

Reprinted from: PROTEIN-METAL INTERACTIONS

Edited by M. Friedman

Book available from: Plenum Publishing Corporation  
227 West 17th Street, New York, New York 10011

LACTOFERRIN CONFORMATION AND METAL BINDING PROPERTIES

R. M. Parry, Jr. and E. M. Brown\*

Eastern Regional Research Center, Agricultural Research

Service, U.S. Department of Agriculture, Phila., Pa. 19118

Lactoferrin<sup>†</sup> is an iron binding protein found in milk and other mammalian secretions. It has properties similar to the serum transferrins and to ovotransferrin of avian egg-white (reviewed by Feeney and Komatsu, 1966). These proteins specifically bind two moles of ferric ion per mole of protein. For each Fe(III) bound, one molecule of bicarbonate is incorporated into the complex (Masson and Heremans, 1968). All of these Fe(III) binding proteins have been found to have similar amino acid compositions, optical spectra and electron paramagnetic resonance spectra.

Two physiological roles have been proposed for the transferrin-like proteins. The primary role for serum transferrin is believed to be transport of iron to the reticulocytes for incorporation into the hemoglobin of red blood cells. In vivo, where the iron binding sites generally are not saturated, the protein may act also as a bacteriostatic agent. The same two functions have been proposed for ovotransferrin, but with the bacteriostatic function considered to be the more important. Evidence for the bacteriostatic function of lactoferrin has been provided by Bullen et al. (1972), but while direct evidence for an iron transport function is lacking, it has not been disproved.

Studies by Welty et al. (1972) on the lactoferrin concentration in the cow mammary gland during involution and mastitis infection indicated that lactoferrin rapidly increases under these

---

\*National Research Council Postdoctoral Research Fellow.

<sup>†</sup>Lactoferrin refers to the metal-free protein; iron(III)lactoferrin is the protein saturated with 2 moles ferric ion per mole protein.

conditions. Lactoferrin appears to be unique among the whey proteins in that its concentration changes roughly parallel the periods of resistance to infection in the mammary gland, so that its concentration is very low in normal milk whereas it is much higher in the lacteal secretion of the non-producing gland.

The iron binding proteins such as lactoferrin, ovotransferrin and transferrin are characteristically composed of a single polypeptide chain (Mann et al., 1970; Querinjean et al., 1971) and the body of evidence accumulated to date does not support the hypothesis that a repeating sequence occurs in the proteins (Bezkorovainy and Grohlich, 1973; Elleman and Williams, 1970). The proteins have been reported to be between 70,000 and 90,000 molecular weight. Several laboratories have found molecular weights near 76,000 for bovine lactoferrin (Castellino et al., 1971), human lactoferrin (Querinjean et al., 1971), ovotransferrin (Greene and Feeney, 1968), and human transferrin (Mann et al., 1970). Other workers have shown 86,000 molecular weights for bovine lactoferrin (Groves, 1960) and ovotransferrin (Fuller and Briggs, 1956; Elleman and Williams, 1970). The iron binding proteins are also characterized as having many intramolecular disulfide bonds, no free sulfhydryls, but sufficient bound carbohydrate to put them in the class of glycoproteins (Feeney and Komatsu, 1966).

The most apparent difference between these metal-binding proteins is their relative affinity for iron and the conditions necessary for in vitro removal of iron. The lactoferrins (bovine and human milk) apparently have a higher affinity for iron than ovotransferrin and transferrin (Aisen and Leibman, 1972). Furthermore, removal of the iron from lactoferrin requires exposure to pH 2, while metal-free forms of transferrin and ovotransferrin can be prepared at pH 4. Exposing the protein to pH 2 causes a distinct conformational change in transferrin and ovotransferrin (Feeney and Komatsu, 1966; Phelps and Cann, 1956). Dielectric dispersion measurements reported by Rosseneu-Motreff (1971) have shown that the saturation of transferrin with iron modifies the parameters of the ellipsoidal shape so that the molecule expands slightly, becoming more spherical after metal incorporation. Thus, the protein conformation appears to play a role in the metal complexation mechanism and needs to be considered in an explanation of the binding.

Studies on the amino acids involved in the metal binding site have shown that the more important residues include tyrosines and nitrogen ligands from either histidine or tryptophan residues. Warner and Weber (1953) first postulated that the tyrosyl phenolic groups were involved in this complex because of the release of three protons for each Fe(III) bound to transferrin. More recently, Michaud (1968) and Luk (1971) have found, by pH difference spectroscopy experiments, that four tyrosyl residues are involved in the

two metal binding sites of transferrin and ovotransferrin. The EPR studies of Aisen and Liebman (1972) have shown that at least one nitrogen plays a role at each metal binding site in lactoferrin and transferrin. The Mossbauer studies of Spertalian and Öosterhuis (1973) indicate that there are two or three nitrogen ligands for each iron in transferrin. At this writing, experimental methodology has not yielded any definitive evidence for which residue(s) contribute the nitrogen ligands.

Luk's (1971) novel studies using the fluorescent Tb(III)-Fe-(III) transferrin complex indicated that the two binding sites are about 43 Å apart. Recent reports by Aisen et al. (1973, see also his work in this volume) and Aasa (1972) have given the first evidence that the two metal-combining sites are nonequivalent in ovotransferrin and that the protein does not bind metals in a simple random fashion.

We have undertaken this study of bovine lactoferrin to obtain further information about its iron binding sites and the protein conformational changes which occur on metal complexation.

#### ISOLATION METHOD

Lactoferrin from bovine milk has not been studied as extensively as transferrin and ovotransferrin, probably because of its low concentration in normal milk. It has been reported (Groves, 1960) that lactoferrin concentration may be as low as 20 mg/l. of milk. Our experience with isolation of the protein from milk indicated considerable variation in the amount of lactoferrin present. It appeared that the level of lactoferrin present may be directly proportional to the level of other minor whey proteins such as bovine serum albumin and immune globulin proteins. Higher amounts of these proteins are often found in late lactation milk or in milk from a cow with a mastitis infection of the mammary gland. Higher levels of these proteins are also found when the mammary gland secretes colostrum immediately after calving. The lactoferrin concentration is at least ten-fold higher in colostrum but rapidly decreases in the following three to four days as the gland begins to produce normal milk.

The fluid expressed from the mammary gland of non-lactating cows was found to be the richest source of lactoferrin. This pre-lacteal secretion yielded purified protein in gram quantities (2 to 4 g/l. of fluid). A further advantage in using pre-lacteal secretion is that the lactoperoxidase concentration in this fluid is negligible. This enzyme, which has a molecular weight and an isoelectric point similar to lactoferrin, is a particularly difficult contaminant to remove from preparations. Samples of lactoferrin

prepared from milk or colostrum, in some cases, would be sufficiently contaminated with lactoperoxidase after purification to render them of questionable value in characterization studies.

The purification procedure is essentially that described by Groves (1965). Some modifications were made to speed this isolation. The nitrilotriacetate complex of iron(III) was added to most preparations at the beginning stages of purification so that all lactoferrin was saturated. Isolation from milk was begun by warming the milk to 30°, adjusting the pH to 4.6 to precipitate the casein, readjusting the whey to pH 6.5 and absorption of the positively charged proteins on Amberlite IRC-50 (approximately 50 g/l.). After stirring several hours at 4° the resin was recovered, washed with water and the proteins eluted with 0.2 M phosphate, pH 6.5. Solutions were dialyzed and concentrated by ultrafiltration.

The initial stages of the purification from colostrum differed slightly from that used for milk. First day post-partum colostrum was obtained and stored frozen until used; the viscous liquid was diluted with two volumes water per volume colostrum, the casein precipitated and the supernatant containing iron(III)lactoferrin was fractionated as described below.

Preliminary treatment of the prelacteal secretion with iron caused the viscous opaque fluid to turn distinctly pink. Addition of ammonium sulfate to 50% saturation precipitated unwanted protein. Solid ammonium sulfate was added to the supernatant to make it 75% saturated at 2°, a pink precipitate containing iron(III)lactoferrin was recovered and the precipitate was analyzed.

After these preliminary treatments for the three mammary secretions, all preparations were pumped through diethylaminoethyl (DEAE) cellulose columns, preequilibrated with 0.005 M phosphate, pH 8.2. This treatment effectively adsorbed most contaminating whey proteins and allowed the lactoferrin to pass through unadsorbed. The effluent was concentrated by ultrafiltration and chromatographed on a phosphocellulose column equilibrated with 0.1 M phosphate, pH 6.0 (2 x 40 cm) using a step-wise gradient of increasing pH and phosphate buffer concentration. Lactoferrin was eluted on the addition of 0.2 M phosphate - 0.1 M NaCl, pH 7.5, dialyzed and concentrated if necessary. Some iron was apparently removed from the protein during this chromatography and iron was again added to the preparation with the excess being removed by gel filtration on Sephadex G-25.

Metal-free lactoferrin was prepared according to the method of Johanson (1960) by titrating the solution to pH 2, whereupon the solution became colorless. The free iron was then adsorbed on Dowex 50-12X resin. Rapid titration of the protein back to pH 6.5 gave good spectroscopic-quality lactoferrin solutions. This method

leaves not more than 0.1 mole of iron per mole of lactoferrin. Iron was determined colorimetrically as the 1,10-phenanthroline complex after protein digestion with sulfuric acid and iron reduction with hydroxylamine hydrochloride (Snell and Snell, 1959).

#### MATERIALS AND METHODS

Sedimentation velocity experiments were carried out in a Spinco Model E ultracentrifuge with schlieren optics. Normally 15 mm, 4° sector, Kel F centerpieces were used, but protein concentrations below 2 g/l. were studied with 30 mm centerpieces. Stock protein solutions for all ultracentrifuge experiments were passed through Sephadex G-25, previously equilibrated with the appropriate solvent, and then dialyzed 18-24 hr against that solvent. Dilutions of the protein stock solution were made with the dialyzate.

Sedimentation equilibrium experiments were carried out using the Yphantis method (1964). The ultracentrifuge was equipped with interference optics, focused at the 2/3 plane of the cell, and photographs made on II G spectroscopic glass plates. The sample cell was modified for external loading as described by Ansevin et al. (1970). Fringe measurements were made on a Nikon comparator and a pre-blank and post-blank measured to insure no change in cell distortion during the experiment. Commonly the final speed chosen for the run was 20,410 RPM. At the start of the experiment the sample was run at  $2\omega^2$  for 1/2 hr and  $2/3\omega^2$  for 1/2 hr before setting the final speed. Under these conditions, equilibrium was reached within 18 hr. The molecular weights were calculated according to the method of Roark and Yphantis (1969) using Dr. Roark's computer program.

Extinction coefficients were determined for lactoferrin in water using optical density measurements on a Zeiss PMQ II spectrophotometer. The protein was dried to a constant weight in vacuo at 50°, usually for about 48 hr.

Partial specific volume measurements were made at 25° on a Paar densimeter in water. Solvent densities were also measured during the time of the ultracentrifuge experiments. Solvent viscosities were taken from the International Critical Tables.

Gel electrophoresis did not prove to be a reliable test for purity of lactoferrin, particularly with regard to lactoperoxidase contamination. The critical test of purity used for this study was based upon the visible and ultraviolet spectra because of the distinctive Soret band of lactoperoxidase. Absorbance ratios of  $A_{280}/A_{465} = 27$  to 28 and  $A_{410}/A_{465} = 0.8$  to 0.85 were considered acceptable values for purified lactoferrin.

Long-term storage has been a continuing problem with lactoferrin. Storage as the frozen 2% protein solution either with or without metal for two months or more caused the formation of turbidity which increased with time. Lyophilization also produced turbid solutions when the protein was redissolved, but the instability did not depend on the length of storage. We chose the lyophilization method for iron(III)lactoferrin which was stored desiccated at 4°, removing the turbidity with Millipore filters (3  $\mu$  pore size). Metal-free protein was prepared immediately before use.

Ultra Pure urea and guanidine hydrochloride were purchased from Schwarz-Mann Biochemicals. Mercaptoacetic acid (Aldrich) was freshly distilled immediately before use. Other chemicals were the best available grades and were used without further purification. Glassware was cleaned with chromic acid-sulfuric acid cleaning solution, rinsed with water, then soaked in concentrated nitric acid and rinsed with copious amounts of water to avoid heavy metal contamination. The water used in this study was doubly deionized and was the best available with respect to conductivity, metal contamination, fluorescence, and absorbance. It compared favorably with water which was deionized and then glass distilled.

Cobalt-, chromium-, and manganese-protein complexes were prepared by the method of Aisen et al. (1969), except that chromic chloride rather than chromous chloride was used for the formation of Cr(III)lactoferrin.

For spectrophotometric experiments, solutions were 0.1 molar in KCl in addition to the specified buffers or perturbants. Neutral, aqueous solutions were either unbuffered at pH 6.5-7.5, or buffered at pH 7.0 with 0.005 M N-(2-hydroxyethyl piperazine)-N'-2-ethane sulfonic acid (HEPES). No differences were noted in the presence or absence of the buffer. Adjustments of pH were made by dilution with appropriate amounts of KCl-KOH or KCl-HCl mixtures.

Absorption spectra were obtained with a Cary Model 14 recording spectrophotometer and single wavelength absorbances with a Zeiss PMQ-II. Beer's law was shown to hold over all practical concentrations. Difference spectra involving only pH changes were obtained using matched 1 cm cuvettes. The effects of urea, guanidine and mercaptoethanol on the difference spectrum were obtained using tandem 1 cm cells so that protein and solvent concentrations could be matched.

Solvent perturbation difference spectra were obtained using a minimum of two different concentrations of iron(III)lactoferrin in aqueous solution, lactoferrin in aqueous solution and in 8 M urea, and 8 M urea solution of lactoferrin which had been disulfide cleaved with mercaptoacetic acid by the method of Herskovits and

Laskowski (1962). Concentrations were selected so that  $A_{280}$  was between 1.0 and 2.2. The perturbants used were sucrose, ethylene glycol, glycerol, and dimethylsulfoxide in aqueous and 8 M urea solutions with a final perturbant concentration of 20%. The data were obtained and analyzed using the method of Herskovits and Sorensen (1968a,b). Spectra were recorded with the Cary 14 at 5 Å per second, dynode setting = 3, slit control = 25; slit width was not more than 0.7 mm at wavelengths greater than 250 nm.

The circular dichroism (C.D.) spectra were recorded using a Jasco Model ORD/UV/CD-5 or a Cary 60 recording spectropolarimeter with a Model 6002 circular dichroism attachment. Protein concentrations and optical path lengths were adjusted to keep the photomultiplier voltage below 900 volts. C.D. data in the 600-300 nm region is expressed as the mean molar ellipticity  $[\theta]$  deg cm<sup>2</sup> per decimole of iron bound, and below 300 nm in terms of protein concentration as the mean molar ellipticity  $[\theta]_{MRW}$ , using 113 as the mean residue weight.

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer with excitation at 280 nm and emission 345-360 nm.

## RESULTS AND DISCUSSION

### Sedimentation Velocity

Sedimentation velocity experiments were made on lactoferrin in three different states: iron(III)lactoferrin in 0.1 M KCl-HEPES at pH 6.5; lactoferrin in 0.1 M KCl-HEPES at pH 6.5; and lactoferrin in 0.1 M KCl-HCl at pH 2. These results, corrected to 20° and water and extrapolated to zero protein concentration are presented in Figure 1. Non-ideal molecular electrostatic repulsion effects are quite apparent at lactoferrin concentrations below 3 g/l., but these effects could be negated by the use of an effective buffer in the experiments done at neutral pH. The lactoferrin appears to resist further conformational changes with time as a result of the pH 2 treatment since two of these experiments were repeated after 48 hr at 4° and gave essentially the same  $S_{20,w}$  values (within 0.01 svedbergs) as the freshly prepared solutions. Varying the temperature and/or the ionic strength of the solvent had no effect upon the corrected sedimentation coefficient.

The decrease in  $S_{20,w}$  from the neutral to the acid protein solutions indicates marked conformation change for the lactoferrin. Spectroscopic studies showed the shape change to be completely reversible and there is no loss of iron binding capability when the lactoferrin is returned to neutrality. A similar change in sedimentation coefficient of ovotransferrin when studied as a

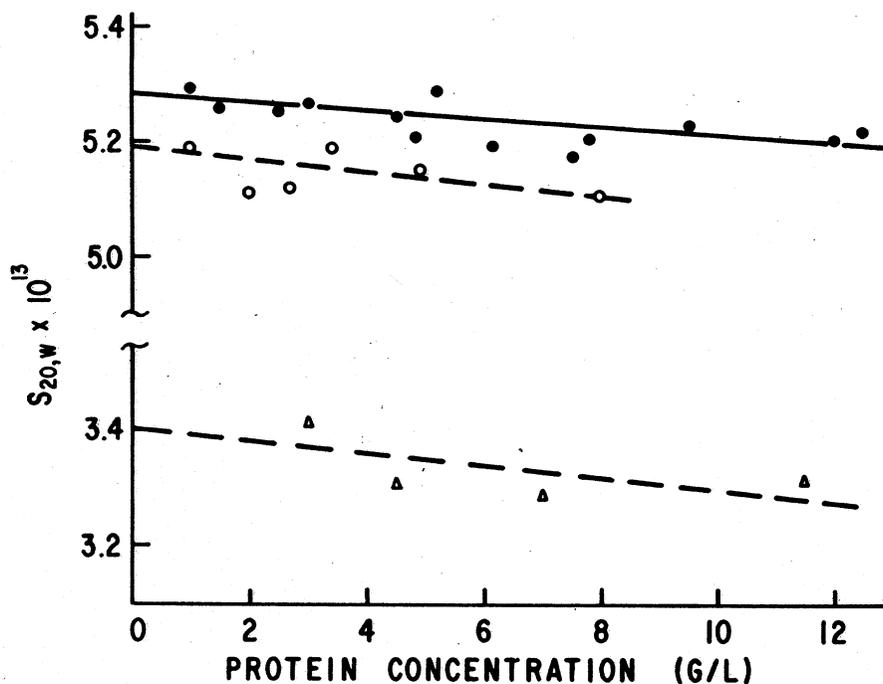


Figure 1. Sedimentation coefficients corrected for solvent and temperature plotted versus protein concentration. Top curve (—) is iron(III)lactoferrin at pH 6.5 with the data indicated by filled circles. Middle curve (---) is lactoferrin at pH 6.5 with the data indicated by open circles. Bottom curve (- - -) is lactoferrin at pH 2.0 with data indicated by open triangles.

function of pH has been reported (Glazer and McKenzie, 1963; Phelps and Cann, 1956).

A small but experimentally consistent difference in the  $S_{20,w}^{c=0}$  between lactoferrin solutions with and without iron was observed (see Table 1). Under our experimental conditions, we were able to achieve an experimental precision of  $\pm 0.05$  svedbergs. Frictional coefficients were calculated according to the equation of Svedberg and Pedersen (1940) for the lactoferrin in the three states and are reported in Table 1. These data indicate a deviation from a spherical shape in agreement with Fuller and Briggs (1956) who

TABLE I

## Physical Measurements of Lactoferrin

	Iron(III)lactoferrin pH 6.5	Lactoferrin pH 6.5	Lactoferrin pH 2
$\epsilon_m$	$1.35 \times 10^5$	$1.09 \times 10^5$	-
$\bar{V}_{25^\circ, \text{app.}}$	0.729 ml/g	0.723 ml/g	-
$S_{20, w}^{c=0}$	$5.27 \pm .05 \text{ S}$	$5.19 \pm 0.05 \text{ S}$	$3.40 \pm 0.05 \text{ S}$
$f/f_o$	1.34	1.40	$2.13^a$
$M_w$	$86,000 \pm 800$	$86,000 \pm 900$	-

$\epsilon_m$ , molar extinction coefficient;  $\bar{V}_{25, \text{app.}}$ , apparent partial specific volume at 25°;  $S_{20, w}^{c=0}$ , sedimentation coefficient corrected to 20°, water and extrapolated to infinite dilution ( $S=10^{-13} \text{ sec}^{-1}$ );  $f/f_o$ , frictional coefficient;  $M_w$ , weight average molecular weight.

<sup>a</sup>Calculated using the  $\bar{V}_{25, \text{app.}}$  of lactoferrin at pH 6.5.

found iron(III)ovotransferrin to be more spherical than the metal-free protein. Rosseneu-Motreff et al. (1971) have also observed a shape difference between iron saturated and metal-free human serum transferrins. Using dielectric dispersion measurements, they found that the iron binding to transferrin resulted in a slight expansion of the molecule causing it to become more spherical as the axial ratio decreased from 2.5 to 2. The actual nature of the shape change seen with lactoferrin can not be assessed at this time, except to note that the metal does affect the protein structure.

## Sedimentation Equilibrium

The molecular weight of iron(III)lactoferrin and lactoferrin in 0.1 M KCl-HEPES, pH 6.5 was determined in sedimentation equilibrium experiments. A plot of the raw apparent weight average

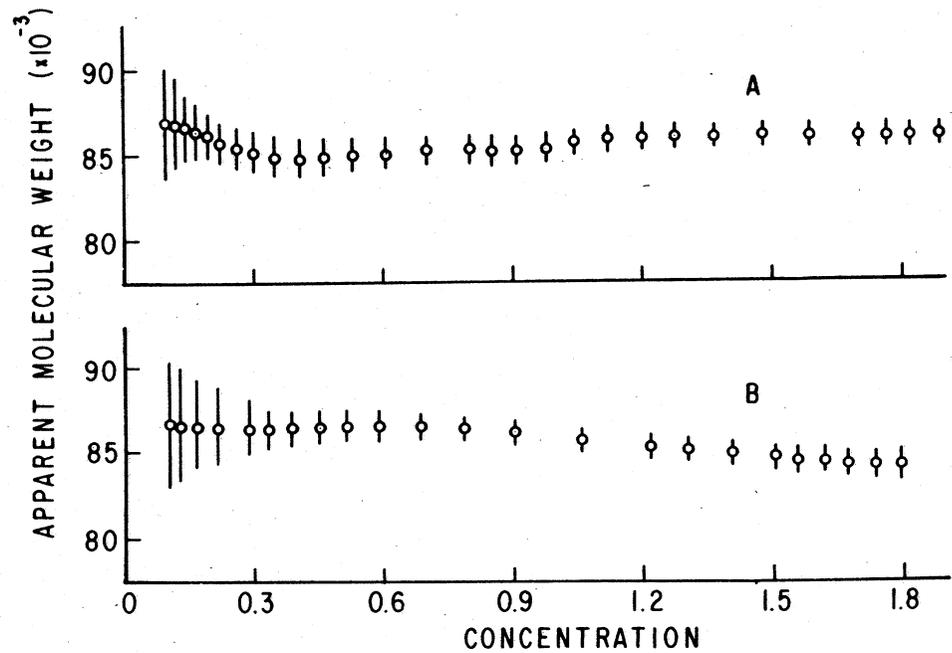


Figure 2. Sedimentation equilibrium plot of the apparent weight average molecular weight versus protein concentration expressed as fringe displacement. Curve A is lactoferrin, pH 6.5, and curve B is iron(III)lactoferrin, pH 6.5.

molecular weight data versus protein concentration in arbitrary fringe displacement units is presented in Figure 2. Each value represents the mid-point molecular weight moment at that position in the ultracentrifuge cell and the range indicated about each point has a confidence level of about 90%. Lactoferrin with and without iron(III) gave a molecular weight of 86,000. The molecular weight is in agreement with the value reported by Groves (1960) but is higher than the values obtained by Castellino et al. (1970) and Querinjean et al. (1971) who reported values of about 76,000 g. Similar flat curves were also obtained for the N and Z average molecular weights, indicating that lactoferrin exists as monomeric non-aggregating protein species at pH 6.5 either with or without Fe(III).

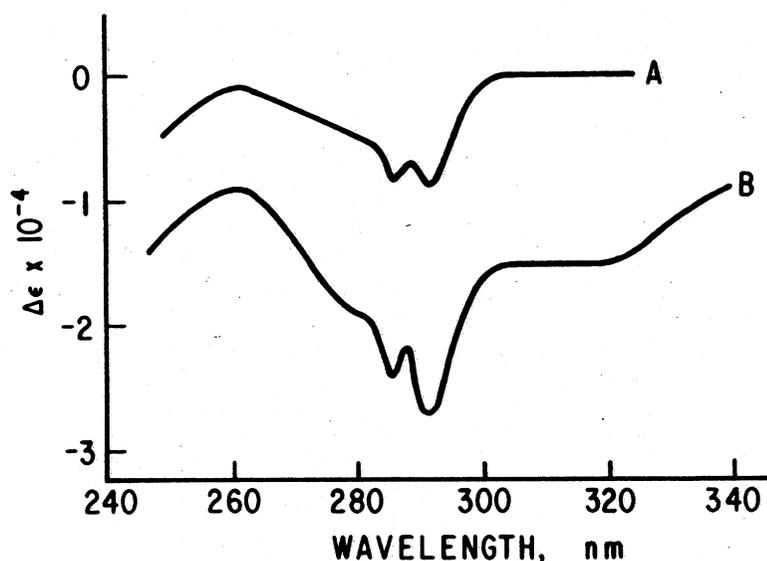


Figure 3. Difference spectra of (A) lactoferrin and (B) iron(III)-lactoferrin in acid solution. In each case the sample solution is at pH 2 and the reference at pH 6.5-7.5. Protein concentrations are  $10^{-5}$  to  $10^{-6}$  molar.

#### Absorption Spectra

Iron(III)lactoferrin and lactoferrin have an absorption maxima at 280 nm in 0.1 M KCl, pH 6.5. This maximum is shifted to 277-8 nm when the solution is made in 6 M guanidine, 8 M urea or has been titrated to pH 2 with HCl. The blue shift in the aromatic region is accompanied by a general decrease in the absorptivity in the 330-250 nm region.

Difference spectra show minima at 292 and 286 nm, characteristic of the transfer of tryptophan and tyrosine residues from a hydrophobic portion of the molecule into the aqueous phase. Acid difference spectra for the two forms are shown in Figure 3. The effects of guanidine on the spectrum of lactoferrin are immediate, but in the case of iron(III)lactoferrin spectral changes are seen to increase over a several hour period. An additional shoulder

appears in the 315-300 nm region of the difference spectrum of iron(III)lactoferrin which may be due to distortion of disulfide bonds (Tan, 1971) or perturbation of tryptophan residues by the iron (Beychok, 1966), or may simply be characteristic of an iron-oxygen bonding. The maximum change as a function of guanidine concentration was achieved with a 2 M solution in the case of lactoferrin and a 6 M solution for iron(III)lactoferrin. The guanidine, urea, and acid effects on the spectrum of lactoferrin were completely reversible. Glazer and McKenzie (1963) found similar results with ovotransferrin and interpreted them as corresponding to a reversible conformational change or unfolding of the molecule.

#### Circular Dichroism

The 600-300 nm region of the C.D. spectrum of iron(III)lactoferrin in 0.1 M KCl, pH 6.5 showed a broad negative band centered at 455 nm and a narrower positive band or possibly an unresolved

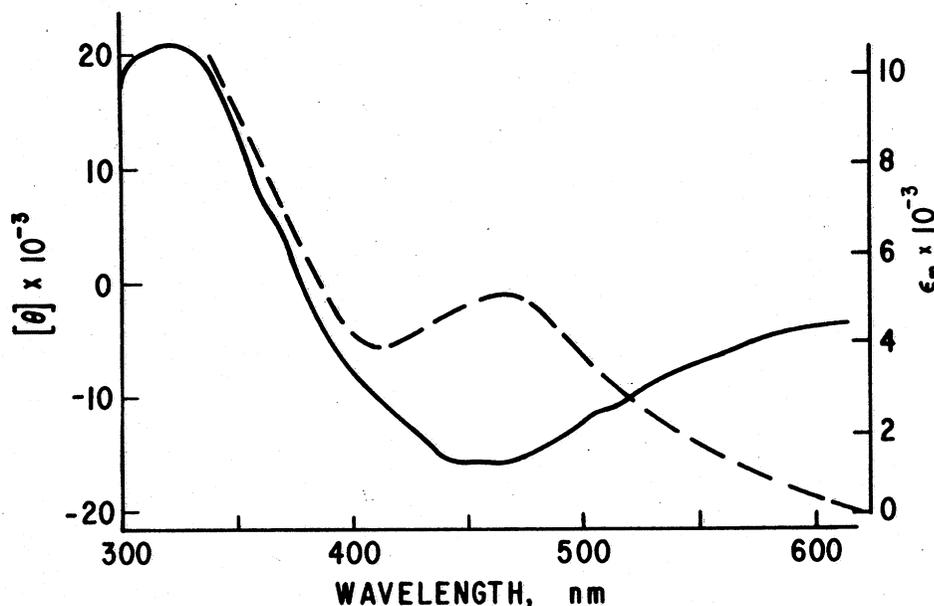


Figure 4. Visible C.D. spectrum (—) and absorption spectrum (---) of iron(III)lactoferrin in aqueous solution. The C.D. spectrum is the average of six experiments; two each in water, 0.1 M KCl, and 0.005 M HEPES all at pH 6.5-7.5.

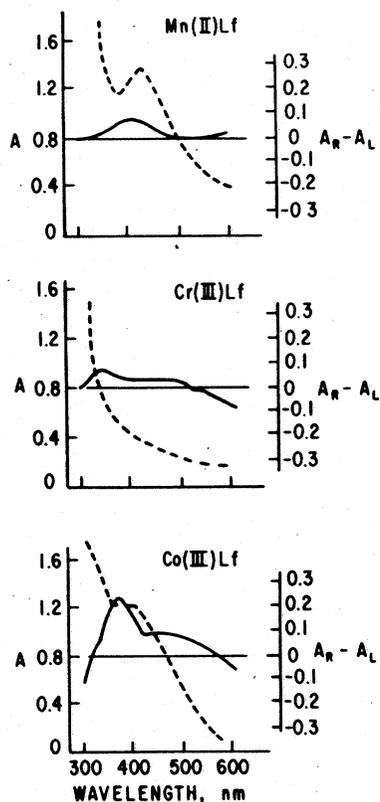


Figure 5. Visible absorption (---) and C.D. (—) spectra of manganese(II), cobalt(III), and chromium(III)lactoferrin complexes. The left axis is labeled in absorption units (A) and the right in C.D. units ( $A_R - A_L$ ). These are relative values only.

doublet in the 330-310 nm region (Figure 4). The 455 nm band corresponds to the absorption maximum near 460 nm and is similar both in position and intensity to those reported for iron(III)ovotransferrin (Tan, 1971) and iron(III)transferrin (Nagy and Lehrer, 1972). The visible C.D. spectra of the manganese, cobalt and chromium lactoferrin derivatives with their respective absorption spectra are shown in Figure 5. The shape of the visible absorption spectra of these various metal derivatives are characteristic of some aqueous octahedral complexes of Mn(II), Co(III), and Cr(III) (Cotton and Wilkinson, 1966).

Tan (1971) has suggested that the 320 nm C.D. band in iron(III)ovotransferrin may be due to a change in the dihedral angle of one or more of the disulfide bonds when iron is bound. Beychok (1966) has observed C.D. spectra of perturbed tryptophan in proteins at wavelengths as high as 320 nm. Though either of these explanations is possible, one should consider that if the manganese, cobalt, and chromium are bound to the protein at the same site as the iron, similar disulfide or tryptophan perturbations would be expected which were not found here.

The C.D. spectra of lactoferrin and iron(III)lactoferrin in the aromatic region of 310-250 nm closely resemble those of ovotransferrin and its iron complex as reported by Tan (1971) and Gaffield et al. (1966). Iron binding has little effect on the shape of the curve (Figure 6). Ellipticities for iron(III)lactoferrin are more positive than those for lactoferrin, particularly above 290 nm where the ellipticities for iron(III)lactoferrin are themselves positive. The aromatic spectra of the manganese, cobalt, and chromium complexes of lactoferrin are identical, within experimental error, with that of metal-free lactoferrin. Studies by Tan (1971), Tomimatsu and Vickery (1972) and Nagy and Lehrer (1972)

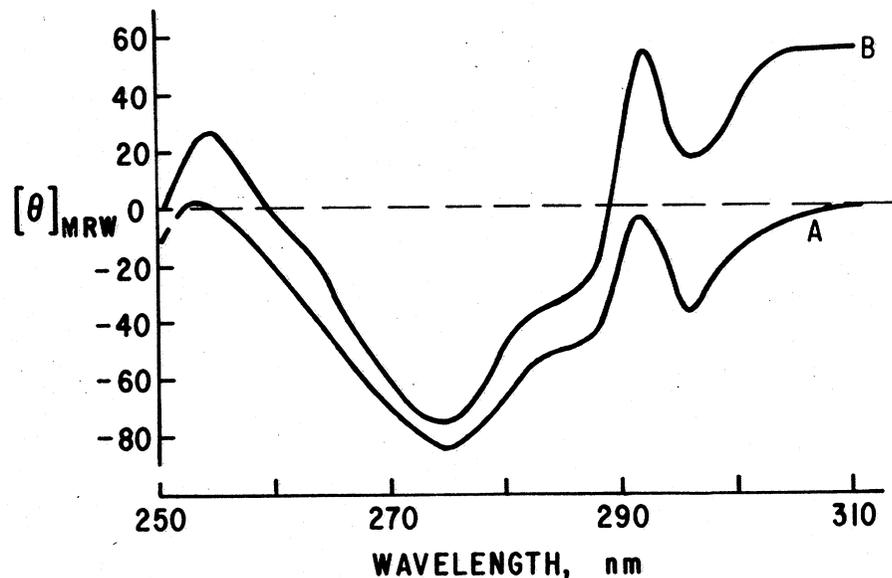


Figure 6. Near ultraviolet C.D. spectra of iron(III)lactoferrin (B) and lactoferrin (A) in neutral aqueous solution. Each curve is an average of 8 to 10 experiments with varying protein concentrations and buffer conditions.

showed that the binding of non-ferrous metals by ovotransferrin and transferrin did not affect the shape of the aromatic C.D. spectra. Analysis of the 290-250 nm portion of the C.D. spectrum in terms of specific residues is complicated by the lack of resolution, but it is a composite of the effects of tyrosine, tryptophan, phenylalanine, and disulfide linkages. Caution must be used in attributing the positive ellipticities in the 300-290 nm region to any direct iron-tryptophan interaction as they may well be due to overlap of the strong 320 nm band.

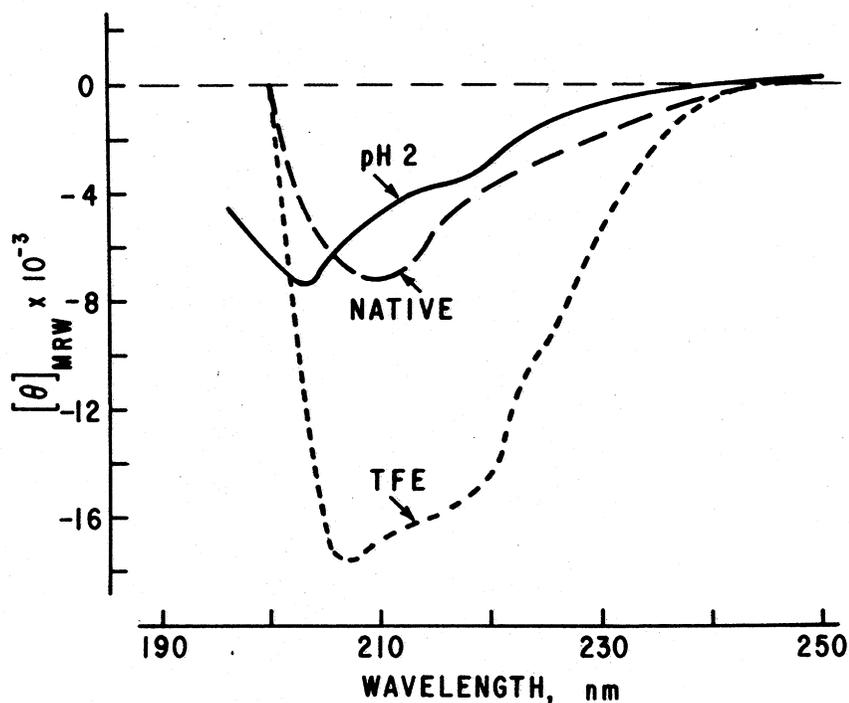


Figure 7. Far ultraviolet C.D. spectra of lactoferrin. Native (—) is either lactoferrin or iron(III)lactoferrin in neutral aqueous solution, an average of 10 experiments each. The pH 2 spectrum (---) is for lactoferrin in an aqueous 0.1 M KCl-HCl mixture at pH 2.0, an average of 6 experiments. The TFE spectrum (· · ·) is for lactoferrin in neutral aqueous solution diluted to give a solution which is 90% trifluoroethanol, an average of 2 experiments.

In the far ultraviolet (240-190 nm) where the C.D. spectrum is dependent primarily on protein conformation, the spectra of iron(III)lactoferrin and lactoferrin in neutral aqueous solution are indistinguishable (Figure 7). The computational method of Chen et al. (1972) using a mean residue weight of 113 gives about 15% helix and 50% unordered structure. The helical content of transferrin and ovotransferrin as calculated by this method range between 15% and 21% (Tomimatsu and Vickery, 1972). Addition of acidic methanol and trifluoroethanol to lactoferrin solutions increases the helical content to about 25% and 40%, respectively, showing that the low helicity of the native protein is not due entirely to the rigidity imparted by disulfide bonds. In solutions of 6 M guanidine, 8 M urea, or in aqueous acid solution (pH 2), the far ultraviolet C.D. spectrum is that of a completely unordered protein. These solvents also affect the near ultraviolet (aromatic) region of the C.D. spectrum by decreasing the magnitude and resolution of the bands. The effect of guanidine on the 300-290 nm portion of the C.D. spectra is shown in Figure 8. The iron(III)lactoferrin is more resistant to the effects of guanidine than is the metal-free lactoferrin.

#### Fluorescence

The intensity of the fluorescent emission from iron(III)lactoferrin in 0.1 M KCl-HEPES at pH 6.5 was about 50% of that for lactoferrin under the same conditions. The emission shifted to the red and became more intense at low pH or when guanidine was added to the solution. The increase in the relative fluorescence intensity for lactoferrin was linear with decreasing pH to pH 2, or when increasing the guanidine concentration to 6 M. On the other hand, the fluorescence of iron(III)lactoferrin showed no linear response when studied as a function of pH or of guanidine concentration. A greater apparent structural stability was seen with the metal-bound protein since no change in fluorescent intensity was observed until pH 4 at which point it rapidly increased to a maximum at pH 2. Similarly no change was observed until the concentration of guanidine reached 3 M; maximum change was seen at a 6 M concentration.

#### CONCLUSIONS

The current study on the physical and conformational properties of bovine lactoferrin has shown that this protein exists as a non-aggregating species with a molecular weight of 86,000 in 0.1 M KCl-HEPES at pH 6.5.

The evidence presented here suggests some conformation differences between lactoferrin and iron(III)lactoferrin. Sedimentation

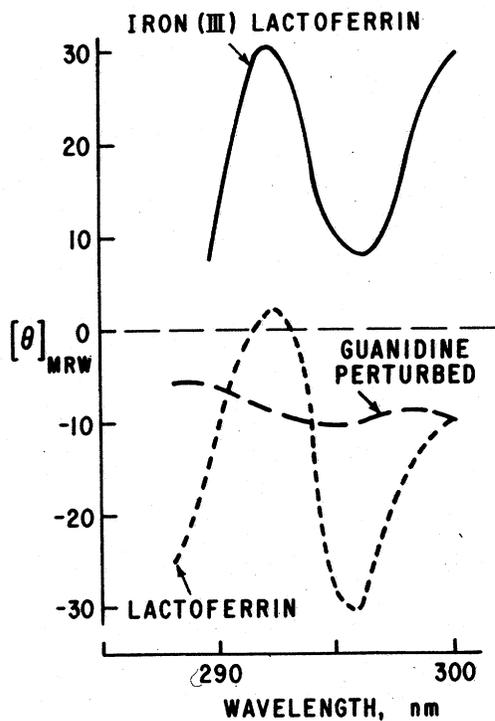


Figure 8. The effect of guanidine on the C.D. bands 300-290 nm of lactoferrin. The guanidine perturbed spectrum (— · —) represents lactoferrin in 2 M guanidine or iron(III)lactoferrin in 6 M guanidine.

velocity experiments showed a small change in the  $S_{20,w}^{C=O}$  and  $f/f_0$  indicating a more spherical shape for iron(III)lactoferrin. Spectroscopic and fluorescence experiments showed that the metal-bound protein was much more resistant to perturbation by acid (on approach to pH 2) or by increasing guanidine concentrations. The metal apparently does not greatly change the spectroscopically observed native conformation, but it does produce a more stable conformation.

No conclusive evidence was obtained about the role of tryptophan as being the residue contributing the nitrogen ligand for iron coordination. Other studies from this laboratory (Brown and Parry) using pH difference spectroscopy indicated four tyrosine residues may be involved in the metal-binding site.

No significant difference in secondary structure was found between iron(III)lactoferrin and lactoferrin, and the calculated helical content of 15% was similar to that reported for ovotransferrin and transferrin. The three iron binding proteins thus have remarkably similar secondary structure which was little affected by metal binding.

A much larger reversible conformational change, seen as an apparent unfolding of lactoferrin, occurred in the presence of acid, urea or guanidine. This change could be observed spectroscopically when access of solvent to chromophoric groups increased, as seen in a hypsochromic shift in the absorption maximum, a negative difference spectra, a decrease in helical structure, and a lowering in the sedimentation coefficient from 5.3 to 3.4.

The fluorescence spectrum of lactoferrin appears to be primarily due to the tryptophan residues. The increase in relative fluorescence intensity which occurred upon lowering the pH, also indicated a protein conformational change. The emission red shift may indicate less tyrosine-tryptophan interaction, i.e. a more "pure" tryptophan fluorescence.

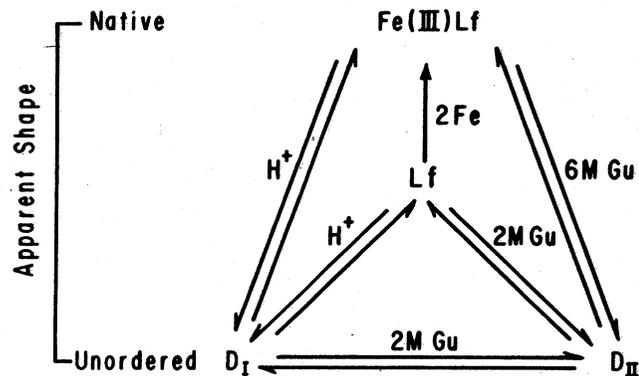


Figure 9. A summary flow sheet of bovine lactoferrin conformation changes. See text for a detailed explanation.

The shape changes observed in this study are summarized by way of a flow diagram in Figure 9. Acid addition to iron(III)lactoferrin to pH 2 causes an unfolding of the molecule and expedites iron removal shown as D<sub>I</sub>. Similarly, 6 M guanidine unfolds the protein yielding protein form D<sub>II</sub>. Our spectroscopic work showed no structural difference between D<sub>I</sub> and D<sub>II</sub>. Reduction of the disulfide bonds did not yield any evidence of further structure perturbation according to C.D. or U.V. difference spectroscopy. The metal-free protein unfolds at a lower concentration of guanidine and a small shape change was observed in the presence of bound iron as discussed above. Hence, iron binding capability of lactoferrin appears to be related to the molecular shape of the protein.

#### ACKNOWLEDGMENTS

We wish to express our thanks to T. T. Herskovits, Fordham University, for the use of his Cary 60-6002 and many helpful discussions, to V. G. Metzger, ERRC, for her help with the computer analysis of the data, and to Kathleen Gentilcore for her excellent technical assistance.

#### REFERENCES

- Aasa, R.(1972). *Biochem. Biophys. Res. Commun.* 49:806.  
Aisen, P., Aasa, R. and Redfield, A.G.(1969). *J. Biol. Chem.* 244: 4628.  
Aisen, P., Lang, G. and Woodworth, R.C.(1973). *J. Biol. Chem.* 248:649.  
Aisen, P. and Leibman, A.(1972). *Biochim. Biophys. Acta* 257:3144.  
Ansevin, A.T., Roark, D.E. and Yphantis, D.A.(1970). *Anal. Biochem.* 34:237.  
Beychok, S.(1966). *Science* 154:1288.  
Bezkorovainy, A. and Grohlich, D.(1973). *Biochim. Biophys. Acta* 310:365.  
Brown, E. M. and Parry, R. M., Jr. Submitted to *Biochemistry*.  
Bullen, J.J., Rogers, H.J. and Leigh, L.(1972). *Brit. Med. J.* 1:69.  
Castellino, F.J., Fish, W.W. and Mann, K.G.(1970). *J. Biol. Chem.* 245:4269.  
Chen, Y.-H., Yang, J.T. and Martinez, H.M.(1972). *Biochemistry* 11:4120.  
Cotton, F.A. and Wilkinson, G.(1966). *Advanced Inorgan. Chemistry*, 2nd rev. ed., Interscience, New York, N. Y., chapter 29.  
Elleman, T.C. and Williams, J.(1970). *Biochem. J.* 116:515.  
Feeney, R.E. and Komatsu, S.K.(1966). *Struct. Bonding* 1:149.  
Fuller, R.A. and Briggs, D.R.(1956). *J. Am. Chem. Soc.* 78:5253.  
Gaffield, W., Vitello, L. and Tomimatsu, Y.(1966). *Biochem. Biophys. Res. Commun.* 25:35.

- Glazer, A.N. and McKenzie, H.A.(1963). *Biochim. Biophys. Acta* 71: 109.
- Greene, F.C. and Feeney, R.E.(1968). *Biochemistry* 7:1366.
- Groves, M.L.(1960). *J. Am. Chem. Soc.* 82:3345.
- Groves, M.L.(1965). *Biochim. Biophys. Acta* 100:154.
- Herskovits, T.T. and Sorensen, M.(1968a). *Biochemistry* 7:2523.
- Herskovits, T.T. and Sorensen, M.(1968b). *Biochemistry* 7:2533.
- Johanson, B.(1960). *Acta Chem. Scand.* 14:510.
- Luk, C.K.(1971). *Biochemistry* 10:2838.
- Mann, K.G., Fish, W.W., Cox, A.C. and Tanford, C.(1970). *Biochemistry* 6:1348.
- Masson, P.L. and Heremans, J.F.(1968). *Eur. J. Biochem.* 6:579.
- Michaud, R.L.L.(1968). Ph.D. thesis, University of Vermont, Burlington, Vt.
- Nagy, B. and Lehrer, S.S.(1972). *Arch. Biochem. Biophys.* 148:27.
- Phelps, R.A. and Cann, J.R.(1956). *Arch. Biochem. Biophys.* 61:51.
- Querijnjean, R., Masson, P.L. and Heremans, J.F.(1971). *Eur. J. Biochem.* 20:420.
- Roark, D.E. and Yphantis, D.A.(1969). *Ann. N.Y. Acad. Sci.* 164:245.
- Rosseneu-Motreff, M.Y., Soetewey, F., Lemote, R. and Peeters, H. *Biopolymers* 10:1039.
- Snell, F.D. and Snell, C.T.(1959). *Colorimetric Methods of Analysis*, Vol. 2A, 3rd ed., D. Van Nostrand Co., Princeton, N.J., p. 231.
- Spartalian, K. and Oosterhuis, W.T.(1973). *J. Chem. Phy.* 59:617.
- Svedberg, T. and Pedersen, K.O.(1940). *The Ultracentrifuge*, Oxford Press, London, p. 40.
- Tan, A.-T.(1971). *Can. J. Biochem.* 49:1071.
- Tomimatsu, Y. and Vickery, L.E.(1972). *Biochim. Biophys. Acta* 285:72.
- Warner, R.C. and Weber, I.(1953). *J. Am. Chem. Soc.* 75:5094.
- Welty, F.K., Schanbacher, F.L. and Smith, K.L.(1972). *Dairy Research*, Ohio Agri. Res. Dev. Center. Research summary No. 59, 23.
- Yphantis, D.A.(1964). *Biochemistry* 3:297.

Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.