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Biochemical Characterization of Gastroferrin*

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ABSTRACT: Gastroferrin is a glycoprotein of human gastric juice which may be operative in the regulation of iron absorption. This protein has been purified and characterized using a variety of physical and chemical techniques. Although it migrates as a single peak in the analytical ultracentrifuge, it can be fractionated using DEAE-cellulose chromatography into several macromolecular species which appear to differ only in their sialic acid content. Gastroferrin binds 6% by weight of iron, and removal of sialic acid does not affect its iron binding capacity. The polypeptide portion of the glycoprotein, 15% by weight of the molecule, is rich in threonine, proline, and serine. The remaining 85% of the molecule is predominantly carbohydrate and consists of galactose, fucose and the acetyl derivatives of sialic acid, glucosamine, and galactosamine. Analytical ultracentrifugation gave a molecular weight of 2.63×10^5 and a sedimentation constant, $s^0_{20,w}$, of 8.9 S. Osmometry, however, yielded a slightly lower value of 2.35×10^5 for the number-average molecular weight indicating a small degree of microheterogeneity. Viscosity measurements demonstrated that gastroferrin was very asymmetric with an intrinsic viscosity, $[\eta]$, of 0.39 dl/g, and a frictional ratio of 2.2. Combination of viscosity data and sedimentation constant provided another independent estimate of molecular weight, 2.78×10^5, in good agreement with the value from sedimentation equilibrium. The immune behavior and iron binding of gastroferrin have been compared with those of soluble blood group substances from ovarian cyst fluid.

Equilibrium binding and chelation of ferric iron appear to be the primary factors in the regulation and control of the transport of iron by intestinal mucosa (Forth *et al.*, 1965; Hopping and Ruliffson, 1966; Helbock and Saltman, 1967; Spiro and Saltman, 1969). Both Fe^{2+} and Fe^{3+} are utilized when bound to a small molecular weight chelate. The amount of iron available for absorption is a function of the competition for dietary iron between the low molecular weight chelates and macromolecular ligands in the lumen of the small intestine. Only the former are utilized by the body for iron absorption. The role of endogenous ligands secreted into the digestive tract in man was studied by Davis *et al.* (1967), who found in gastric juice a high molecular weight component excluded by Sephadex G-200 and capable of binding all the iron present in a typical daily diet. This component was called gastroferrin. Of particular interest was their observation that gastroferrin was absent or low in several cases of iron storage associated with hemochromatosis (Davis *et al.*, 1966), suggesting that the amount of this high molecular weight ligand regulated the uptake of dietary iron. Further, gastroferrin was present in lower concentrations in patients with iron deficiency anemia (Luke *et al.*, 1967) where enhanced iron uptake is observed.

We have been concerned with the isolation and purification of gastroferrin together with its biochemical characterization and the nature of its iron binding. In a preliminary communication (Davis *et al.*, 1969), we reported a simple method for its isolation from human gastric juice. This method is based on the coprecipitation of iron gastroferrin with ferric hydroxide followed by removal of iron and molecular sieving

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on Sephadex G-200. This article focuses on the biochemical nature of gastroferrin.

Experimental Section

Gastroferrin Preparation. Gastroferrin was prepared from pooled human gastric juice by the procedure of Davis *et al.* (1969). To the pooled gastric juice at pH 2.0 was added twice the amount of iron (as solid $FeCl_3 \cdot 6H_2O$) required to saturate its iron binding capacity. The mixture was titrated slowly with concentrated NH_4OH to pH 8.0. The precipitate was collected by centrifugation at 18,000*g* for 15 min and dissolved in a small volume of 12 N HCl. To this solution was added nitrilotriacetate sufficient to chelate all the iron used in the preparation. After titration to pH 7.0, the solution was dialyzed against running water to remove the Fe–nitrilotriacetate complex. This treatment with nitrilotriacetate was repeated twice and the removal of Fe–nitrilotriacetate completed by gel filtration on a Sephadex G-200 column equilibrated with 0.01 M $NaHCO_3$. Gastroferrin was collected in the excluded fraction. This method was also employed to isolate a high molecular weight iron binding material from hog gastric mucin powder (Wilson and Co. lot no. 111513).

Protein Concentration. The specific refractive increment for gastroferrin was determined as 1.50×10^{-5} dl/g using purified and lyophilized material. Subsequent gastroferrin concentrations were estimated using this value. Refractive index measurements were made on a Bausch and Lomb refractometer.

Analytical Ultracentrifugation. Sedimentation velocity and molecular weight analyses were carried out in a Beckman Spinco Model E analytical ultracentrifuge equipped with schleiren optics and RTIC temperature unit. Gastroferrin solutions of varying concentrations were prepared in 0.01 M Tris buffer (pH 7.5). Velocity sedimentation was performed at 59,780 rpm at 20° using a standard analytical cell fitted with a 12-mm, 4° KelF centerpiece, in an AN-D rotor. Metallographic plates were employed for recording the schleiren patterns.

For sedimentation equilibrium, the six-channel centerpiece described by Yphantis (1964) was used. Solutions of gastroferrin were centrifuged for 36 hr at 6800 rpm using the heavier AN-H rotor to maintain inertial stability at low speeds. The x and y coordinates of the refractive index gradients were determined from the photographic plate with a Scherr Tumico optical comparator. Molecular weights were calculated from the equilibrium data according to

$$M = \frac{\mathrm{d}(\ln H - \ln r)}{\mathrm{d}(r^2)} \frac{2RT}{(1 - \bar{v}\rho)\omega^2}$$

where r is the radial distance, ω is the angular velocity, $\bar{v}$ is the partial specific volume, ρ is the density of solvent, R is the gas constant, T is the absolute temperature, and M is the molecular weight. The values of H at increasing values of r were determined by measuring the height of the schleiren refractive index gradient relative to the solvent blank. Molecular weights, z average, were determined from the least-squares plot of $\ln H - \ln r$ *vs.* r^2. The equilibrium data were analyzed using a FORTRAN IV computer program (D. W. Hopkins, unpublished results).

The partial specific volume of gastroferrin was determined by both pycnometery and from its chemical composition (Cohn and Edsall, 1943; Gottschalk, 1966). Both methods were in excellent agreement and gave a value of 0.66 ml/g for the partial specific volume.

Osmometry. All osmotic pressure measurements were performed in a Mechrolab Model 503 high-speed membrane osmometer. The membranes were equilibrated with the solvent buffer (0.01 M Tris–0.1 M NaCl, pH 7.5) for at least 24 hr before installation in the osmometer. The quality of the membrane was judged by the reproducibility of solvent readings. Osmotic pressure was determined twice for each concentration and solvent readings were taken between each set of concentrations. All measurements were made at 20°. The number-average molecular weight, $\bar{M}_n$, was calculated from the Van't Hoff equation

$$\lim_{C \to 0} \pi/C = \frac{RT}{\bar{M}_n}$$

where C is the concentration in grams per liter, and π is the osmotic pressure in centimeters of height of solvent column.

Viscosity. These measurements were made with an Ostwald viscometer at 20° in 0.01 M Tris buffer (pH 7.5). Both the solvent and solution were made dust free by passing through a Millipore filter. Relative viscosity, η_{rel}, and specific viscosity, η_{sp}, were determined from the average flow times, t, and the densities, ρ

$$\eta_{sp} = \eta_{rel} - 1 = \frac{\rho t - \rho_0 t_0}{\rho_0 t_0}$$

where the subscript zero refers to the solvent. Intrinsic viscosity, $[\eta]$, was determined from the relationship

$$[\eta] = \lim_{C \to 0} \eta_{sp}/C$$

where C is the concentration of the protein in g/100 ml.

Carbohydrate Analysis. Gastroferrin was hydrolyzed with 2 N HCl for 10 hr in a sealed tube under N_2 atmosphere. Descending chromatography on Whatman No. 3MM paper was used for identification of sugars in the hydrolysate following the separation of amino and neutral sugars by ion-exchange chromatography on a Dowex 50-X-4 (H^+ form) column coupled to a Dowex 1-X-10 (formate form) column (Spiro, 1966). The solvent system used for development was 1-butanol–pyridine–0.1 N HCl (5:3:2, v/v). Development was usually carried out for 30 hr and the sugars were located on the chromatograms by the silver nitrate method (Neuberger and Marshall, 1966).

Total carbohydrate was determined colorimetrically by the phenol–sulfuric acid method (Dubois *et al.*, 1956). Neutral sugars were estimated on the intact protein with the anthrone reagent of Roe (1955). Galactose was used as a standard. Sialic acid was determined after hydrolysis with 0.1 M H_2SO_4 at 80° for 1 hr by the method of Warren (1959). It was also determined by the same method after release with neuraminidase in 0.1 M acetate–0.001 M $CaCl_2$ buffer (pH 5.6). Fucose was determined on unhydrolyzed

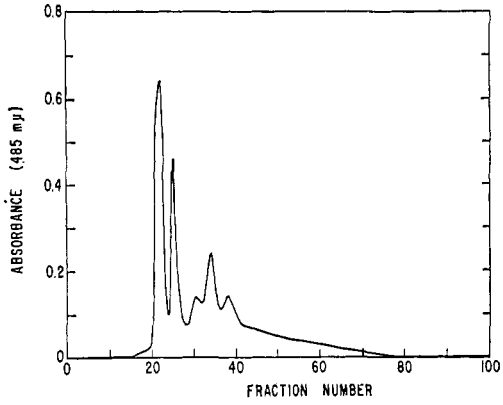

FIGURE 1: Elution profile of gastroferrin from DEAE-cellulose chromatography. The column (20 × 1.5 cm) was equilibrated with 5×10^{-3} M Tris buffer (pH 7.5) The protein was eluted with a 2-l. linear gradient of 0–0.5 M NaCl in the Tris buffer after washing the column with 100 ml of equilibration buffer. The wash and the eluent from the gradient were collected in 10-ml fractions and assayed colorimetrically for total carbohydrate by absorbance at 485 nm.

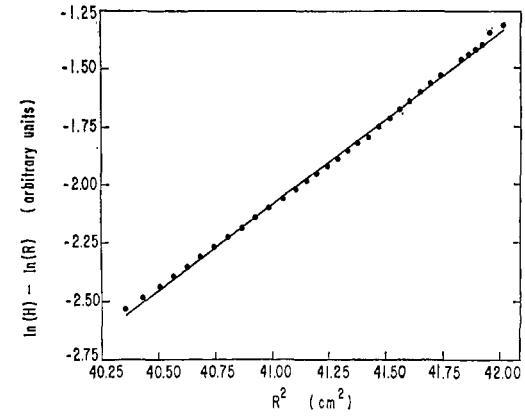

FIGURE 3: Computer plot of sedimentation equilibrium data for gastroferrin. The logarithm of the height of the gradient, H, at a given radial distance, R, minus the logarithim of R (in arbitrary units) is plotted *vs.* R^2.

samples by the Dische–Schettles method (1948). The amino sugars were determined after hydrolysis of the sample with 4 N HCl at 100° for 5 hr. The amino sugars in the hydrolysate were separated on a short Dowex 50 ion-exchange column and determined by the modified Nelson–Morgan method (Boas, 1953). Acetyl analysis was carried out by the procedure of Ludoweig and Dorfman (1960). All colorimetric analyses were done on a Beckman DU spectrophotometer using 1-cm path-length cells.

Amino Acid Analysis. Samples of gastroferrin were hydrolyzed with constant-boiling 6 N HCl at 110° for 24, 36, and 48 hr and amino acid analysis performed by the procedure of Spackmann *et al.* (1958) on the short and long columns of a Beckman Spinco amino acid analyzer Model 120B. All solutions were clarified by passing through a Millipore filter before analysis. Cysteine content was determined after oxidation with performic acid (Hirs, 1956). Tryptophan was determined by the colorimetric procedure of Spies and Chambers (1948).

Iron Binding. The iron chelating ability was determined by the radioiron solubility method of Davis *et al.* (1966). Serial dilution of gastroferrin solutions was used to determine the percentage iron binding capacity of gastroferrin. Two-milliliter aliquots of gastroferrin (1–2 mg/ml) at pH 2.0 were mixed with 4 ml of [^{59}Fe]$FeCl_3$ solution (pH < 2) containing 2 μmoles of Fe and the mixture titrated to pH 8.0 with 0.3 N NH_4OH. A Radiometer automatic titrator was used for pH measurements. The solution was centrifuged at 10,000 rpm for 15 min and the supernatant was analyzed for soluble iron.

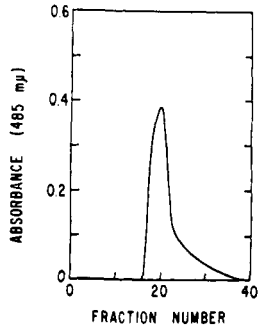

FIGURE 2: Elution profile of sialic acid free gastroferrin from DEAE-cellulose. Conditions same as in Figure 1.

Blood Group Activity. Blood group activity was measured by inhibition of agglutination of erythrocytes by human antiserum. Serial dilutions of the samples were prepared in 0.85% saline. Suitably diluted serum was mixed with each and after a 15-min incubation at room temperature, one drop of a 2% suspension of human erythrocytes was added. Agglutination was read after 15 min and reported as the minimum sample concentration which completely inhibited agglutination of the test cells. Standards for activity were samples of purified human cyst blood group substances.

Results

DEAE-cellulose Chromatography. Gastroferrin was applied to a DEAE-cellulose column (20 × 1.5 cm) equilibrated with 5×10^{-3} M Tris buffer (pH 7.5). The column was washed with 100 ml of the starting buffer and the wash collected in 10-ml fractions. Gastroferrin was eluted from the column with a 2-l. linear gradient from 0 to 0.5 M NaCl in the Tris buffer. The wash and the 10-ml fractions collected from the column were assayed for total carbohydrate. The elution profile of gastroferrin from the column is shown in Figure 1. The fractions comprising each peak were also pooled separately and assayed for both total carbohydrate and iron binding. All peaks were similar in the ratio of iron bound to carbohydrate present. Gastroferrin made free of sialic acid by neuraminidase treatment was also subjected to DEAE-cellulose chromatography in an identical manner. Its elution profile is shown in Figure 2.

Sedimentation Velocity. Gastroferrin showed a single peak in the analytical ultracentrifuge. The sedimentation coefficients of gastroferrin were determined at five concentrations and plotted as a function of concentration. Extrapolation of the plot to zero concentration gave a value for $s^0_{20,w}$ of 8.9 S. From the slope of the plot and its intercept,

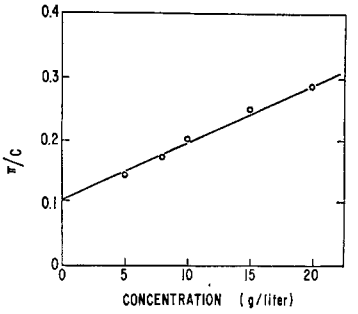

FIGURE 4: Reduced osmotic pressure of gastroferrin as a function of concentration.

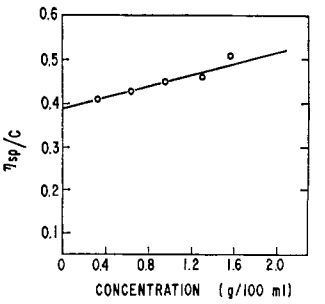

FIGURE 5: Concentration dependence of reduced specific viscosity of gastroferrin.

the following relationship was obtained for the variation of sedimentation coefficient with concentration: $s = 8.9 - 0.374C$, where C is the concentration in milligrams per milliliter.

Sedimentation Equilibrium Centrifugation. Molecular weight determinations on gastroferrin by the short-column technique of Yphantis gave an average value of 2.63×10^5 using 0.66 ml/g as the partial specific volume. The slope of the computer plot of $\ln H - \ln r$ *vs.* r^2 (Figure 3) was used to calculate the molecular weight. A small degree of microheterogeneity was indicated from the computer analysis of the equilibrium data.

Osmometry. The concentration dependence of the osmotic pressure for gastroferrin is shown in Figure 4. Least-squares fit to the data gave

$$\lim_{C \to 0} \pi/C = 0.105$$

Substitution of this value in the Van't Hoff equation resulted in a number-average molecular weight, $\bar{M}_n$, of 2.35×10^5.

Viscosity Measurements. Because of a small degree of microheterogeneity, diffusion studies were not attempted, and viscosity measurements were used in conjunction with the sedimentation constant to obtain an independent estimate for the molecular weight of gastroferrin. The concentration dependence of reduced specific viscosity is presented in Figure 5. Least-squares fit of the data gave a value of intrinsic viscosity, $[\eta]$, of 0.39 dl/g. Molecular weight was estimated from the sedimentation and viscosity data in the manner proposed by Schachman (1957). The value of β used in this formulation was 2.72×10^6 to correct for the asymmetry of the molecule. With this value of β, a $s^0_{20,w}$ value of 8.9 S and $[\eta]$ of 0.39 dl/g the molecular weight was calculated to be 2.78×10^5.

Carbohydrate Composition. Paper chromatographic analysis of sugars from various gastroferrin preparations showed only galactose, fucose, glucosamine, and galactosamine. *N*-Acetylneuraminic acid was also found to be present by colorimetric analysis. The percentage composition of various sugars present in two preparations of gastroferrin are given in Table I. The values of amino sugars are given as their acetyl derivatives. Carbohydrate accounts for 82% of the total weight of the gastroferrin. The acetyl and sulfate content of gastroferrin are also shown. No phosphorous was found.

Amino Acid Composition. Quantitative amino acid analyses of two preparations of gastroferrin showed the presence of all common amino acids (Table II). Amino acids constitute about 15% by weight of the total gastroferrin weight.

Iron Binding and Blood Group Activity. Radioactive iron binding studies together with elemental analysis indicated that gastroferrin binds 6% by weight of iron. Removal of sialic acid does not affect its iron binding capacity. The high molecular weight material, 2×10^5, isolated from hog gastric mucin by the iron precipitation method was found to have iron binding capacity comparable to gastroferrin as was ovarian cyst blood group substances of type A and B. The inhibition of agglutination by various preparations of gastroferrin and other glycoproteins is presented in Table III. The two samples of gastroferrin have an appreciable amount of activity. The variation in these two preparations presumably reflects the blood group type and secretor status of the gastric juice donors. The gastroferrin isolated from hog gastric mucin has an A-type activity stronger than that exhibited by purified blood group substance A.

Discussion

Although a single peak was observed for gastroferrin during sedimentation velocity ultracentrifugation, this is not sufficient evidence for claiming homogeneity of the preparation. Indeed, DEAE-cellulose chromatography permitted resolution of a number of peaks, which were all similar in one respect; the ratio of iron binding to carbohydrate present under each peak was constant. Despite differences in net charge, the relative iron binding capacity of the macromolecule remained the same. When sialic acid was removed from gastroferrin by neuraminidase treatment, chromatog-

TABLE I: Carbohydrate Composition of Gastroferrin.[a]

	Sample I	Sample II	Av
Galactose	27.11	31.60	29.36
Fucose	12.15	15.60	13.90
Sialic acid	4.52	3.42	3.97
Glucosamine, galactosamine	36.53	34.20	35.37
Acetyl		0.90	0.90
Sulfate		1.46	1.46

[a] As g/100 g of gastroferrin.

TABLE II: Amino Acid Composition of Gastroferrin.[a]

	Sample I	Sample II	Av
Lysine	0.54	0.70	0.62
Histidine	0.53	0.57	0.55
Arginine	0.55	0.62	0.59
Cysteine[d]	0.48	0.53	0.51
Aspartic acid	0.84	0.77	0.81
Threonine[c]	3.32	3.56	3.44
Serine	1.54	1.66	1.60
Glutamic acid	1.18	0.95	1.07
Proline	2.26	2.04	2.15
Glycine	0.66	0.60	0.63
Alanine	0.82	0.87	0.85
Valine	0.67	0.70	0.69
Methionine	0.17	0.17	0.17
Isoleucine	0.38	0.42	0.40
Leucine	0.62	0.69	0.66
Tyrosine	0.26	0.24	0.25
Phenylalanine	0.38	0.39	0.39
Tryptophan[d]	0.33	0.33	0.33

[a] As g/100 g of gastroferrin. [b] After performic oxidation to cysteic acid. [c] Values determined by extrapolation to zero time of hydrolysis. [d] Determined from the average of spectrophotometric analysis.

TABLE III: Blood Group Activity.[a]

	A Act.	B Act.
Human cyst type A	2	
Human cyst type B		0.25
Gastroferrin pool 1	16	8
Gastroferrin pool 2	4	>500
Hog gastroferrin	1	>500

[a] Expressed as minimum concentration in micrograms per milliliter inhibiting agglutination.

raphy on DEAE-cellulose column resulted in a homogeneous preparation. Thus, the heterogeneity of gastroferrin on DEAE-cellulose appeared to be a function of varying sialic acid content. Desialogastroferrin, however, retained its initial iron binding ability suggesting that sialic acid, *per se*, is not essential to the iron binding property of gastroferrin. Complete removal of sialic acid by enzyme treatment demonstrated that sialic acid is present in the terminal positions of the sugar chain(s).

Determination of molecular weight by sedimentation equilibrium yielded an average value of 2.63×10^5. The computer program used in the analysis of the data also provided molecular weights at each point of the concentration gradient. Although most of the macromolecular species have a molecular weight around 2.6×10^5, there were a few points along the concentration gradient where the molecular weights were slightly lower or higher than the average value. The range of molecular weights was from 2.4×10^5 to 2.9×10^5. The microheterogeneity of the preparation was confirmed by determination of the number-average molecular weight from osmotic pressure measurements which gave a value of 2.35×10^5. The polydisperse nature of gastroferrin precluded determination of its diffusion constant. The heterogeneity of gastroferrin is similar to that observed for many other glycoproteins. Such carbohydrate–protein macromolecules have variations of composition, structure, and molecular size which make difficult the isolation of a truly homogeneous preparation. These variations may arise at the biosynthetic level, by differential degradation by enzymes present in the tissue fluids, or by alteration during isolation (Gibbons, 1966). The use of cooled gastric juice samples obtained from various individual donors for the preparation of gastroferrin may also have contributed to the microheterogeneity.

The value of the sedimentation constant for gastroferrin, 8.9 S, corresponds to a globular protein of molecular weight equal to 120,000 (Scheraga and Mandelkern, 1953). This is not only at odds with the observation that gastroferrin is excluded by Sephadex G-200 but is also in disagreement with the molecular weights obtained from sedimentation equilibrium and osmotic pressure measurements. The low value of $s^0_{20,w}$ suggests a highly asymmetric molecule. The high value of $[\eta]$, 0.39 dl/g, supports this assumption. Results of sedimentation and viscosity measurements were combined to obtain an independent measure of molecular weight. This value, 2.78×10^5, is in good agreement with the value from sedimentation equilibrium.

The shape of the molecule was deduced from viscosity measurements. The viscosity increment, ν, obtained from intrinsic viscosity (Cohn and Edsall, 1943) was employed to calculate the axial ratio, a/b, of 25.8 (Simha, 1940) for a prolate ellipsoid. The β value of 2.72×10^6 excludes an oblate ellipsoid. A frictional ratio, f/f_0, of 2.2 was obtained from the axial ratio using Perrin's factor (1936). Calculation of molecular dimensions from molecular weight and partial specific volume (Holtzer and Lowey, 1959) provided a length of 712 Å and diameter of 27.6 Å. An independent estimate of 2.15 for f/f_0 was also obtained from the Svedberg equation (Schachman, 1957) and the Stokes relationship (Tanford, 1965). Thus, the two calculations for f/f_0 are in excellent agreement with each other, and demonstrate the internal consistency of the results. Some preliminary electron microscope studies indicate that gastroferrin does approximate a rod-like structure.

Gastroferrin is predominantly carbohydrate. The acetyl content suggests that its amino sugars are present in the N-acetylated form. Although all the common amino acids are present in gastroferrin, a distinguishing feature is its high content of threonine, serine, and proline. The hydroxyamino acids probably provide the linkage sites for the carbohydrate. The physical and chemical characteristics of gastroferrin are summarized in Table IV.

This glycoprotein binds 6% by weight of iron corresponding to a molar ratio of 4:1 for carbohydrate:iron. Sugars such as fructose are effective chelating agents for iron (Charley *et al.*, 1963), but molar ratios of 20:1 or higher are required to prevent precipitation of iron at physiological pH. Thus,

gastroferrin appears to bind iron more effectively than free sugars by proper orientation of binding groups on the protein.

The general chemical composition of gastroferrin is quite similar to that of blood group substances from ovarian cyst fluid (Gottschalk, 1966) both in terms of relative carbohydrate and protein content and also in the distribution of various sugars and amino acids. The blood group substances contain 7 to 16% by weight of amino acids. The presence of sialic acid in comparatively smaller quantities in blood group substances may be due to the prolonged action of neuraminidase since the blood group substances accumulate over a long period in the cyst fluid. The two types of macromolecules, however, differ in size. While the upper limit for the molecular weight of gastroferrin is approximately 2.9×10^5, the average particle weights of blood group substances have been reported from a low of 2×10^5 to a high of 1×10^6 (Kekwick, 1952a,b; Caspary, 1954). Even the most highly purified preparations of blood group substances show a moderate degree of polydispersity.

The behavior of gastroferrin and blood group substances was examined with respect to their immunological activity and iron binding. Gastroferrin does possess blood group activity at a level similar to that of purified blood group substance from ovarian cysts as shown by inhibition of agglutination. Blood group substances of A and B type from cyst fluids, on the other hand, have iron binding comparable to that of gastroferrin. The biological significance of these relationships is not clear at present.

Direct experiments to elucidate the role of gastroferrin in iron absorption by addition of exogenous glycoprotein to the diet are in progress. It may be pointed out that gastric juice is not an ideal source for obtaining large quantities of this material. We have, therefore, investigated the possibility of isolating a similar material from commercially available pig gastric mucin by the iron precipitation method. From all preliminary observations and iron binding studies, this material behaves in a manner similar to that of gastroferrin. The spectral and magnetic properties of iron gastroferrin are under investigation in order to understand the nature of iron binding to this macromolecule.

TABLE IV: Summary of Physiocochemical and Chemical Characteristics of Gastroferrin.

Property	Value
$s^0_{20,w}$	8.9
$[\eta]$ (dl/g)	0.39
$\bar{v}$	0.66
M_{op}[a]	2.35×10^5
M_{se}[b]	2.63×10^5
$M_{s^0_{20,w}}$ & $[\eta]$	2.78×10^5
f/f_0	2.2
Amino acids (w/w) (%)	15
Carbohydrate (w/w) (%)	82
Moles (acetyl)/moles (amino sugars + sialic acid)	1.1

[a] op = osmotic pressure. [b] se = sedimentation equilibrium.

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Nuclear Magnetic Resonance Spectral Studies on Actinomycin D. Preliminary Observations on the Effect of Complex Formation with 5′-Deoxyguanylic Acid*

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ABSTRACT: The complete assignment of the nuclear magnetic resonance spectrum of actinomycin D is described. This compound was studied in various organic solvents, in water at low temperature, at room temperature, and in aqueous solutions containing various small amounts of dimethylformamide.

Solvent effects, unusual chemical shifts, and the behavior of the active hydrogens were used to establish specific geometric relationships in the pentapeptide configuration. Spectral studies of this compound in aqueous solutions in the presence of 5′-deoxyguanylic acid have shown that complex formation occurs by base stacking; the pyrimidine ring of 5′-deoxyguanylic acid is located above and below the phenoxazone chromophore of actinomycin D.

Actinomycin D (Figure 1) is of considerable biochemical interest. It binds strongly to double-stranded DNA and inhibits the DNA-dependent RNA synthesis (Waksman, 1968). Two models have been proposed for the mode of binding to DNA, one by Hamilton *et al.* (1963), where the molecule binds externally to the DNA molecule with the NH_2 group and the quinone oxygen of the phenoxazone ring system forming three hydrogen bonds with deoxyguanine. A different model, which involves intercalation of the phenoxazone ring between the base pairs in DNA has been proposed by Müller and Crothers (1968). As the first step to arrive by nuclear magnetic resonance techniques at a more detailed understanding of the mode of binding of actinomycin D we have completed the assignment of its nuclear magnetic resonance spectrum.

Because the solubility of actinomycin D in water at room temperature is too low for direct spectral observation, various organic solvents were used first. Additional spectra were taken in water at low temperatures, at room temperature in supersaturated solution and in aqueous solutions containing various small amounts of dimethylformamide. Finally, the extrapolated chemical shifts in water were used to study the effect of 5′-deoxyguanylic acid on the spectrum of actinomycin.

Experimental Section

All spectra were determined on a Varian HA 100D spectrometer operating at a probe temperature of 32°. Concentrations were in the 5–10% range. Chemical shifts are reported in parts per million relative to internal tetramethylsilane (TMSi) (organic solvents) and internal 2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt (aqueous media). Peak positions were determined by measuring the differences between the manual and sweep frequency oscillator settings and are reliable to ±1 Hz. Actinomycin D was obtained from Merck and Co. A purity of >98% was indicated by thin-layer chromatography and phase solubility analyses.

Results

The 220-MHz spectrum of actinomycin D in $CDCl_3$ is illustrated in Figure 2.[1] Although the 100-MHz spectrum was sufficiently resolved for a complete analysis, the 220 was chosen for illustration because of the improvement in resolution of the sarcosine and Thr-α-CH protons (τ 5.2–5.5) and for a somewhat better definition of the proline α- and γ-proton resonances. All chemical shifts in this report refer to the 100-MHz spectrum.

The nuclear magnetic resonance parameters of actinomycin D in $CDCl_3$, acetone, pyridine, dimethylformamide,

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