reason, tryptophan and cystine (as cysteic acid) were not determined, nor were moisture, nitrogen, or ash estimated. When analyses were run on such approximately weighed samples, equal aliquots of hydrolysate were added to the 150- and 15-cm columns so that the molar percentage of each amino acid could be calculated from total micromoles of all amino acids actually found in the complete analysis. These results are listed in Table II. Also included for comparison are figures for the "red protein" calculated in the same way from the analyses of 24-hour hydrolysates.

Identification of Amino-terminal Residue.—The "red protein" was dinitrophenylated, hydrolyzed, and analyzed for DNP-amino acids according to the directions of Fraenkel-Conrat *et al.* (1955). Only DNP-alanine was found as an amino terminal group in amounts of about 0.55 μmole per 86.1 mg protein uncorrected. With correction factors determined as recommended by Fraenkel-Conrat *et al.*, this approximates one residue per 86,100 molecular weight.

DISCUSSION

The distribution of amino acids in the "red protein" shows few unusual features. The low methionine and histidine and the somewhat high arginine contents may be noted. An excess per molecule of 27 cationic residues (lysine + histidine + arginine = 95) over anionic residues (aspartic + glutamic acid − amide = 68) is the reason for the basic character of the protein.

The total nitrogen of the protein is accounted for reasonably well in terms of amino acids and ammonia. The weight recovery of 85.7% shown in Table I does not include the 7.1% carbohydrate and 1.4% ash found to be present in the protein (Groves, 1960); when these are added, the total becomes 94.2%. We have no information regarding the missing few per cent of, presumably, nonnitrogenous components or impurities.

The almost perfect agreement between the analyses of "red protein" and iron-free apoprotein shown in Table II, together with the evidence reported previously (Gordon *et al.*, 1962), leaves little doubt that these proteins are identical. In this and subsequent comparisons of the values in Table II, the half-cystine figures should be disregarded because of variable destruction during hydrolysis.

Possible relationships between bovine milk and blood proteins have been explored by various investigators and it has been demonstrated that the immune globulins (Smith, 1948) and serum albumin (Polis *et al.*, 1950; Coulson and Stevens, 1950) of both biological fluids are closely related, if not identical. Our comparison of "red protein" with bovine transferrin suggests that "red protein" occurs only in milk. The principal fractions of commercial transferrin are quite different from "red protein" in chromatographic behavior, electrophoretic mobility, and rate of sedimentation, and also in amino acid composition, as shown in Table II. Fraction T_{2a}, the purest bovine transferrin available for comparison, is not only strikingly different in electrophoretic mobility, but also in content of methionine, histidine, alanine, aspartic acid, and arginine. The small fraction, T_1, likewise differs from "red protein" in content of many amino acids, in spite of similarities in chromatographic and electrophoretic behavior. Here, the conclusion that the proteins differ cannot be unequivocal because of the heterogeneity of T_1, but it is safe to say that only traces of "red protein" could be present in T_1. It should be noted also that in properties and amino acid composition, fraction T_3 resembles serum albumin, and it is almost certain that T_3 is largely this protein rather than transferrin. The results of these experiments imply that "red protein" is absent from blood, but it is obvious that our findings depend on the commercial transferrin sample used.

T_{2a}, although fairly pure and clearly a strong iron-binding protein, cannot yet be considered to be a definitive preparation of bovine transferrin. The iron-

binding capacity of 0.10%, and absorptivity of the Fe-transferrin at 465 mμ of 0.42, are lower than the figures of 0.125% and 0.57, respectively, reported for crystalline human transferrin by Surgenor *et al.*, (1949) and the 0.126% and 0.48 to 0.55 found for crystalline swine transferrin by Laurell (1953). Laurell has also mentioned the difficulty in preparing swine transferrin free from a heme-containing globulin characterized by high absorptivity at 408 mμ. A small amount of a similar impurity is present in T_{2a}, and our values for iron-binding capacity and absorptivity at 465 mμ may be a little low for this reason. It is also possible, of course, that these constants are characteristically lower in the bovine variety.

The problem of preparing a purified bovine blood β-globulin (transferrin) which can be considered homogeneous by current standards is further complicated by the occurrence of genetic variants of this protein (Ashton, 1958, 1959; Gahne, 1961). We have not attempted to deal with this aspect of the problem in this paper, since we have used mixed herd milk and, we assume, pooled blood as sources of protein. As has been stated previously, our conclusion regarding the nonidentity of "red protein" and transferrin is in accord with the results reported by Derechin and Johnson (1962).

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Action of α-Chymotrypsin on Diethyl *N*-Acetylaspartate and on Diethyl *N*-Methyl-*N*-Acetylaspartate*

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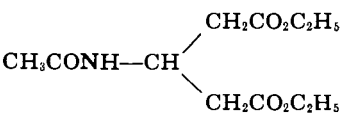
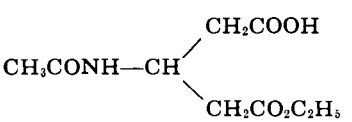
Study of glutarate esters had indicated that a β-carbalkoxyl group would be an activating substituent in hydrolyses catalyzed by α-chymotrypsin. L(−)Diethyl-*N*-acetylaspartate is hydrolyzed effectively by α-chymotrypsin, K_m = 0.023 M, k_3 = 22 sec^{-1}, leading to L(+)β-ethyl-α-hydrogen-*N*-acetylaspartate. The L-enantiomorph is also hydrolyzed rapidly from the DL material, leading to L(+)β-ethyl-α-hydrogen-*N*-acetylaspartate and D(+)diethyl-*N*-acetylaspartate. The D-enantiomorph does not inhibit hydrolysis of the L, and is itself hydrolyzed very slowly. Diethyl-*N*-methyl-*N*-acetylaspartate is not hydrolyzed by α-chymotrypsin. The results are compared with those for derivatives of alanine and β-phenylalanine, placing L(−)diethyl-*N*-acetylaspartate among the good substrates for α-chymotrypsin. It is proposed that the β-carbethoxyl group of the aspartate associates at the β-aryl site of α-chymotrypsin.

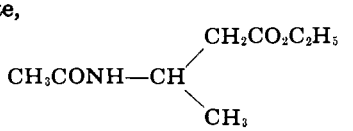
We have reported (Cohen and Khedouri, 1961a) that diethyl-β-acetamidoglutarate,



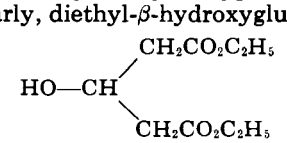
is hydrolyzed by α-chymotrypsin, slowly but with stereospecificity, leading to (+)ethyl-hydrogen-β-acetamidoglutarate,



of high optical purity, and this hydrolysis probably proceeds in the L sense (Cohen and Khedouri, 1961b). The related asymmetric compound, ethyl-*dl*-β-acetamidobutyrate,



is not hydrolyzed by α-chymotrypsin (Cohen *et al.*, 1961). Similarly, diethyl-β-hydroxyglutarate



is slowly hydrolyzed by α-chymotrypsin, in the L sense and with high stereospecificity, while the related asymmetric compound, ethyl-*dl*-β-hydroxybutyrate,

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