

ever, it is impossible to tell whether there is a glu-glu sequence in the  $\beta^A$  chain or val-glu in the  $\beta^S$  chain because of the complexity of the material from the degradation. The evidence of this investigation, though less conclusive than might be desired, leads to the conclusion that hemoglobins A and S differ in the sixth amino acid residue from the N-terminus of the  $\beta$  chains.

NOTE ADDED IN PROOF.—Among the peptides in a tryptic hydrolysate of hemoglobin A, we have found one for which all evidence points to the N-terminus of the  $\alpha$  chains as the source. The sequence is val-leu-ser-pro-ala-asp-lys-thr-aspNH<sub>2</sub>-val-lys. It contains aspartic acid, however, instead of asparagine in the sixth position from the N-terminus as reported in the present paper for  $\alpha$  chains. The assignment of asparagine to the sixth position of the  $\alpha$  chains is based on indirect evidence whereas in the peptide the sixth position

is definitely occupied by aspartic acid. Experiments to resolve the discrepancy are in progress. N. Hilschmann and G. Braunitzer<sup>22</sup> have concluded on the basis of indirect evidence that the N-terminal sequence of the  $\beta^A$  chains is val-his-leu-thr-pro-glu-glu-lys-(ser, ala, ala, thr, val, leu)-(try, gly, lys)-.

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(22) N. Hilschmann and G. Braunitzer, *Z. physiol. Chem.*, **317**, 285 (1959).

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## The Isolation of a Red Protein from Milk<sup>2</sup>

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A method is described for the preparation of the red protein of milk. It was separated from casein by acid extraction, then fractionated with ammonium sulfate and DEAE cellulose chromatography. The isolated red protein is homogeneous by electrophoresis and the ultracentrifuge. It has a molecular weight of 86,100 and contains two atoms of iron. The molecular weight and absorption spectra of the red protein are similar to those given by the complex of iron with conalbumin and the B<sub>1</sub>-metal combining protein of plasma. It is a glycoprotein with an isoelectric point at about pH 7.8. It is possible to remove the iron at pH 2 with Dowex 50. Addition of ferric ions to the apoprotein appears to restore the complex.

The occurrence of a red protein in bovine milk was reported in 1939 by Sørensen and Sørensen<sup>3</sup> who found it to be present in a small concentration and were able, by careful fractionation of the whey protein, to prepare it in a partially purified form. Polis and Shmukler<sup>4</sup> at this Laboratory also partially purified a red protein during the course of preparation of lactoperoxidase by column chromatography of whey protein fractions. In none of this work was the red protein purified sufficiently to permit accurate characterization, however, it was reported to contain iron, an unusually high content of tryptophan and some phosphorus and carbohydrate. Recently, Johansson<sup>5</sup> isolated a salmon-colored component from human milk whey by calcium phosphate chromatography and like the red protein of bovine milk, it contains a small amount of iron.

In the present study, the red protein was prepared from casein rather than from the whey. During the preparation of  $\alpha_2$ -casein<sup>6</sup> by the acid extraction of casein, it was found that the red protein was also extracted. The isolation and characterization of the red protein is described.

**Preparation of the Red Protein.**—Three preparations of the pure red protein were made and the method used for

their preparation varied only slightly. They will be referred to as preparations A, B and C.

The casein from 15 gallons of fresh unpasteurized skim milk was precipitated at 25° by the addition of 1 N HCl to pH 4.6. After filtration through a cloth bag and draining overnight at 2°, the casein was either stored at about -20° until needed or worked up immediately by washing four to five times with distilled water in a 20 gallon crock. The supernatant was removed by decantation. Resuspension of the acid precipitated casein in distilled water was always accompanied by a drop in pH which was adjusted to 4.6-4.7 by the addition of 0.1 N sodium hydroxide. The amount of alkali required decreased on successive washings until it became negligible. Thorough washing of the casein is necessary to remove the whey proteins that are carried down with the occluded water of the casein.

**Acid Extraction of the Casein.**—The washed casein was suspended in 16 l. of water and the pH adjusted to 4.0 with 1 N acetic acid. After 2 hr. of stirring during which the pH was kept at 4.0, the casein was removed by filtration on large Buchner funnels. The filtrate, which contains about 2% of the original protein, was then adjusted to pH 6.0 and a casein fraction was precipitated.<sup>6</sup> It was removed by centrifugation. A proteolytic enzyme<sup>7</sup> was associated with this fraction. On saturation of the filtrate with ammonium sulfate, the protein which precipitated was filtered off by gravity. It contained the red protein and phosphatase among other proteins. The above fractionation was carried out in the presence of liberal amounts of toluene as a preservative.

**Ammonium Sulfate Fractionation.**—The fraction containing the red protein was next dialyzed free of ammonium sulfate at 2° giving a brown solution with some precipitate. After lyophilization, water was added to give a 2% protein concentration, the pH adjusted to 7.6 and a saturated solution of ammonium sulfate also adjusted to pH 7.6 was then added. The red protein precipitated between 40 and 65% of saturation and for preparation B this fraction contained 2.59 g. protein. In earlier experiments, the fractionation with ammonium sulfate was repeated. On the second fractionation of preparation A, the red fraction precipitated

(1) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(2) Presented at the 135th Meeting of the American Chemical Society, Boston, Mass., April 1959.

(3) M. Sørensen and S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg*, **23**, 55 (1939).

(4) B. D. Polis and H. W. Shmukler, *J. Biol. Chem.*, **201**, 475 (1953).

(5) B. Johansson, *Nature*, **181**, 996 (1958).

(6) T. L. McMeekin, N. J. Hipp and M. L. Groves, 131st Meeting of American Chemical Society, Miami, Fla., April 1957.

(7) R. C. Warner and E. Polis, *THIS JOURNAL*, **67**, 520 (1945)

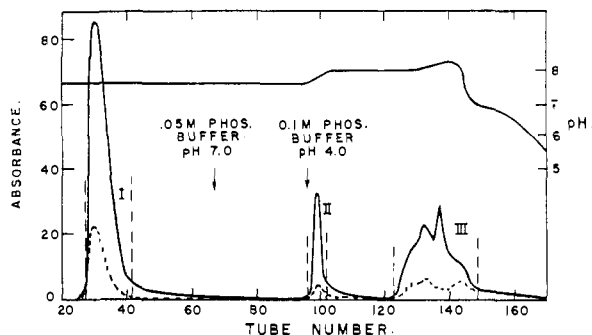


Fig. 1.—Stepwise elution diagram of the red protein fraction: 2.59 g. protein in 30 ml. applied to a 2 by 32 cm. column, effluent collected in 3 ml. fractions. Absorbance; 280  $m\mu$ , solid line, 412  $m\mu$ , broken line. Values at 412  $m\mu$  were multiplied by 10. Arrows indicate points of change in buffer.  $pH$  is shown by solid line (top) with ordinate at the right. The tubes that were combined to yield fractions I, II and III are shown by the broken vertical lines.

between 50 and 65% of saturation and amounted to 2.14 g. Because of the characteristic color, it was easy to determine the amount of ammonium sulfate required to precipitate the red protein. The fractions were recovered after dialyzing free of salt by lyophilization.

**DEAE Cellulose Chromatography.**—Diethylaminoethyl cellulose, type 20, was obtained from Brown Company, Berlin, New Hampshire,<sup>8</sup> and a column, 2 by 32 cm., was poured as described by Sober, *et al.*,<sup>9</sup> using a 0.005 *M* sodium phosphate buffer,  $pH$  8.2. A concentrated solution in phosphate buffer of the red fraction was adjusted to  $pH$  8.2 and dialyzed against buffer overnight at 2°.

Chromatography was carried out at 25° using a fraction collector. Fractions of 3 ml. were taken with a flow rate of about 11 ml./hr. The effluent fractions were examined in a Beckman spectrophotometer at 280  $m\mu$  and in some experiments at 412  $m\mu$ . In Fig. 1 is shown the elution diagram for the red fraction, preparation B (2.59 g.). The starting buffer 0.005 *M* sodium phosphate,  $pH$  8.2 was followed by 0.05 *M* sodium phosphate,  $pH$  7.0 and finally by 0.1 *M* sodium phosphate, 0.5 *M* NaCl,  $pH$  4.0 at the points indicated by the arrows. Thymol was added to all the buffers. The protein was recovered from combined fractions by dialysis and lyophilization. The three major fractions are included between the broken vertical lines in Fig. 1. Most of the red protein was found in fraction I and amounted to 1.03 g. The two tubes immediately preceding this fraction contained a greenish yellow protein. With the second buffer, a small red band (fraction II) moved down the column and contained 0.14 g. protein. Paper electrophoresis indicated one component with a different mobility than the red protein. However, analysis of this fraction by free-flowing electrophoresis showed it to be two components of equal amount, one of which had the mobility of the purified red protein. In a similar manner, Tiselius<sup>10</sup> has noted with calcium phosphate columns, a single protein may give rise to several zones on stepwise elution. Fraction III was a mixture of several proteins as determined by paper electrophoresis. Weight recovery of the total protein put on the column was 80%. Paper electrophoresis in veronal buffer of the various combined fractions showed that the more basic proteins were only slightly retarded by the column while the more acidic proteins required higher salt for elution.

The red protein from Fig. 1, fraction I (1.03 g.) was next dissolved in 14 ml. buffer, dialyzed and rechromatographed on a second DEAE cellulose column, 2 by 27 cm., prepared as described above. The effluent pattern is shown in Fig. 2, and 80% of the protein put on the column was eluted in the first peak. This compares with 40% for the first chro-

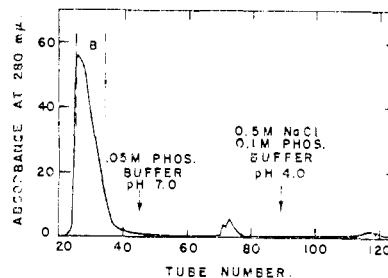


Fig. 2.—Effluent diagram on rechromatography of the red protein (Fig. 1, fraction I): 1.03 g. protein in 14 ml. applied to a 2 by 27 cm. column, effluent collected in 3 ml. fractions.

matogram. The reproducibility of the positions of the red protein peak shows good agreement on rechromatography. The center of the red peak in Fig. 1 emerged at tube No. 30 and this position for the 32 cm. column agrees with the corresponding tube No. 26 in Fig. 2 with a 27 cm. column. On rechromatography, as in the first run, the two tubes immediately preceding the red protein contained small amounts of a greenish yellow protein. The fraction indicated between the broken vertical lines, Fig. 2, amounted to 0.65 g. of the red protein, B, and it was used for further characterization. On the rechromatography experiment, 95% of the protein put on the column was recovered.

The crude red fraction of preparation A obtained from ammonium sulfate fractionation, 2.14 g., was also chromatographed on a DEAE cellulose column as described above. A yield of 0.74 g. of the red protein was obtained. This preparation was chromatographed only once. Recovery of the total protein put on the column in this instance amounted to about 85% by weight.

In preparation C, various fractions from the DEAE cellulose experiments, rich in the red protein, together with the red fractions from previous ammonium sulfate fractionations were combined and chromatographed on DEAE cellulose as described. In this experiment, 2.78 g. of protein was put on the column and 1.29 g. of the purified red protein obtained.

The red protein is very soluble in water and a concentrated solution is highly colored, the crude preparations being red in color while the purified protein tends to be more of a salmon red. Yields of about 1.0 g. from 15 gallons of milk can be expected when corrections are made for losses due to fractionation.

**Minor Components Associated with the Red Fraction. Phosphatase.**—Acid extraction of casein also resulted in the extraction of phosphatase which was found to be associated with the red fraction. Qualitative determinations of alkaline phosphatase showed high activity in the fractions precipitated between 40 and 65% of saturation with ammonium sulfate. On DEAE cellulose chromatography of the red fraction, alkaline phosphatase activity was low where the red protein was eluted, Fig. 1, fraction I, and high for fractions II and III. On the other hand, the acid phosphatase activity was eluted with fraction I and concentrated in the first two tubes containing the greenish yellow protein, while the red protein was relatively low in activity.

**Crystalline Protein.**—Another minor impurity associated with the red fraction is a crystallizable protein. In early work it was found that on ammonium sulfate fractionation, the fraction precipitated between 40 and 65% of saturation, on dialysis at 2° showed birefringence and tiny needle-shaped crystals were observed under the microscope. It was later noted that if salt-free solutions of the red protein from an ammonium sulfate fractionation were adjusted to a  $pH$  of about 8, character-

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

(9) H. A. Sober, F. J. Gutter, M. M. Wyckoff, E. A. Peterson, *THIS JOURNAL*, **78**, 756 (1956).

(10) A. Tiselius, S. Hjerten and Ö. Levin, *Arch. Biochem. Biophys.*, **65**, 132 (1956).

istic crystals formed at 2° and grew in size with time. On chromatography of the red protein on DEAE cellulose with 0.005 M phosphate buffer, pH 8.2, a fraction immediately following the red protein peak (Fig. 1, tubes 45-80) was found to give the protein crystals. Cooling the tubes increased the yields, and recovery of the crystals followed by recrystallization resulted in a yield of about 35 mg. for 15 gallons of milk. The crystalline protein is colorless, low in acid and alkaline phosphatase activity and differs from the red protein in electrophoretic and ultracentrifugation patterns. Further work on this interesting protein will be described at a later date.

**Composition and Properties of the Red Protein. Elementary Composition.**—The elementary composition of the red protein preparations is shown in Table I. Moisture was determined by heating at 70° in a vacuum oven for four hours. Nitrogen was determined by a modification of the A.O.A.C. Kjeldahl method<sup>11</sup> and ash by the magnesium acetate method.<sup>12</sup> Phosphorus was determined by the A.O.A.C. method.<sup>13</sup> Iron was determined by the 1,10-phenanthroline method<sup>14</sup> using a wet digestion. Distilled water passed through an ion exchange column was used and all glassware was cleaned with hot 6 N hydrochloric acid.

TABLE I  
ELEMENTARY COMPOSITION OF THE RED PROTEIN PREPARATIONS<sup>a</sup>

Prep.	% N	% P	% Fe	% Ash <sup>b</sup>	Theor. ash % <sup>c</sup>	Excess ash % <sup>d</sup>	% N (corr.)
A	14.64	0.25	0.10	2.68	0.71	1.97	14.93
B	15.13	0.22	0.12	1.36	0.67	0.69	15.24
C	...	..	0.11	..			

<sup>a</sup> On moisture free basis. <sup>b</sup> Determined after adding magnesium acetate and corrected for magnesium oxide. <sup>c</sup> Calculated as P<sub>2</sub>O<sub>5</sub> from % P and as Fe<sub>2</sub>O<sub>3</sub> from % Fe. <sup>d</sup> Ash by difference, attributed to inorganic contamination.

**Carbohydrate Content.**—The red protein contains a significant amount of hexose and hexosamine, and a little sialic acid, but no fucose (Table II). Hexose, hexosamine and fucose were determined by the methods described by Winzler.<sup>15</sup> Sialic acid was determined by the resorcinol-hydrochloric acid method of Svennerholm.<sup>16</sup> I am indebted to Dr. Schmid for an acid  $\alpha_1$ -glycoprotein preparation<sup>17</sup> which was used as a standard for the sialic acid determination.

**Mobility.**—Electrophoresis experiments were performed in a Tiselius apparatus and mobilities were calculated as described by Warner.<sup>18</sup> A comparison of electrophoretic patterns in glycine buffer,<sup>19</sup> 0.1 ionic strength, of the red protein, preparation B, with the crude fraction before

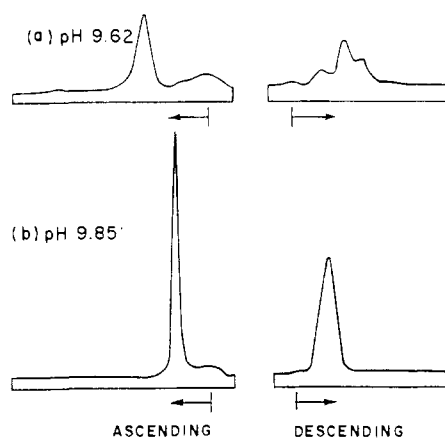


Fig. 3.—Electrophoretic patterns obtained in glycine buffer, 0.1 ionic strength, protein concentration about 1%. (a) Red fraction, preparation B, before chromatography on DEAE cellulose, after electrophoresis for 4 hr. at a field strength of 4.72 volts/cm. (b) Red protein, preparation B, after chromatography on DEAE cellulose, after electrophoresis for three hours at a field strength of 4.89 volts/cm.

chromatography is shown in Fig. 3. Homogeneous patterns for the red protein were also obtained for preparations A and C in glycine buffer pH 9.85 with mobilities of -2.18, -2.13 and -2.31  $\times 10^{-5}$  cm.<sup>2</sup>/volt second for A, B and C, respectively.

TABLE II  
CARBOHYDRATE CONTENT<sup>a</sup> OF THE RED PROTEIN PREPARATIONS

Prep.	% Hexose <sup>b</sup>	% Hexosamine	% Sialic acid <sup>b</sup>	% Total carbohydrate <sup>c</sup>
A	4.73	2.28	0.3	7.31
B	4.58	2.22	0.3	7.10
C	4.30	2.12	..	..

<sup>a</sup> Moisture free basis. <sup>b</sup> Corrected for a protein blank. <sup>c</sup> No fucose was found to be present.

Electrophoretic patterns of the red protein were found to be homogeneous at several pH values and a plot of the pH against mobility at 0.1 ionic strength (Fig. 4) shows the isoelectric point at about pH 7.8.

**Molecular Weight.**—Sedimentation studies were made using the Spinco Model E ultracentrifuge<sup>8</sup> at 59,780 r.p.m. (259,700  $\times$  g.) using Kel-F cells. Diffusion measurements were made in a Claesson diffusion cell.<sup>20</sup> Measurements made on sucrose at 1° and calculated by the height area method agreed well with values in the literature.<sup>21</sup> Both diffusion measurements and sedimentation constants were corrected to standard conditions by the method recently summarized by Schachman.<sup>22</sup> Sodium phosphate buffer, 0.05 molar sodium chloride, 0.1 ionic strength, pH 7.0 was used. All determinations were made on protein solutions after dialysis with stirring against buffer at 2° overnight.

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(21) L. G. Longworth, *THIS JOURNAL*, **74**, 4155 (1952).

(22) N. K. Schachman, "Methods in Enzymology," Vol. 1V, Academic Press, Inc., New York, N. Y., 1957, pp. 55, 93.

(11) C. L. Ogg, *J. Assoc. Official Agric. Chemists*, in press.  
 (12) "Official Method of Analysis," 8th Ed., Assoc. Official Agric. Chemists, Washington, D. C., 1955, p. 207.  
 (13) *Ibid.*, p. 115.  
 (14) H. Pyenson and P. H. Tracy, *J. Dairy Sci.*, **28**, 401 (1945).  
 (15) R. J. Winzler, "Methods of Biochemical Analysis," Vol. 11, Interscience Publishers, Inc., New York, N. Y., 1955, p. 293.  
 (16) L. Svennerholm, *Biochem. et Biophys. Acta.*, **24**, 604 (1957).  
 (17) K. Schmid, W. L. Benze, T. Nassbaumer and J. G. Wehrmüller, *J. Biol. Chem.*, **234**, 529 (1959).  
 (18) R. C. Warner, *THIS JOURNAL*, **66**, 1725 (1944).  
 (19) G. L. Miller and R. H. Golder, *Arch. Biochem. Biophys.*, **29**, 420 (1950).

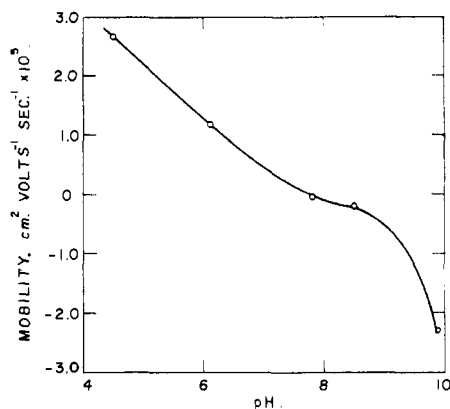


Fig. 4.—Effect of  $pH$  on mobility of the red protein at  $1.0^\circ$ , an ionic strength of 0.1 and a protein concentration of about 1.0%. Sodium acetate at  $pH$  4.5 and 6.1 and veronal buffer at  $pH$  7.8 and 8.5 each with 0.05  $M$  sodium chloride and glycine buffer<sup>19</sup> at  $pH$  9.9 were used.

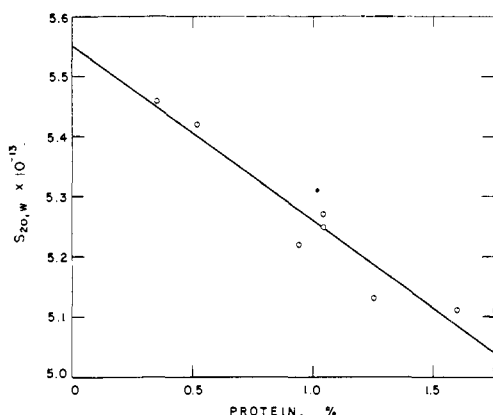


Fig. 5.—Effect of concentration on the sedimentation coefficient, phosphate buffer, 0.1 ionic strength,  $pH$  7.0. Open circles were determined at about  $25^\circ$ , closed circle at  $8.85^\circ$ .

A single symmetrical sedimenting boundary was observed for preparations A and B. Preparation C showed a slight amount of slow-moving material. Sedimentation coefficients at about 1.0% concentration of preparations A, B and C are 5.25, 5.27 and 5.22 S, respectively. The concentration dependence of the sedimentation coefficient is shown in Fig. 5. A curve, drawn by the method of least squares on extrapolation to zero concentration, gives a sedimentation coefficient of 5.55 S. It is probable from the figure that temperature does not significantly effect the sedimentation coefficient.

Diffusion measurements were made on the red protein at two concentrations in phosphate buffer 0.1 ionic strength,  $pH$  7.0. At 1% concentration, the value  $D$  (uncorrected) with the standard deviation for seven determinations is  $3.07 \pm 0.07 \times 10^{-7}$  cm.<sup>2</sup>/sec. at  $0.9^\circ$ . This compares with a value of  $3.09 \pm .08$  on five determinations at 0.6% protein concentration. In order to calculate molecular weight, the diffusion coefficient was corrected to standard conditions,<sup>22</sup> resulting in a value  $D_{20,w} = 5.75 \times 10^{-7}$ .

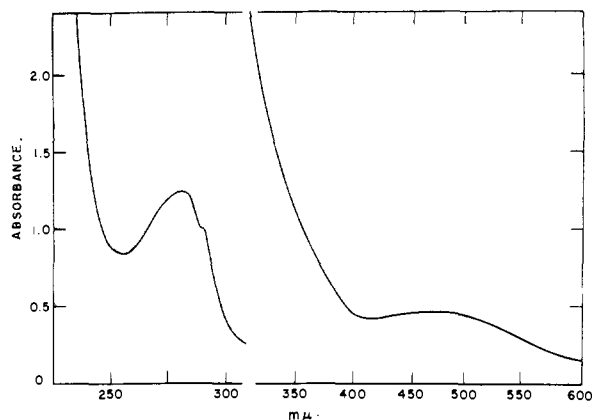


Fig. 6.—Absorption spectrum, in glycine buffer, 0.1 ionic strength,  $pH$  9.9, obtained with the recording spectrophotometer. Concentration of protein: visible range about 0.9%, ultraviolet range 0.09%.

The partial specific volume of a 1% solution of the red protein was found to be  $\bar{V} = 0.725$ . Calculation of the molecular weight using the sedimentation and diffusion coefficients, together with the partial specific volume, results in a molecular weight of 86,100.

**Absorption Spectra.**—The absorption spectra were determined with a Beckman spectrophotometer or the Cary recording spectrophotometer in cells with absorbing path length of 1 cm. Absorptivity,  $a$ ,<sup>23</sup> is expressed with concentration units of g. per 100 ml. of solution. The shape of the absorption spectrum in the ultraviolet and visible regions is shown in Fig. 6. Absorptivity at 280  $m\mu$  for preparation C was 15.1 at  $pH$  7 to 10. Absorbance of the red protein in the wave length range of 350–765  $m\mu$  shows only a broad maximum around 470  $m\mu$ . No Soret band was found for the purified preparations, however, in crude preparations after fractionation with ammonium sulfate but before chromatography, a very small peak about 410  $m\mu$  was evident. This peak is probably due to the greenish yellow protein separated in the first few tubes on chromatography, since it was found to give a strong absorption about 410  $m\mu$ . This was the fraction, as already mentioned, that was rich in acid phosphatase. Lactoperoxidase is a green protein that also shows a strong absorbance around 410  $m\mu$ . Lactoperoxidase has not been determined on this fraction, however, estimations on preparations A, B and a crude red fraction, using pyrogallol,<sup>24</sup> showed that there was less than 0.1% lactoperoxidase.

The effect of  $pH$  on the absorbance of preparation C at 465  $m\mu$  is shown in Fig. 7. Sodium hydroxide or hydrochloric acid was added to solutions of the red protein in water and readings were made about one hour after making the solutions to the desired  $pH$ . Maximum absorption was found around  $pH$  10 with a rapid decrease in color both above this value and below  $pH$  4.0. At  $pH$  2, the solution became colorless and at  $pH$  12.1, a yellow color persisted. Adjusting the  $pH$  of the latter solution to

(23) H. K. Hughes, *Anal. Chem.*, **24**, 1351 (1952).

(24) J. B. Sumner and E. C. Gjessing, *Arch. Biochem. Biophys.*, **2**, 291 (1943).

neutrality gave a precipitate. On regeneration of the color from the *pH* 2 sample, determinations were made on solutions after 24 hours at 2° at the desired *pH*. It was found that at *pH* 6, 60% and *pH* 9, 80% of the color at 465 *mμ* was regained. On recovery of the protein by dialysis, followed by lyophilization and then redissolving at *pH* 7.9, 100% recovery of the absorbance was attained.

A change in the *pH* of red protein solutions also resulted in a small but perceptible shift in the absorption maxima. At *pH* 4.3, the maximum was around 465 *mμ* while at *pH* 10.4, it increased to 475 *mμ*.

In order to compare the absorbance at 465 *mμ* of the three preparations A, B and C, 0.99% solutions were prepared in 0.1 *M* sodium phosphate, *pH* 7.9. Readings were made after one hour and 24 hours, the latter solutions were stored at 2°. The values were found to increase with time from 5 to 10%. The 24 hour values gave absorptivity, *a*, at 465 *mμ* of 0.55, 0.55 and 0.49, respectively, for A, B and C. The small increase in absorptivity with time may be explained as resulting from a slow uptake of carbon dioxide. Schade<sup>25</sup> reported that carbon dioxide has a role in the formation of the salmon-pink complex of siderophilin and conalbumin.

**Iron Removal and Recombination.**—Figure 7 shows the absorbance of the red protein at 465 *mμ* to be negligible at *pH* 2 indicating that under these conditions, the iron is dissociated from the protein. Johansson<sup>5</sup> found that at *pH* 6.9 the iron could not be removed by Dowex 50 from a red protein prepared from human milk. Dr. Johansson has since informed me it can be removed by the resin at *pH* 2. In order to remove the iron, a 1% solution of preparation C was made to *pH* 2.2 with hydrochloric acid and after standing about 3 hours was passed through Dowex 50-12x<sup>8</sup> in the hydrogen form. The iron-free protein was recovered by adjusting the solution to *pH* 8 with dilute ammonium hydroxide. A turbidity in the solution was noted on the first addition of ammonia up to a *pH* of about 4.5 but above this *pH*, a clear solution was obtained. Analysis of the protein after this treatment showed that it contained only 0.016% iron compared to a value of 0.11% for the original protein. The ultracentrifuge pattern of the iron-free protein was essentially unchanged with a slightly higher sedimentation value of 5.5 compared to 5.2 for the red protein. The electrophoretic pattern after 3 hours in glycine buffer, *pH* 9.85, was comparable to that of the red protein. Electrophoresis after 4.5 hours, however, indicated the presence of a second smaller component with a mobility slightly less than that of the major component. The mobilities of the iron-free and red protein were -2.73 and -2.31, respectively. The difference in mobilities of the two components in the Dowex treated protein is consistent with the idea that a small amount of the protein contains iron and is similar to the original red protein. At *pH* 4.5 the electrophoretic patterns and mobilities of the two proteins were in good agreement. Warner and Weber<sup>26</sup> found by

(25) A. L. Schade, R. W. Reinhart and H. Levy, *Arch. Biochem. Biophys.* **20**, 170 (1949).

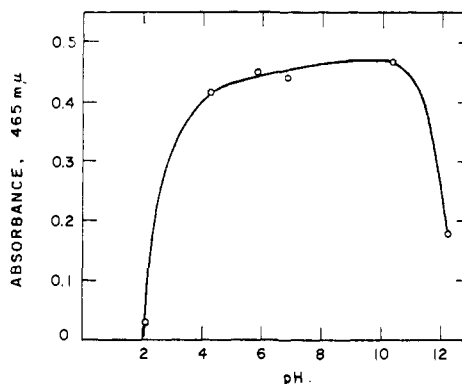


Fig. 7.—Effect of *pH* on the absorbance at 465 *mμ*, 1.01% protein.

electrophoresis that the iron-conalbumin complex was more negative than the metal-free protein at any *pH* between 5.5 and 8.5 and suggested that the greater negativity of the metal complex results from the binding of the bicarbonate ion and from displacement of protons from the groups of the binding site in which the metal coordinates. They also found that at *pH* 11 the net charge of the two species was equal, which indicated the combining groups were partially ionized and concluded that the protein phenolic groups were involved. In contrast to this, with the red protein a greater negative mobility was found for the metal-free protein at *pH* 10, suggesting that perhaps other groups are involved.

In order to reconstitute the iron complex, the metal-free protein was dissolved in veronal buffer, 0.1 ionic strength, *pH* 8.4. Ferrous ammonium sulfate was dissolved in 0.001 *M* acetic acid and an aliquot was added to the protein solution equivalent to 0.2% iron based on the protein. The final protein concentration was 1.0%. A metal-free protein control was also run together with the red protein before Dowex treatment. After standing overnight at 2°, absorption spectra were determined in the visible region. At 465 *mμ*, the absorptivity of the reformed iron complex was 0.60 compared to a value of 0.47 for the original red protein. The reason for the higher absorptivity of the reformed complex is not clear. However, it was found that the iron-free protein, although essentially colorless in solution, became yellow with time and this absorption might be reflected in the higher value for the reformed complex.

### Discussion

It appears likely that the red protein, prepared from milk by Sørensen and Sørensen using salt fractionation and by Polis and Shmukler from rennet whey, is the same protein as that obtained from acid precipitated casein. The pure red protein herein described has a mobility of -0.2 at *pH* 8.4 as compared to the value of -2.2 reported by Polis and Shmukler under similar conditions. Our studies on impure preparations of the red protein from whey indicate that this difference in mobility may be due to a difference in purity. The distribution of the red protein between acid precipitated casein and the whey fraction is

(26) R. C. Warner and I. Weber, *This Journal*, **75**, 5094 (1953).

probably affected by such factors as the  $pH$  and salt content.

The iron complex of the red protein is similar in many respects to those of the  $B_1$ -metal combining protein<sup>27</sup> (siderophilin, transferrin) from blood plasma and conalbumin.<sup>28</sup> They all show maximum absorbance at 460 to 470  $m\mu$ . Absorbance between 310 and 700  $m\mu$  of the red protein closely parallels that shown by the iron-transferrin complex of Laurell.<sup>29</sup> Absorptivity at 470  $m\mu$ ,  $pH$  7.0 varied between 0.48 and 0.55 for the iron-transferrin complex and compares with values of 0.49 to 0.55 for the red protein at  $pH$  7.9. The red protein contains 0.10 to 0.12% iron, which, compares with a value of 0.126% for the iron-transferrin reported by Laurell.

The absorptivity of 0.62 and iron content of 0.159%<sup>28</sup> for the conalbumin complex, however, are significantly higher. It was also found that the ratio between the absorptivity maximum at 470  $m\mu$  to the minimum at 410  $m\mu$  is higher for conalbumin<sup>28</sup> and transferrin<sup>29</sup> complex than for the red protein, namely 1.46 and 1.41 compared to 1.1.

Both the conalbumin<sup>28</sup> and the  $B_1$ -metal combining protein<sup>27</sup> are found to combine with two molecules of iron per mole of protein, giving molecular weights estimated at 76,600 and 90,000. Using 0.12% iron for the red protein, a minimum molecular weight of 46,500 is calculated, giving a value of 93,000 which compares with a determined value of 86,100.

(27) B. A. Koechlin, *THIS JOURNAL*, **74**, 2649 (1952).

(28) R. C. Warner, *J. Biol. Chem.*, **191**, 173 (1951).

(29) C. B. Laurell, *Acta Chem. Scand.*, **7**, 1407 (1953).

The iron complex of the red protein appears to be more stable than those of conalbumin and transferrin. Warner<sup>28</sup> removed the iron from conalbumin at  $pH$  4.7 with citrate buffer. The red protein complex, however, remained stable in the presence of an iron chelating resin at  $pH$  4.0. With the  $B_1$ -metal combining complex, Inman<sup>30</sup> reports that EDTA rapidly dissociates the iron complex at  $pH$  5.6. The red protein complex was stable under these conditions.

It is interesting to note that Schäfer, *et al.*,<sup>31</sup> found that radioactive iron added to a solution of milk whey proteins produced a pale red color and that the labeled iron moved as a single band in paper electrophoresis with a mobility similar to serum siderophilin. However, it has been reported by Gugler, *et al.*,<sup>32</sup> that the red protein of milk is not present in bovine serum.

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(30) J. K. Inman, 10th Conference on Plasma Protein, p. 46, January 1956.

(31) K. H. Schäfer, A. M. Brejer, W. Horst, H. Karte and W. Lenz, *Klin. Wochschr.*, **34**, 300 (1956).

(32) E. Gugler, M. Bein and G. vonMural, *Schweiz. Medizinische Wochenschrift*, **89**, 1172 (1959).

[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE, NEW YORK, N. Y.]

## Distribution Studies with Bovine Plasma Albumin<sup>1</sup>

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A solvent system composed of ethanol, *n*-propanol, ammonium sulfate and water is described for the countercurrent distribution of bovine plasma albumin. Due to the reactive sulfhydryl group of the albumin, it was found necessary to carry out the distribution with its *p*-mercuribenzoate derivative. The apparent inhomogeneity of the albumin was shown to be due to the presence of dimers and the dimer content could be lowered by treatment with thioglycolate.

Previous studies from this Laboratory have shown<sup>2</sup> that both bovine and human plasma albumin can be studied by countercurrent distribution in an acid system containing 2-butanol, ethanol, water, acetic acid and trichloroacetic acid. Although the  $pH$  of the system was low, 2.54, stabilization appeared at that time to be achieved by the effect of trichloroacetic acid.

Another interesting observation, apparently also an effect caused by the trichloroacetic acid, was a decided narrowing of the main band in the countercurrent distribution run. This occurred both with bovine and human plasma albumin. However, the partition coefficient ( $K$ ) value for each

of these protein preparations appeared to be very similar.

Shortly after this work was completed, another type of system made from a mixture of ethanol and ammonium sulfate solution<sup>3</sup> which provided a higher  $pH$  was studied with ribonuclease and lysozyme. The separations accomplished indicated this type of system to have considerable selectivity. More recent work (D. Eaker and T. P. King, unpublished results) has now shown a structural basis for the separation in that one of the ribonucleases separated differs from the main component by lack of the N-terminal lysine. Preliminary studies with this type of system modified slightly by addition of 1-propanol<sup>4</sup> on bovine and human

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(2) W. Hausmann and L. C. Craig, *THIS JOURNAL*, **80**, 2703 (1958).

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