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Physical Properties of Human Follicle-Stimulating Hormone*

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ABSTRACT: The molecular weight of human follicle-stimulating hormone has been determined by both analytical ultracentrifugation at moderate concentrations and a combination of gel filtration and density gradient centrifugation at extremely low concentrations. The latter approach is by far the more discriminatory and useful for these studies. The results reveal that this glycoprotein hormone, as normally isolated from human pituitaries, is an extremely stable dimer of mol wt 35,000 ± 1000.

The hormone can be partially and irreversibly dissociated into its constitutive subunits by a variety of denaturing conditions (2–4 M guanidine hydrochloride, freezing and thawing) as well as prolonged standing in the cold in aqueous solution. At moderate concentrations, the latter process also results in the increased production of high molecular weight aggregates (*ca.* 500,000) of the glycoprotein.

The quantities of hormonal proteins of the pituitary which can be isolated as homogeneous preparations are normally small. In the past, this has prohibited extensive investigations of the molecular weight and other physical properties of these molecules. During recent years, however, advances in gel filtration chromatography as well as ultracentrifugation have made it possible to obtain an extensive amount of physical data on remarkably small quantities of material. With the advent of isolation procedures for homogeneous preparations of several of the gonadotropins (Squire *et al.*, 1962; Hartree *et al.*, 1964; Parlow *et al.*, 1965; Reichert and Jiang, 1965; Amir *et al.*, 1966; Roos, 1967; Saxena and Rathnam, 1967; Ryan, 1968; Peckham and Parlow, 1969) as well as convenient assay procedures (Faiman and Ryan, 1967a,b), we felt that an examination of the size, shape, and other physical characteristics of these proteins by the dual chromatographic and ultracentrifugal approach would be profitable. In this communication, we wish to report the results with one of these proteins, follicle-stimulating hormone (FSH),[1] isolated from human pituitaries.

Experimental Section

The preparations of human FSH employed in these studies are listed in Table I along with their biologic potencies and the methods of preparation. The biologic assays were performed using the rat ovarian weight augmentation assay for FSH (Steelman and Pohley, 1953) and the rat ovarian ascorbic acid depletion assay for human luteinizing hormone (Parlow, 1961) as previously described (Ryan, 1968). Radioimmunoassays were performed by the method of Faiman and Ryan (1967a,b).

Protein determinations were made either by obtaining the dry weight of newly lyophilized material or from solutions using the Lowry procedure (Lowry *et al.*, 1951). Protein determined by the Lowry method accounts for 76–80% of the dry weight of human FSH.

Radioiodination Procedure. Protein (1–2 μg) was labeled with ^{131}I by the method of Greenwood *et al.* (1963). Specific activities of 90–500 μCi/μg were obtained. ^{131}I-Labeled protein (2–5 ng) was applied to the gel filtration columns. ^{131}I activity was determined in an automatic gamma well counting system.

Treatment with Guanidine Hydrochloride. These experiments employed Ultra Pure guanidine hydrochloride, obtained from Mann Research Laboratories. The FSH was dissolved or diluted in the guanidine solutions at pH 6.5 and allowed to stand at room temperature for varying periods of time. The concentrations of guanidine hydrochloride and the incubation times are given in the Results section. Guanidine hydrochloride was not removed from the solutions prior to gel filtration, sedimentation, or assay but the concentration was lowered by dilution.

Analytical ultracentrifugation experiments were conducted with a Spinco Model E analytical ultracentrifuge equipped with Rayleigh and schlieren optics. Sedimentation velocity experiments with the standard proteins employed as calibrat-

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[1] Abbreviations used are: FSH, follicle-stimulating hormone; BSA, bovine serum albumin.

TABLE I: Characteristics of FSH Preparations Studied.

Preparation	Biologic Potency[a] FSH (S1 U/mg)	LH[b] (S1 U/mg)	Method of Preparation
5765B	100	0.08	Ryan (1968)
11867C	105	0.21	Ryan (1968)
41267B	115	0.12	Ryan (1968)
LER 869-2[c]	116	0.15	Reichert *et al.* (1968)
WDP-1[d]	304	0.007	Peckham and Parlow (1969)

[a] Per milligram dry weight. When assays were based on protein determined by Lowry method, a factor of 0.78 was used to convert into dry weight. Although preparations 11867C and 41267B are listed as approximately 100 NIH S1 U/mg, both had potencies in excess of 220 S1 U/mg soon after preparation. The liability of FSH biologic activity is well recognized. All preparations were of nearly identical potency when assayed radioimmunologically. [b] LH = lutenizing hormone. [c] Gift of the National Pituitary Agency, Baltimore, Md. [d] Gift of Dr. W. D. Peckham, University of Pittsburgh, Pa.

TABLE II: Hydrodynamic Properties of Human FSH Determined by Analytical Ultracentrifugation.

Sample	Concn (g/dl)	$s_{20,w}$ (Peak) (S)	$D_{20,w} \times 10^7$ (cm^2/sec)
11867C	0 (extrapolated)	3.04	7.66[b]
	0.0125	3.04[a]	
	0.130	2.99	
	0.250	2.86	7.64
	0.430	2.90	7.68
	0.430	$\bar{s}_{20,w}$ = 2.99	
41267B	0.157	3.07	5.93
	0.157	$\bar{s}_{20,w}$ = 3.64	

[a] Sedimentation coefficient determined as $s_{50\%}$ with absorption optics at 280 mμ. [b] Since the concentration dependence of the diffusion coefficient of most substances is usually small, this value reported for 0 concentration was simply the average of the values determined at 0.250 and 0.430 g per dl.

ing solutes in the sucrose gradient centrifugation experiments were performed with 1% protein solutions in a 0.05 M Tris-HCl buffer at pH 7.5 at room temperature. The sedimentation coefficients of these proteins were measured as the rate of movement of the schlieren peak and corrected to 20° and water by applying the appropriate factors containing the density and viscosity of the solvent and water as well as the partial specific volume, $\bar{v}$, of the proteins. These latter values were also employed in the correction of the sucrose gradient sedimentation data and are given in Table VI.

Solutions of FSH examined in the analytical ultracentrifuge were prepared by either dissolving salt-free, lyophilized samples in 0.10 M NaCl–0.05 M CH_3COONa–CH_3COOH (pH 4.9), or else exhaustively dialyzing a salt-free solution against successive changes of this solvent. Initial protein concentrations were between 0.1 and 0.5% and the temperatures of the experiments were usually between 4 and 10°. A single sedimentation velocity experiment at 0.0125% FSH was conducted at 25° with an instrument equipped with a photoelectric scanning system. Standard 12-mm 2.5-deg double-sector cells with epoxy centerpieces were employed in the sedimentation velocity experiments at 59,780 rpm. Diffusion coefficients were determined at 12,590 rpm in a double-sector capillary-type synthetic boundary cell. Sedimentation equilibrium experiments were conducted at 9945, 12,590, 31,410, and 39,460 rpm using a Yphantis multichannel Kel F centerpiece and cell assembly. Column heights were usually 2.8 mm. Two types of oils, Kel F and FC-43, were employed to provide a sharp interface at the base of the liquid column so that the position of the latter could be viewed optically. Rayleigh optics were routinely used. The duration of the experiments at a given speed was sufficient to ensure the fact that fringe patterns obtained were invariant over a 6- to 9-hr period. Generally, this amounted to approximately 24 hr.

For the calculation of molecular weights as well as the correction of the sedimentation velocity data, a partial specific volume of FSH of 0.696 cc/g was used. This value was calculated from the amino acid and carbohydrate composition (Reichert *et al.*, 1968) according to the procedure of McMeekin and Marshall (1952). The partial specific volumes of the carbohydrate moieties were obtained from Gibbons (1966).

Stokes Radius. The method for determination of Stokes radius by gel filtration, and the justification for the use of radioiodinated proteins, has previously been published (Ryan, 1969). In brief, Sephadex G-100 columns, of known volume, V_t, were calibrated during each analysis with Blue Dextran-2000 to determine the void volume, V_0, and with standard proteins of known Stokes radii. The standard proteins, obtained from Mann Research Laboratories, were BSA, chymotrypsinogen, and cytochrome *c*. Their Stokes radii were taken to be 36, 22.4, and 16.4 Å, respectively (Laurent and Killander, 1964). The standard proteins, in 1–2-mg quantities, Blue Dextran, and FSH were applied to the columns in 0.5–1.0-ml volume and elution positions, V_e, were determined by monitoring the adsorbancy of column eluates at 225 mμ.

In most experiments FSH was labeled with ^{131}I and the elution position of the 2–5 ng of hormone applied to the column was monitored by assaying radioactivity in the eluates. In one experiment, 2 μg of unlabeled FSH was applied to the column and the elution position was determined by a specific radioimmunoassay for FSH (Faiman and Ryan, 1967a,b).

The fraction of the volume of the gel bed available for diffusion, K_{av}, for each standard protein or FSH was calculated using the equation of Laurent and Killander (1964)

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (1)$$

The Stokes radius of the standard proteins was then plotted against $(-\log K_{av})^{1/2}$ and the Stokes radius of FSH was then read from the standard curve.

Sucrose Density Gradient Centrifugation. These experiments were performed with radioiodinated FSH, before and after treatment with guanidine hydrochloride, using a modification of the method of Martin and Ames (1961). A Spinco Model L2-65 ultracentrifuge with a SW65 rotor was employed. The

TABLE III: Characteristic Molecular Weights of Human FSH Determined by Analytical Ultracentrifugation.

Sample	Sedimentation Equilibrium Experiments				$Ms^0_{20,w}$ (D)
	Loading Concn (g/dl)	Speed (rpm)	$M_w \pm 1000$	$\bar{M}_A$	
11867C	0		33,600	27,000 ± 1000[b]	33,300[a]
	0.127	9,945	32,700	27,100 ± 1000	
	0.242	12,590	32,700	24,900 ± 1000	
	0.434	9,945	31,100	23,300 ± 1000	
41267B	0.157	9,945	47,500	38,115 ± 1000	49,000
	(low speeds)	12,590	47,000	36,914 ± 1000	
	0.157	31,410		31,700 ± 3000	
	(high speeds)	39,447		31,500 ± 3000	
		39,447		16,000 ± 3000	
		(After 5 days at 10°)			

[a] $s^0_{20,w}$ was obtained for this calculation by multiplying $s^0_{20,w}$ (peak) by the ratio, ($s_{20,w}$ at 0.439%/$s_{20,w}$ (peak) at 0.439%). [b] See text for explanation.

sucrose density gradients were formed in 0.05 M Tris buffer at pH 7.4, using a Büchler apparatus to make a linear gradient from 5 to 27% sucrose (1.146–2.708-Cp viscosity or 1.017–1.101-g/cc density at 20°). Gradient solution (4.9 ml) was placed in the centrifuge tube and overlayed with 0.1 ml of sample solutions containing 2–5 ng of ^{131}I-labeled FSH and 1 mg of a standard protein. Runs were conducted at 60,000 rpm for 7 hr at a temperature of 20°. Upon completion of the run, successive 0.1-ml portions were removed from the top of tube and collected separately using an Isco density gradient fractionator and collector. The radioactivity and optical absorbance of each fraction were measured and plotted. The fraction at which a maximum of radioactivity or optical absorbancy occurred was determined by triangulation. The standard proteins, obtained from Mann Research Laboratories, were bovine serum albumin, ovomucoid, lysozyme, and ribonuclease.

Results and Discussion

Experiments at Moderate Concentrations (Analytical Ultracentrifugation). Our first physical studies on FSH were conducted in the analytical ultracentrifuge at moderate concentrations. Sedimentation velocity, diffusion, and sedimentation equilibrium experiments were performed with two preparations, 11867C and 41267B, of approximately equal potency (see Table I). Sample 11867C and a portion of 41267B were lyophilized for these studies. Sedimentation velocity patterns of both preparations revealed a single but broad peak. Sample 11867C was examined over a wide range of concentrations and the sedimentation coefficient was found to vary very little at concentrations below 0.4%. Correspondingly, sample 41267B was examined at only a single concentration, 0.157%. Routinely, only the rate of movement of the schlieren peak was followed. For two solutions, the weight-average sedimentation coefficient, $s_{20,w}$, was also calculated. The sedimentation coefficients of these various solutions as well as some diffusion coefficients (measured by the height–area method (Schachman, 1957)) are given in Table II.

The sedimentation velocity patterns of the two samples, 41267B and 11867C, differed in shape. The pattern for 41267B was definitely skewed toward the leading edge of the boundary, and its weight-average sedimentation coefficient (3.64 S) was, as expected, significantly higher than that of the schlieren peak (3.07 S). No significant difference was observed between the behavior of the lyophilized and the unlyophilized portions of this preparation. The pattern for 11867C appeared to be symmetrical and the weight-average sedimentation coefficient at 0.434% (2.99 S) was only slightly higher than the sedimentation coefficient of the peak (2.90 S) at this concentration. This apparent agreement, however, was due to a fortuitous combination of effects as this preparation (11867C) upon further examination could be clearly shown to be heterogeneous: an apparent diffusion coefficient of 11×10^{-7} cm^2/sec obtained from an analysis of the sedimentation velocity patterns of 11867C at high speeds (59,780 rpm) was appreciably higher than the value of 7.66×10^{-7} cm^2/sec determined at low speeds in the absence of sedimentation. Sedimentation equilibrium experiments confirmed this conclusion of heterogeneity (see below). In the light of these data and the experimental results at lower concentrations (see following section), it was concluded that the symmetrical appearance of the sedimentation patterns of this sample was due to the presence of high molecular weight aggregates together with parent protein and dissociated subunits. The close agreement in $s^0_{20,w}$ for the two samples (3.04 S for sample 11867C and 3.07 S for 41267B) is probably fortuitous since both samples were polydisperse.

Sedimentation equilibrium experiments revealed that both samples were polydisperse. The results of the low-speed equilibrium experiments (9945 and 12,590 rpm) are given in Table III as weight-average molecular weights of the entire cell contents, M_w, as well as the weight-average molecular weight of the species at the air–water interface, $\bar{M}_A$. The molecular weights obtained by combining the weight-average sedimentation and diffusion coefficients, $M_{s,D}$, in the Svedberg equation (Svedberg and Pedersen, 1940) are also included for comparison.

The molecular weight behavior of sample 11867C was determined at several concentrations. The weight-average molecular weights, M_w, of the entire centrifuge cell contents of separate solutions exhibited only the characteristic slight increase with decreasing concentration of protein due to activity effects. As the data in Figure 1 demonstrate, this sample was clearly polydisperse. The weight-average molecular weight, $\bar{M}$, at a specific point and concentration in the

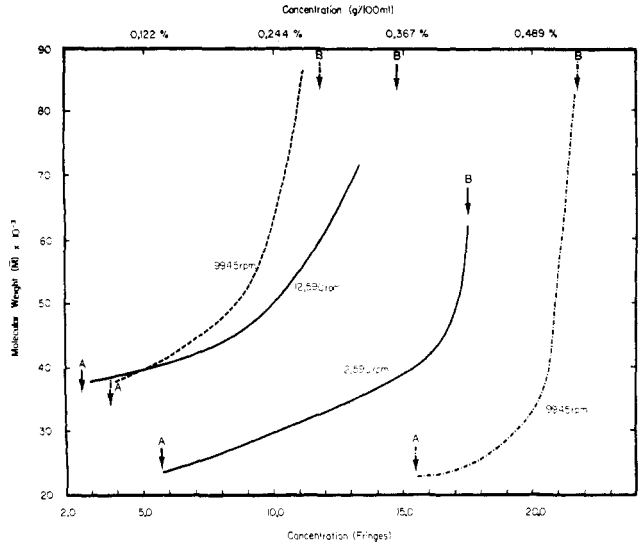

FIGURE 1: Molecular weight distribution across the centrifugal column in low-speed sedimentation equilibrium experiments in the analytical ultracentrifuge. Curves at the left in the above figure, (- - -) and (——), represent data from experiments with sample 41267B at the indicated speeds; curves to the right of the figure, (——) and (· - · -), represent data from experiments with sample 11867C at the indicated speeds. Coded arrows indicate the positions of the centripetal (A) and the centrifugal (B) ends of the solution columns.

column in the experiment shown ranged from 23,000 at the air–water interface (A) to 70,000–90,000, and perhaps even higher, at the base (B). In view of the small concentration dependence of M_w, a similar small dependence of $\bar{M}_A$ might also be expected. In the absence of suitable experimental data which would permit an accurate extrapolation to 0 concentration, we have assumed that the value of $\bar{M}_A$ at 0.127% (27,000) represents the value at infinite dilution within experimental error.

Characteristic molecular weights of sample 41267B were all higher than those of sample 11867C although no significant difference was observed between the lyophilized and the unlyophilized portions of the former. This sample also exhibited a spectrum of molecular weights across the column, the lowest of which was 37,000 in the low-speed experiments (see Table III). The change in $\bar{M}$ across the column for this sample is also shown in Figure 1. Again, the molecular weights at the base of the column are quite high and approach no apparent limit.

In the case of both 11867C and 41267B, the distribution in $\bar{M}$ across the column in two sets of concentration ranges of the same sample do not overlap or form a common curve. This demonstrates that the heterogeneity observed in both preparations is *not* the result of a reversible association process. Rather, it seems to be the result of an irreversible self-association as in other experiments it could be demonstrated that the high molecular weight species in these solutions were indeed aggregates of FSH (see following section). Although the oils at the base of the columns in the equilibrium experiments may have contributed somewhat to the production of these high molecular weight species, significant amounts must also be present in the absence of oil as is demonstrated by the reasonably good correspondence between M_w from the sedimentation equilibrium experiments and $M_{s,D}$ calculated from the weight-average sedimentation and diffusion coefficients determined under conditions where no oil was employed.

In order to ascertain whether the 37,000 molecular weight species was the lowest molecular weight component in sample 41267B, some sedimentation equilibrium experiments were also continued at the higher speeds of 31,410 and 39,447 rpm, according to the method of Yphantis (1964). At the initial loading concentrations employed (appropriate for the low-speed experiments), only the data at the centripetal end of the column could be analyzed. This represented only 15% of the total weight of FSH present in the column. The fringe displacements were small and the precision is thus very poor. The results of these experiments are also shown in Table III. If the high-speed experiments immediately followed the low-speed ones, the lowest molecular weight species which could be resolved at either speed was 32,000 ± 3000 for both the unlyophilized and the lyophilized portion of 41267B.

If the solutions in the sedimentation equilibrium experiments described above were allowed to stand at 10° in the cell for 5 days following the high-speed experiments and then sedimented again to equilibrium at the higher speeds, the lowest molecular weight species observed at the centripetal end of the column was now 16,000 ± 3000. The best interpretation of these results is that a species of molecular weight 32,000 to 37,000 has dissociated upon standing to a smaller molecular weight species.

Experiments at Low Concentrations. Although the results from the analytical ultracentrifuge were useful in giving an approximate idea of heterogeneity and molecular size, it was clear that more discriminatory techniques were required for the precise assessment of the various molecular species present. In the interests of economy of material, it was also advisable to employ methods which would permit the detection and determination of the physical properties and biological activity of very low concentrations of FSH. We correspondingly turned to an approach first suggested by Siegel and Monty (1966) in which one combines the data from gel filtration chromatography and sucrose gradient ultracentrifugation. The former technique, as previously discussed (Ryan, 1969), evaluates the effective Stokes radii, a (Laurent and Killander, 1964), and the latter gives, with proper corrections, the sedimentation coefficients, $s_{20,w}$ (Martin and Ames, 1961), of the various molecular weight species which can be resolved. If one assumes that (1) the hydrodynamic shape of the protein unknowns and standards is approximately spherical,[2] (2) the densities of these solutes are comparable, and (3) the sucrose gradient in which the centrifugation experiments are performed affects the hydration (or preferential solvation) of the

[2] Although the assumption of spherical shape may not be necessary for the less highly cross-linked gels such as Sephadex G-200 (Siegel and Monty, 1966), where diffusion predominantly controls the relative rates of elution, it seems intuitively wise to envoke it for the more tightly cross-linked gel, G-100, where steric exclusion and restricted diffusion may make a contribution. Since these latter factors for spheres should bear a mathematical relationship to the sphere's radius (somewhat similar in form to Stokes' equation, $f = 6\pi\eta_0 a$) the elution position for a spherical unknown should correspond to the frictional coefficient of the unknown, when compared to a calibration curve based on the elution position of other spheres. If, however, either the unknown or standards are rodlike then the contributions of steric exclusion and restricted diffusion should be some exponential function of the diameter and length of the rod. The contribution of this term would be dependent on the dimensions of the rod and would not bear the same relationship to the frictional coefficient as seen in Perrin's equation. Correspondingly, the elution position of the rod would not bear a simple relationship to the frictional coefficient as is characteristic of free diffusion.

TABLE IV: Stokes Radii and Distribution of Molecular Species of Human FSH.

Sample	Small Species: Stokes Radius (Å)	Small Species: % Present	Large Species: Stokes Radius (Å)	Large Species: % Present	% High Mol Wt Aggregates
11867C[a]	23.1	46	31.9	54	
41267B[b]		0	31.9	98	2
41267B[c]		5	31.5	92	3
41267B[d]		17	32.3	79	5
41267B[e]		15	31.9	85	
5765B		10	32.4	90	
5765B[f]		10	31.5	90	
WDP-I		20	32.3	80	
LER 869-2		10	33.7	90	
Average	23.1		32.2 ± 0.2		

[a] Pooled solutions of lyophilized FSH recovered from a variety of analytical centrifuge experiments. [b] Original salt-free isoelectric stock solution (0.5 g/dl) of unlyophilized FSH employed in sedimentation equilibrium experiments. [c] Dilution of above stock solution, subjected to conditions of solvent, time, and temperature comparable to those employed in the low-speed sedimentation equilibrium experiments. [d] Same solution in footnote *c* subjected to conditions comparable to those employed in the high-speed sedimentation equilibrium experiments. [e] Salt-free isoelectric dilute solution of FSH repeatedly frozen and thawed. [f] Unlabeled with ^{131}I. Followed by radioimmunoassay.

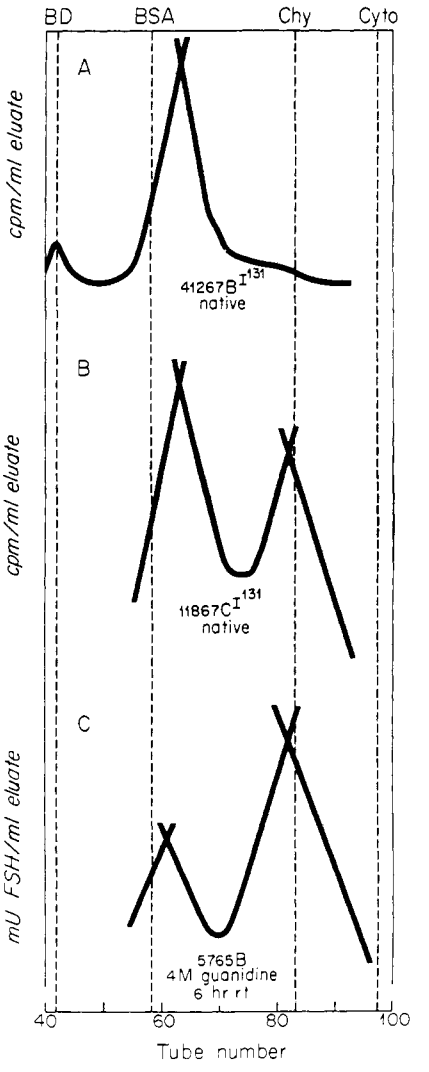

FIGURE 2: Elution profiles from gel filtration studies on human FSH using Sephadex G-100. (A) Preparation 41267B, labeled with ^{131}I, at 5×10^{-6} g/dl. (B) Preparation 11867C, labeled with ^{131}I, at 5×10^{-6} g/dl. The symbols across the top indicate the elution positions of standard markers: Blue Dextran (BD), bovine serum albumin (BSA), chymotrypsinogen (Chy), and cytochrome *c* (Cyto). (C) Preparation 5765B, 2×10^{-3} g/dl, unlabeled but treated with 4 M guanidine hydrochloride for 6 hr at room temperature; determined by radioimmunoassay.

unknown and the standard proteins to the same extent, one may combine these two separate measurements to yield molecular weight, M, according to the equation

$$M = \frac{6\pi\eta_0(N)(a)(s_{20,w})}{(1 - \bar{v}\rho_0)} \quad (2)$$

where η_0 and ρ_0 are the viscosity and the density, respectively, of water at 20°, N is Avogadro's number, and $\bar{v}$ is the partial specific volume of the molecule of molecular weight, M, sedimentation coefficient, $s_{20,w}$, and effective Stokes radii, a, at 20° in water.

If several discrete peaks are observed in each type of experiment (gel filtration and centrifugation), the assumption of sphericality requires that the order of the Stokes radii follow the order of the sedimentation coefficients. Thus, the molecular weight of the several species present may be calculated from such data if these species are not in reversible equilibrium or else the time required to establish equilibrium is long compared to the rate of separation by both techniques. The use of radioiodinated samples of unknowns as well as the availability of specific and sensitive radioimmunoassays for the species separated in such experiments thus permits their size and immunologic activity to be determined at very low concentrations of proteins.

Determination of Stokes Radii. The samples, 11867C and 41267B, employed in the analytical ultracentrifuge studies together with other related preparations were examined by gel filtration chromatography. Elution profiles are illustrated in Figure 2. Sample 41267B (Figure 2A) was examined after recovery from various solutions employed in the analytical ultracentrifuge experiments that were previously discussed. All solutions exhibited at least two species on Sephadex G-100. The major species was eluted at a position corresponding to material of approximately 32-Å radius. Small but variable amounts of material eluted at the void volume of the column and at a position corresponding to material of approximately 23-Å radius. Both of these minor radioiodinated components seemed to be FSH as they were bound by an antiserum to FSH and their binding to that antiserum was competitively inhibited by unlabeled human FSH of 32-Å size. The material eluting with the void volume on Sephadex G-100 was also eluted in the void volume when studied on Sephadex G-200. The amount of this large aggregate as well as the 23-Å material appeared to increase the longer the solution stood in the cold (see Table IV). These elution profiles did not vary over a concentration range of 10^{-3}–10^{-6} g/dl, thus demonstrating

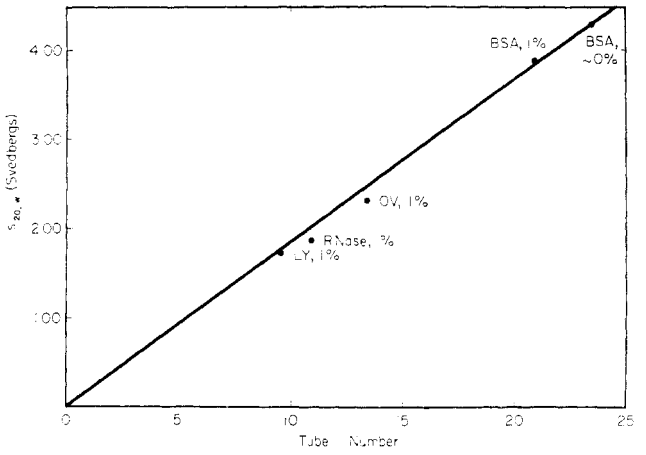

FIGURE 3: Sedimentation behavior of standard proteins in density gradient ultracentrifugation experiments. The straight line (——) represents a plot of eq 3 in the text, using the sedimentation behavior of ^{131}I-labeled BSA at 5×10^{-6} g/dl in a 5–27% sucrose gradient as the reference protein. (See text for explanation.) The points represent the actual values of $s_{20,w}$ (determined in the analytical ultracentrifuge at a concentration of 1 g/dl) of other reference proteins plotted against the fraction number of the peak position when the protein is run in this sucrose gradient at an initial loading concentration of 1% under the same conditions as [^{131}I]BSA. Deviations of the points from the line represent the errors in $s_{20,w}$ determinations by density gradient ultracentrifugation when [^{131}I]BSA is employed as the standard protein. Abbreviations for standard proteins other than BSA are: lysozyme (LY), ribonuclease (RNase), and ovomucoid (OV).

TABLE V: Stokes Radii and Distribution of Molecular Species of Human FSH Treated with 4 M Guanidine Hydrochloride at Room Temperature.

Sample	Treatment Time (hr)	Small Species: Stokes Radius (Å)	Small Species: % Present	Large Species: Stokes Radius (Å)	Large Species: % Present
11867C	4	22.6	59	33.0	39
41267B	2	22.6	49	32.5	51
5765B	5	23.5	62	33.2	33
5765B[a]	6	22.8	70	33.7	30
5765B	24	23.2	71	33.2	29
WDP-I	20	23.6	72	32.5	28
Average Stokes radius		23.2 ± 0.2		33.0 ± 0.2	

[a] Not labeled with ^{131}I. Followed by radioimmunoassay.

that the separate species observed were not in rapid and/or reversible equilibrium with one another.

Preparation 11867C was studied less extensively since less was recovered from the analytical ultracentrifuge experiments. The material examined represented pooled solutions recovered from many different types of centrifuge experiments. Its elution profile, illustrated in Figure 2B, clearly indicates two species, one of 32-Å radius and the other of 23-Å radius. Both species of radioiodinate material were bound by anti FSH serum. Unfortunately, the presence of a large molecular weight aggregate was not sought in this experiment.

Data concerning the gel filtration behavior of the above two preparations and several others are given in Table IV. It is apparent from inspection of these data that, with the exception of 11867C, 80–98% of the material applied to the columns eluted in a position corresponding to 32 Å. Furthermore there did not appear to be a significant difference in radius between the various preparations and therefore an overall average of 32.2 Å was calculated.

The behavior of preparation 11867 suggested that FSH could be dissociated into subunits. This possibility was tested by treating various preparations with guanidine hydrochloride. The results of these experiments are presented in Table V and illustrated in Figure 2C. Treatment with 4 M guanidine hydrochloride resulted in a decrease in 32-Å material and production of a 23-Å component. After 6 hr at room temperature the extent of dissociation appeared to have reached its limit in 4 M guanidine hydrochloride. Higher concentrations of guanidine hydrochloride do result in complete dissociation (unpublished data). It is also possible that partial reassociation occurred upon separation from the guanidine salt during gel filtration. This does not seem probable, however, since the 23-Å species isolated from the G-100 columns was concentrated by ultrafiltration (to 10^{-6}-g/dl concentrations), incubated for various times (2–72 hr) at several temperatures (4–80°), and rechromatographed without evidence of reassociation occurring. This 23-Å material clearly has much of its original tertiary structure intact as it bound very well to FSH antisera.

It is also apparent from the data in Table V that the molecular radius of this small species of FSH did not differ significantly among the several preparations of FSH that were studied or among radioiodinated or unlabeled material. An overall average of 23.2 Å was therefore calculated. This value agrees very well with the radius of 23.1 Å found spontaneously in preparation 11867C. It can also be seen from Table V and Figure 2B that the undissociated species of FSH, after guanidine treatment, has a significantly ($p \leq 0.05$) larger radius (33.0 Å) than native FSH (32.2 Å). This is not unexpected.

Determination of the $s^0_{20,w}$ of Various Molecular Species of FSH.[3] In order to obtain the $s^0_{20,w}$ of the various molecular species of FSH observed in the gel filtration experiments, we conducted a series of density gradient centrifugation studies with these samples as well as with a set of standard proteins whose sedimentation coefficients were also determined in the analytical ultracentrifuge.

The usual procedure (Martin and Ames, 1961) employed in obtaining $s_{20,w}$ of unknowns in experiments of this type is to run the unknown and some appropriate sedimentation standard in the same density gradient. After separation and collection of successive fractions in a series of test tubes, the sedimentation coefficient of the unknown, s_U, is calculated by eq 3

$$s_U = \frac{s_S T_U}{T_S} \tag{3}$$

where S_S is the $s_{20,w}$ of the standard and T_U and T_S are the fraction tube numbers of the unknown and the standard, respectively. ($T = 0$ corresponds to the air–water meniscus at the centripetal end of the density gradient column.)

The validity of this procedure rests on a number of assump-

[3] Since these sucrose gradient experiments were conducted with nanogram quantities of FSH, the sedimentation coefficients obtained are essentially those at infinite dilution.

TABLE VI: Sedimentation Coefficients and Partial Specific Volumes of Standard Proteins Employed in Sucrose Density Gradient Centrifugation Experiments.

Protein at 1 g/dl	$\bar{v}$ (cc/g)	Sucrose Density Gradient Centrifugation: Tube No. ±0.2	Sucrose Density Gradient Centrifugation: $s'_{20,w}$[a] (S)	Anal. Ultracentrifugation $s^{1\%}_{20,w}$ (S)	Δs (S)	$\Delta\bar{v}$ (cc/g)
BSA	0.734[b]	21.0	3.87	3.90	−0.03	0
Ovomucoid	0.685[c]	13.5	2.47	2.32	+0.15	0.049
Ribonuclease	0.695[d]	10.9	1.99	1.89	+0.10	0.039
Lysozyme	0.722[e]	9.6	1.76	1.72	+0.04	0.012

[a] Calculated values based on $s^0_{20,w}$ = 4.31 S for [^{131}I]BSA whose tube no. was 23.5 in these experiments. [b] Dayoff *et al.* (1952). [c] Fredericq and Deutsch (1949). [d] Harrington and Schellman (1956). [e] Wetter and Deutsch (1951).

tions, the ones most relevant to the present discussion being that the partial specific volumes, $\bar{v}$, of the unknown and the standard be the same and, furthermore, be identically affected by the two component solvent, sucrose plus water. Thus, if the unknown is FSH with a $\bar{v}$ = 0.696 cc/g, the standard should be a glycoprotein of comparable $\bar{v}$. For those experiments in which we examined the sedimentation properties of FSH after guanidine treatment, it was possible to employ such a standard, namely, ovomucoid ($\bar{v}$ = 0.685) (Fredericq and Deutsch, 1949). In other experiments, however, this standard overlapped the sedimentation of the unknown and it was more convenient to use bovine serum albumin (BSA) ($\bar{v}$ = 0.734 cc/g) (Dayoff *et al.*, 1952) as it sedimented well ahead of the unknown. This standard protein clearly does not conform to the requirements of the previously mentioned assumptions.

In order to estimate and correct any systematic errors introduced in the determination of $s_{20,w}$ of FSH by the use of BSA as a sedimentation standard, we set up an $s_{20,w}$ calibration curve based on the sedimentation behavior of BSA at infinite dilution and then compared the sedimentation coefficient of 1% solutions of other standard proteins determined in the same run by use of eq 3 with the values determined at the same concentration in the analytical ultracentrifuge. That is, ^{131}I-labeled BSA at 5 × 10^{-6} g/dl and the unlabeled proteins, bovine serum albumin, ovomucoid, lysozyme, and ribonuclease, at 1 g/dl were centrifuged in separate tubes in the same run.[4] The ^{131}I-labeled BSA was designated as the standard whose sedimentation coefficient was taken as 4.31 S, the value for $s^0_{20,w}$ of the peak at infinite dilution determined by Creeth (1952). As demonstrated in Figure 3, a calibration curve was constructed for the apparent sedimentation coefficients, $s_{20,w}$, of the other proteins by plotting $s^0_{20,w}$ of 4.31 S against its tube number and then drawing a line between this one point and the origin. The apparent sedimentation coefficients of the other proteins would correspondingly be located along this line at their respective tube numbers. The equation for this line is, of course, eq 3. On the same coordinate system, the $s_{20,w}$ values of these proteins determined in separate analytical ultracentrifuge experiments were also plotted, as *points*, against their respective tube numbers in the experiment described above. (These various sedimentation coefficients and other relevant data are given in Table VI.)

The extent of the deviation of these points from the line reflects the combined random errors of the techniques employed as well as those systematic errors due to the failure of the assumptions of a uniform $\bar{v}$ and hydration, a linear relationship between distance traveled in the centrifuge tube and sedimentation coefficient, and a number of other effects. As the data in Figure 3 show, the deviation was significant only for ovomucoid ($\bar{v}$ = 0.685 cc/g) and ribonuclease ($\bar{v}$ = 0.695 cc/g) (Harrington and Schellman, 1956). One may thus conclude that only the $\bar{v}$ and preferential solvation effects are somewhat important factors to consider in our experimental determinations.

The extent of the observed difference, Δs, between the sedimentation coefficient determined by sucrose gradient sedimentation, $s'_{20,w}$, in the fashion indicated, and the true value, $s_{20,w}$, determined in the analytical ultracentrifuge, is a function (although not a simple one) of the difference in partial specific volume, $\Delta\bar{v}$, between the centrifugation standard, $\bar{v}_S$, and the unknown, $\bar{v}_U$. These Δ functions are defined as

$$\Delta s = s'_{20,w} - s_{20,w} \quad (4)$$

$$\Delta\bar{v} = \bar{v}_S - \bar{v}_U \quad (5)$$

To obtain an approximate correction of the sedimentation coefficient of an unknown for this difference in $\bar{v}$ effect, one may construct an empirical correction curve from the standard protein data by plotting Δs *vs.* $\Delta\bar{v}$. For a given $\Delta\bar{v}$, the Δs is obtained and applied to the $s'_{20,w}$ determined by sucrose gradient centrifugation to yield the actual sedimentation coefficient, $s_{20,w}$, which would have been observed in the analytical ultracentrifuge. That is

$$s_{20,w} = s'_{20,w} - \Delta s \quad (6)$$

Such a correction curve based on our data is displayed in Figure 4. Although Δs is not a linear function of $\Delta\bar{v}$, the small range covered and the joint precision of the separate techniques employed hardly justify anything more than an average straight line through the four points. The actual data for the

[4] The difference between the $s_{20,w}$ of BSA at 1% (1 g/dl) and [^{131}I]BSA at 5 × 10^{-6} (g/dl) arises because of the concentration dependence of the sedimentation coefficient and is not an artifact of labeling. This could be demonstrated by the fact that when both the labeled preparation at 5 × 10^{-6}% and the unlabeled BSA at 1% were run together in the same tube, the ^{131}I peak coincided with the protein peak. When run in separate tubes, however, the radioiodinated BSA sedimented faster than the unlabeled BSA. The latter point is illustrated in Figure 3 and Table VI.

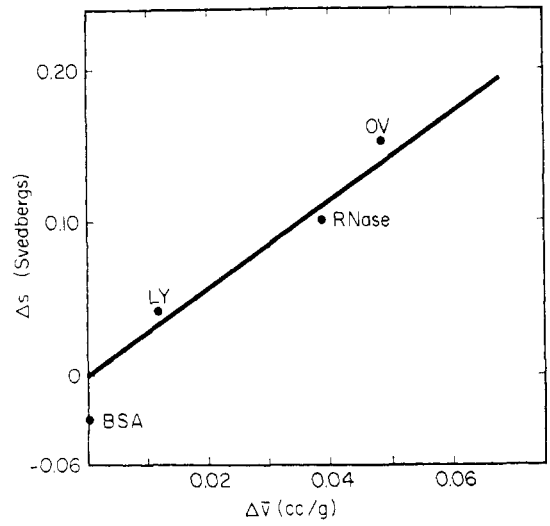

FIGURE 4: Variations in Δs as a function of $\Delta\bar{v}$. For a given protein, the difference in $s_{20,w}$ determined from the straight line function shown in Figure 3 and that value determined in the analytical ultracentrifuge is plotted against the difference between the partial specific volumes of BSA and the protein. Individual proteins are coded as indicated in the legend for Figure 3.

curve is included in Table VI. The empirical correction for $s'_{20,w}$ of FSH when ^{131}I-labeled BSA is employed as a standard protein is 0.11 S.

The results of the $s^0_{20,w}$ determinations[3] of various preparations of FSH are given in Table VII. Column 3 gives the $s'_{20,w}$ values of the major sedimenting species in the absence of guanidine hydrochloride, and column 4 gives the $s^0_{20,w}$ of the major species after guanidine hydrochloride treatment. In contrast to the gel filtration technique, sucrose gradient centrifugation was insufficient for resolving completely the large and the small molecular species of all samples except 11867C. In the absence of guanidine hydrochloride, the latter species could be seen as only a shoulder on the main fast-moving peak. After guanidine hydrochloride treatment, this situation was reversed. These points are illustrated in Figure 5. The average $s^{0'}_{20,w}$ of FSH before guanidine hydrochloride treatment was found to be 2.99 ± 0.06 S and, after guanidine hydrochloride treatment, 2.05 ± 0.04 S. Correction of these sedimentation coefficients yields $s^0_{20,w}$ values of 2.88 and 1.94 S. These values presumably reflect the sedimentation behavior of the 32- and the 23-Å material, respectively. The $s^0_{20,w}$ of the larger FSH species determined in these experiments agrees reasonably well with the values obtained for the sedimentation of the peaks in the analytical ultracentrifugation experiments (see Table II). (The latter data presumably reflect mainly the movement of the species present in greatest concentration.)

Molecular Weights of the Large and Small Species of Human FSH. The molecular weights of the 23- and 32-Å components of FSH were calculated from the data given in Tables IV, V, and VII by the application of eq 2. Table VIII gives these values for both the corrected as well as the uncorrected sedimentation coefficients obtained from the sucrose density gradient experiments. Also included, for comparison, are the results of the appropriate analytical ultracentrifugation experiments. The molecular weight relationships from the several experiments clearly demonstrate a monomer–dimer relationship. The more reliable set of molecular weights are those obtained from the discriminatory gel filtration–density gradient centrifugation experiments which yield values of

TABLE VII: Sedimentation Coefficients of the Major Component of Human FSH Determined by Density Gradient Ultracentrifugation before and after Guanidine Hydrochloride Treatment.

Prepn	Concn (g/dl)	$s^0_{20,w}$ (S) of Major Peak (Rel to [^{131}I]BSA) Before Gdn·HCl[a] ±0.06S	After Gdn·HCl[a] ±0.04S
41267B	5×10^{-6}	2.94	
11867C	5×10^{-6}	3.02	
11867C	2×10^{-6}		1.98[b]
5764B	5×10^{-6}	2.96	2.06[c]
5765B	2×10^{-3}		2.16[d]
WDP-I	5×10^{-6}	*3.02*	*2.00*[e]
	Average	2.99	2.05
	Average, corrected for $\Delta\bar{v}$	2.88	1.94

[a] Gdn·HCl = guanidine hydrochloride. [b] 2 M guanidine hydrochloride, 4 hr. [c] 2–4 M guanidine hydrochloride, 4 hr. [d] 4 M guanidine hydrochloride, 4 hr; unlabeled sample, tube position followed by radioimmunoassay. [e] 4 M guanidine hydrochloride, 12 hr.

35,000 for the larger component and 17,000 for the smaller component.[5]

The agreement between the calculated and the found molecular weights of samples 11867C and 41267B at the air–water meniscus, M_A, in the analytical ultracentrifuge demonstrates the fact that the 23- and the 32-Å species seen at very low concentrations are not in rapid and/or reversible equilibrium with one another. We have calculated M_A for these samples using the distribution data shown in Table IV (entries 1 and 3) together with the molecular weights of both the 23- and the 32-Å species (obtained at low concentrations) and the assumption that molecular weight species above 35,000 are absent from this position in the column. Although this assumption is less than satisfactory for sample 41267B, the agreement is reasonably good. After correcting for the redistribution of the two species in the centrifugal field, the calculated M_A is 26,000 for sample 11867C and 34,000 for sample 41267B. The observed values are 27,000 for the former and 37,000 to 38,000 for the latter. (See Table III, low speed results.)

Conclusion

These experimental results indicate that human FSH, as normally isolated, is a glycoprotein of molecular weight 35,000, with subunit structure. Upon subjecting this molecule, after isolation, to a variety of denaturing conditions, it can be partially and irreversibly dissociated into its constitutive subunits. There appear to be two such subunits of approximately equal molecular weight. Whether the subunits are identical in composition is currently under investigation.

The stability of the 35,000 molecular weight dimer is exceptionally high. Even at the nanogram levels of concentration

[5] The failure to find an exact molecular weight ratio of 2.0 is very likely due to cumulative experimental errors.

TABLE VIII: Summary of the Molecular Weights of the Associated and Dissociated Forms of Human FSH.

Form	Av Stokes Radius (Å)	Av $s^0_{20,w}$[a] (S)	Molecular Weight: Calcd by Eq 2	Molecular Weight: Anal. Ultracentrifuge
Associated	32.2	2.99	36,200	32,000 (high speed)
		2.88[b]	35,100	37,100 (low speed)
Dissociated	23.2	2.05	17,800	16,000 (high speed)
		1.94[b]	16,800	

[a] Determined by density gradient ultracentrifugation. [b] Corrected for $\Delta\bar{v}$ effect in density gradient ultracentrifugation.

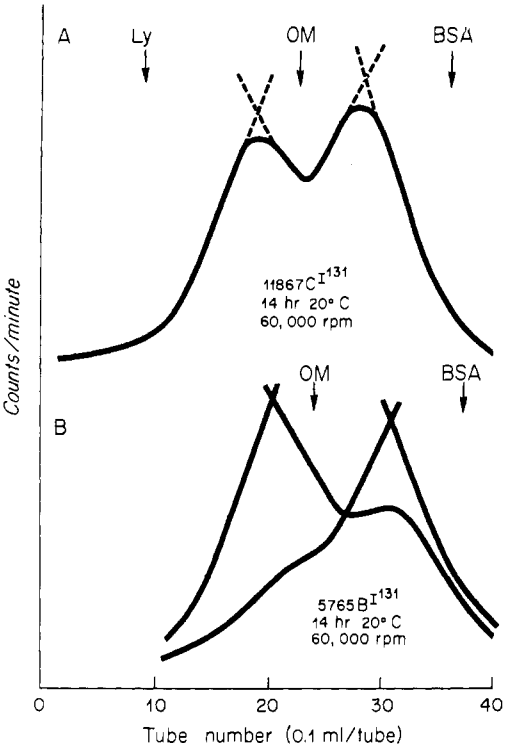

FIGURE 5: Density gradient ultracentrifugation patterns of ^{131}I-labeled FSH. (A) Sample 11867C recovered from analytical centrifugation experiments and labeled with ^{131}I. (See text.) Initial loading concentration was 5×10^{-6} g/dl. (B) Sample 5765B labeled with ^{131}I and applied at an initial concentration of 5×10^{-6} g/dl. The curve with the major peak to right of OM indicates material treated with 4 M guanidine hydrochloride for 2 min and the curve with the major peak to the left of OM indicates material treated with 4 M guanidine hydrochloride for 2 hr at room temperature. The symbols across the top of A and B indicate the peaks for the sedimentation of the standard proteins bovine serum albumin (BSA), ovomucoid (OM), and lysozyme (LY) at a concentration of 1 g/dl.

employed in the gel filtration and sucrose gradient centrifugation studies, dissociation is usually minimal unless the protein is subjected to the denaturing conditions described. The increased amount of irreversible dissociation as a function of time of standing in the absence of denaturants, however, suggests that this stability may be kinetic rather than thermodynamic.

This unusually slow rate of dissociation could perhaps be due to the immobilization of the polypeptide chains by virtue of the interaction of carbohydrate residues of the molecule with the aqueous solvent. This latter interaction could give rise to a large viscous drag when the various segments of the glycoprotein molecule moved through the solvent and would serve to prevent them from rapidly unfolding to a more thermodynamically stable conformation which would favor dissociation. Upon the addition of a denaturant, this interaction with the solvent would be destroyed and dissociation would ensue. Although this is speculative, it suggests a possible role for the carbohydrate residues of this and other glycoproteins. They may serve to kinetically lock in place an otherwise thermodynamically unstable but biologically active conformation of a given polypeptide chain.

Molecular weights, hydrodynamic and dissociation properties of human pituitary FSH have been reported previously by others. Our value for the sedimentation coefficient of the major species of native FSH agrees rather well with the value of 2.96 Å reported by Roos (1967) but differs significantly from the values reported by Saxena and Rathnam (1967) and Papkoff *et al.* (1967). The Stokes radii of FSH reported herein agree well with the values of 34.6 and 22.3 Å reported by Reichert and Midgley (1968) for native and urea-treated FSH, respectively. Gray's data (Gray, 1967; Crooke and Gray, 1968) for the molecular weights of two species of FSH are in good agreement with ours, but this is probably fortuitous since his estimates were based entirely on gel filtration experiments. In the light of our survey of various preparations it is clear that the wide discrepancies in previously reported values of the physical properties of this protein can be attributed to differential amounts of dissociation and aggregation, as well as the pitfalls and errors inherent in calculating molecular weights from a single type of hydrodynamic measurement. We feel that the present study is the first definitive one on human pituitary FSH which yields reliable molecular weights and firmly established the subunit character of this hormone.

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Circular Dichroism of Cyclic Hexapeptides with One and Two Side Chains*

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ABSTRACT: We report ultraviolet circular dichroism curves for several cyclic hexapeptides of glycine, leucine, tyrosine, and histidine in neutral aqueous solution. Our interpretation is assisted by previously reported nuclear magnetic resonance data on these compounds which indicate that they contain two transannular hydrogen bonds. These structures are fairly rigid, but have some conformational mobility. A circular dichroism band at 198 nm is assigned to the amide π–π^* band while bands at 214 and 222 nm are assigned to amide n–π^*.

A weak band seen in *cyclo*-Gly_5-Leu as well as in random coil polypeptides at 230 nm is interpreted as a residual of overlapping n–π bands and not as a result of an absorption band at that wavelength. Circular dichroism bands due to the neutral tyrosyl side chain are identified at 275, 228, and 198 nm and a band due to histidyl side-chain absorption is identified at 211 nm. The amide backbone perturbs the symmetry of the side chain leading to Cotton effects at the wavelengths of side-chain absorptions. In addition, we also observe the effect of the side chain in modifying the optical activity of both the n–π^* and π–π^* transitions of the amide. We propose that the conformation of *cyclo*-Gly_5-Leu is an equilibrium between two or more forms, one of which is preferred in *cyclo*-$(Gly_2\text{-Leu})_2$. The similarity of the circular dichroism curve of the former compound to that of polyglutamic acid in neutral solution leads us to suggest that a similar combination of conformations could contribute in random coil systems.

The optical activity curves for model peptides are often used in the interpretation of protein optical activity spectra. The model structures most commonly used are the α helix, random coil, and more recently the anti-parallel β form (Greenfield *et al.*, 1967). Although these models provide a general framework for the interpretation of protein optical rotatory dispersion and circular dichroism, it is clear that globular proteins contain rigid conformations other than α helix and β forms and that they are unlikely to contain any truly random coils. In the present study, we report circular dichroism curves for four cyclic hexapeptides of relatively rigid conformation for which we have structural information from nuclear magnetic resonance (Kopple *et al.*, 1969a,b). We have studied their optical activity in the hope of providing a better understanding of the contributions to optical activity of various rigid polypeptide structures other than the presently available models, α helix and β forms.

The nuclear magnetic resonance spectra of the cyclic

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