Preliminary Investigations of Intrinsic and Extrinsic Optical Activity as Purity Criterion for Human Serum Albumins

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Abstract □ Induced circular dichroism measurements were made to follow the binding of four acidic drugs to two lots of crystalline and two lots of fraction V human serum albumins. The magnitude of the induced circular dichroism varied with all lots of albumin, suggesting a strong sensitivity of the phenomenon to small changes in purity or secondary structure of the albumins. The circular dichroism of the albumins themselves showed much less variation. The more classical analytical techniques of UV absorption, measurement of absorption following methyl orange binding, gel electrophoresis, and ultracentrifugation were also performed on the albumins for comparison.

Keyphrases □ Albumins, human serum—intrinsic and extrinsic optical activity as a purity criterion, circular dichroism compared to other techniques □ Optical activity, intrinsic and extrinsic—purity criterion for human serum albumins, circular dichroism studies □ Circular dichroism—intrinsic and extrinsic optical activity as a purity criterion for human serum albumins, binding of four acidic drugs

Dicumarol has been shown to become optically active on binding to human serum albumin (1, 2). During a quantitative investigation of this binding, it was found that the shape and magnitude of the induced circular dichroism (CD) curves varied with the lot of albumin (2). Therefore, it was decided to investigate the curves generated by four planar and acidic drugs, dicumarol, sulfaethidole, phenylbutazone, and flufenamic acid, following the binding of two new lots of crystalline and two new lots of fraction V human serum albumin to see whether the phenomenon is purity related.

The CD curves of the albumins themselves were also investigated. More classical analytical techniques such as ultracentrifugation, gel electrophoresis, UV absorption, and absorption following methyl orange binding were performed on these albumins for comparison.

EXPERIMENTAL

Materials—Dicumarol[1] USP, flufenamic acid[2], and phenylbutazone[3] were used as supplied. Sulfaethidole[4] was recrystallized twice from water, mp 185–186°. All other materials were reagent grade, and deionized water was used throughout. Two lots of crystalline and two lots of fraction V human serum albumins were obtained from leading manufacturers. They were stored, refrigerated, and desiccated under identical conditions and were used as supplied by the manufacturers.

Methods—All CD spectra were obtained in a 6003 CD attachment to a spectropolarimeter[5] with a slit programmed for a half bandwidth of 15 Å. All measurements were made in 0.054 *M* sodium phosphate buffer, pH 7.4, adjusted to isotonicity with sodium chloride. All solutions were scanned in 10- or 20-mm cells from regions of high wavelength having no ellipticity to the lower limit of the system.

All albumin samples were subjected to sodium lauryl sulfate gel electrophoresis, similar to that described by Weber and Osborn (3). The samples were dissolved in 0.01 *M* sodium phosphate buffer, pH 7.0, containing sodium lauryl sulfate and 1% β-mercaptoethanol, and were heated at 37° for 2 hr. Electrophoresis was carried out at 3 mamp/gel for 18 hr with the anode in the lower chamber. Gels were then stained for 4 hr with 0.25% Coomassie brilliant in 50% methanol and 9.2% acetic acid; destaining was accomplished by diffusion in 5% methanol and 9.2% acetic acid.

Sedimentation velocity experiments were carried out at 20° in an analytical ultracentrifuge[6]. A Kel F cell with a sapphire window was used in these runs, carried out at 68,000 rpm on 0.9% albumin solutions. The schlieren optical system was used to record the rate of change in the refractive index of the sample as a function of distance from the rotor's axis of rotation.

Spectrophotometric[7] measurements were made in silica cells of 10-mm path length. For the determination of the albumins by methyl orange, the original method of Bracken and Klotz (4) was modified in that twice as much methyl orange was used and measurements were made at 535 and 550 nm.

Table I—$E^{1\%}_{1\,cm}$ of pH 7.4 Solutions of the Albumins

Sample	Absorbance, nm	
	279	210
A, fraction V	5.85	157
B, fraction V	6.13	168
C, crystalline	6.36	190
D, crystalline	3.85	136

Table II—Percentage Purity of Albumins Using the Methyl Orange Method of Bracken and Klotz (4), Taking Sample C as the Standard

Sample	Purity, %	
	550 nm	534 nm
A	96	98
B	99	98
C	100	100
D	72	72

RESULTS AND DISCUSSION

UV Characteristics—The absorbances of 1% solutions of the albumins in 1-cm cells at 279 and 210 nm are shown in Table I. Sample D gave a significantly lower absorption than the others, and this crystalline sample repeatedly behaved as if it contained 25% of a nonabsorbing impurity, perhaps an inorganic salt used in the purification procedures. Even if Sample D is omitted, there was still almost a 10% fluctuation in the absorbances at 279 nm.

Most literature reports give a figure of $E^{1\%}_{1\,cm}$ for "pure" serum albumin of 5.30–5.80 (5–7). The absorption in this region is primarily due to the aromatic amino acid residues. Therefore, contamination due to globulins, which contain more aromatic amino

[1] Abbott Laboratories, North Chicago, Ill.
[2] Parke-Davis, Detroit, Mich.
[3] Geigy Research, Ardsley, N.Y.
[4] Smith Kline and French, Philadelphia, Pa.
[5] Cary 60, Cary Instruments, Monrovia, Calif.
[6] Beckman model E, Beckman Instruments, Palo Alto, Calif.
[7] Cary 14, Cary Instruments, Monrovia, Calif.

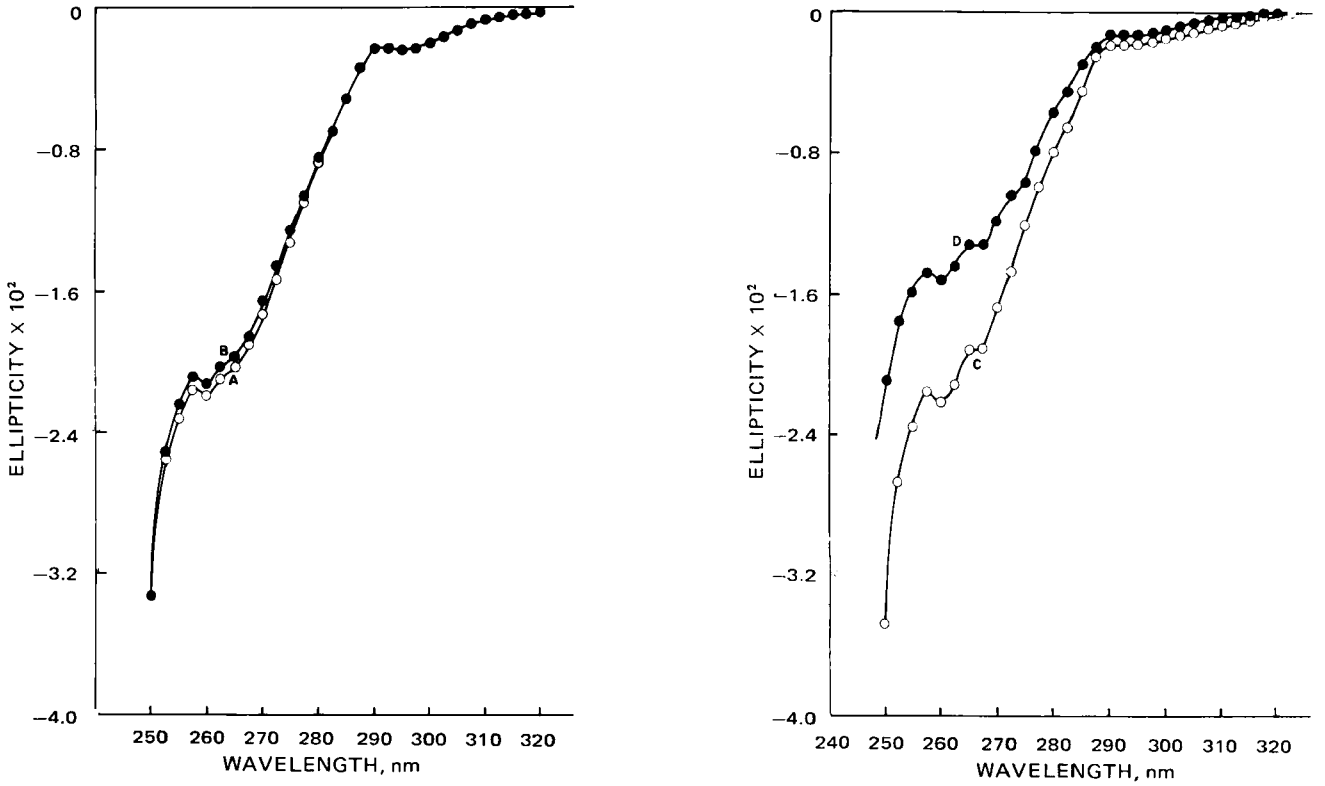

Figure 1—*CD curves of albumin samples alone in the 250–300-nm region. Human serum albumin concentration is 0.2% (2.9 × 10^{-5} M) in 10-mm cells at pH 7.4. (See Table I.)*

acids than do albumins, results in an increased absorbance at this wavelength. Any aromatic impurity acquired during the extraction or purification process or added as a stabilizer also contributes to the absorbance in this region.

Globulins and albumins have been reported to have similar UV absorbances at 210 nm, and a pure sample of albumin was reported to have an $E^{1\%}_{1\,cm}$ of 190 (5). This value was obtained with the crystalline Sample C. The fraction V samples gave significantly lower values than 190. The UV spectrum at 210 nm resulted from aromatic residues and contributions from peptide linkages. The latter contributions are from both the primary and secondary structures of the protein.

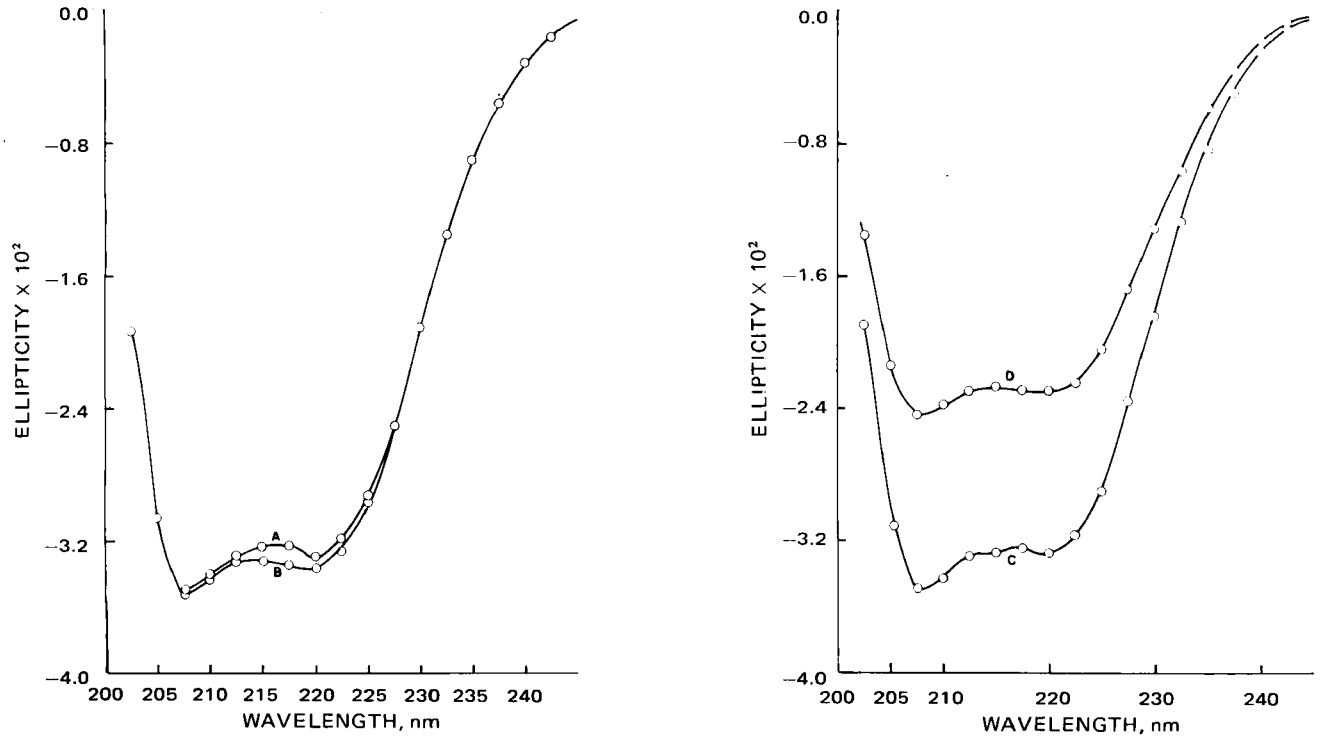

Figure 2—*CD curves for the four albumins in the 200–250-nm region. Human serum albumin concentration is 0.001% (1.45 × 10^{-7} M) in 20-mm cells at pH 7.4. (See Table I.)*

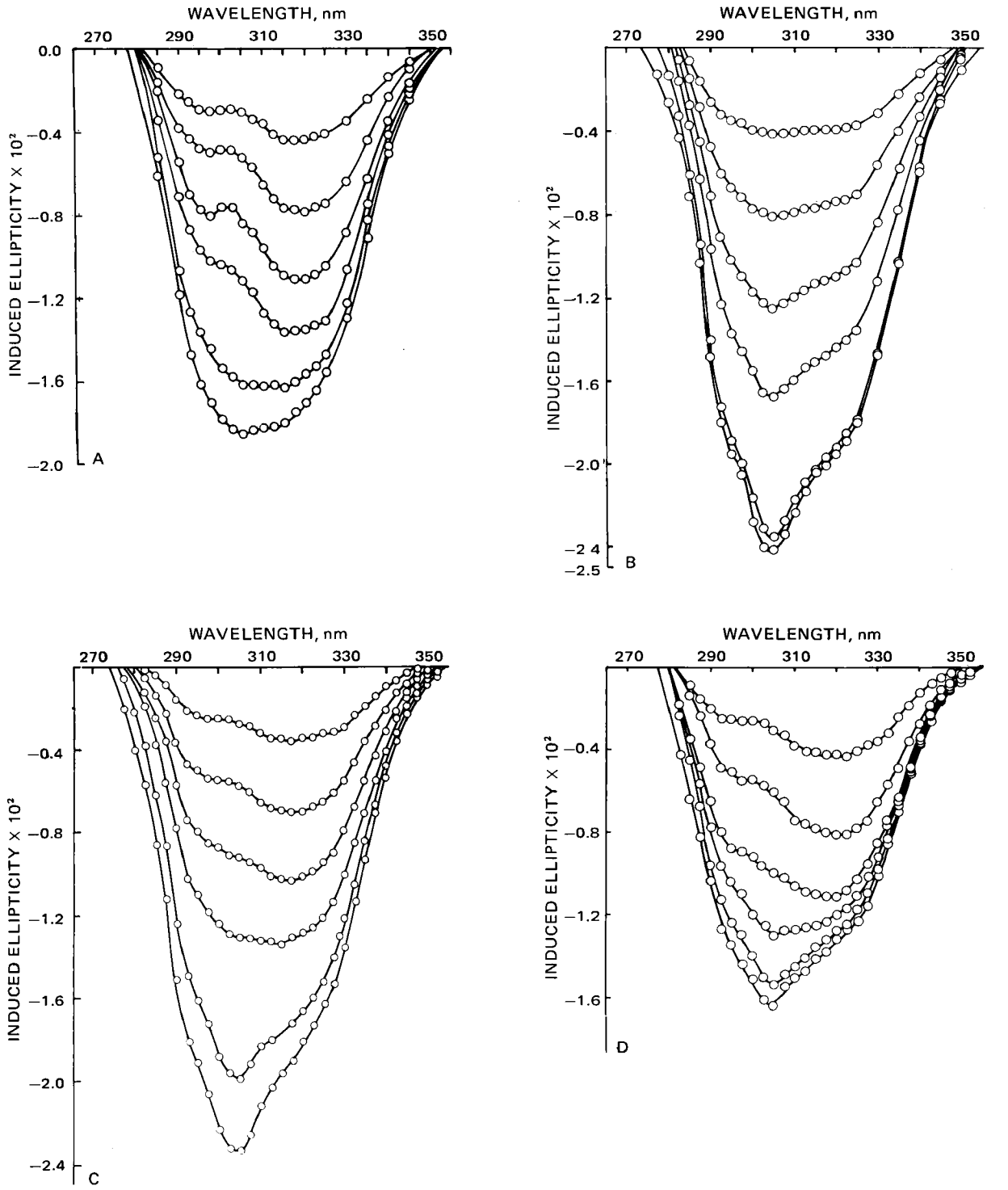

Figure 3—*Induced CD of dicumarol with the four albumin samples. (See Table I.) Human serum albumin concentration is 1.45 × 10⁻⁵ M in 10-mm cells at pH 7.4. Dicumarol concentrations are 0.743, 1.48, 2.23, 2.96, 4.46, and 5.92 × 10⁻⁵ M.*

The decreased absorbance following reaction with methyl orange was measured at 550 and 535 nm. The percentages are expressed taking Sample C as the standard, because this sample gave the largest decrease in absorbance (Table II). Methyl orange does not react with globulins (5). This method, involving the reaction with dyes, is greatly superior to the UV measurement of the albumins, but it demands a correct choice of an albumin standard and it may not be sensitive to changes in the secondary structure of the albumins.

CD Characteristics—CD in the 250–300-nm region is due to the aromatic residues in the primary structure of albumin. As evident from Fig. 1, there is considerable fine structure present in this region due to contributions from various electronic transitions associated with phenylalanine, tryptophan, tyrosine, and histidine residues. Samples A, B, and C were virtually superimposable over this wavelength region, suggesting that they had similar concentrations of albumin. However, Sample D, as expected, showed decreased ellipticity of approximately 30%.

The 200–250-nm region is frequently regarded as being due to the secondary structure of the protein. However, it contains contributions from the aromatic residues as well. The spectra of Samples A, B, and C in this region were again virtually superimposable, having peaks near 220 and 208 nm (Fig. 2). Greenfield and Fasman (8) suggested that the percentage of α-helix in a protein, using

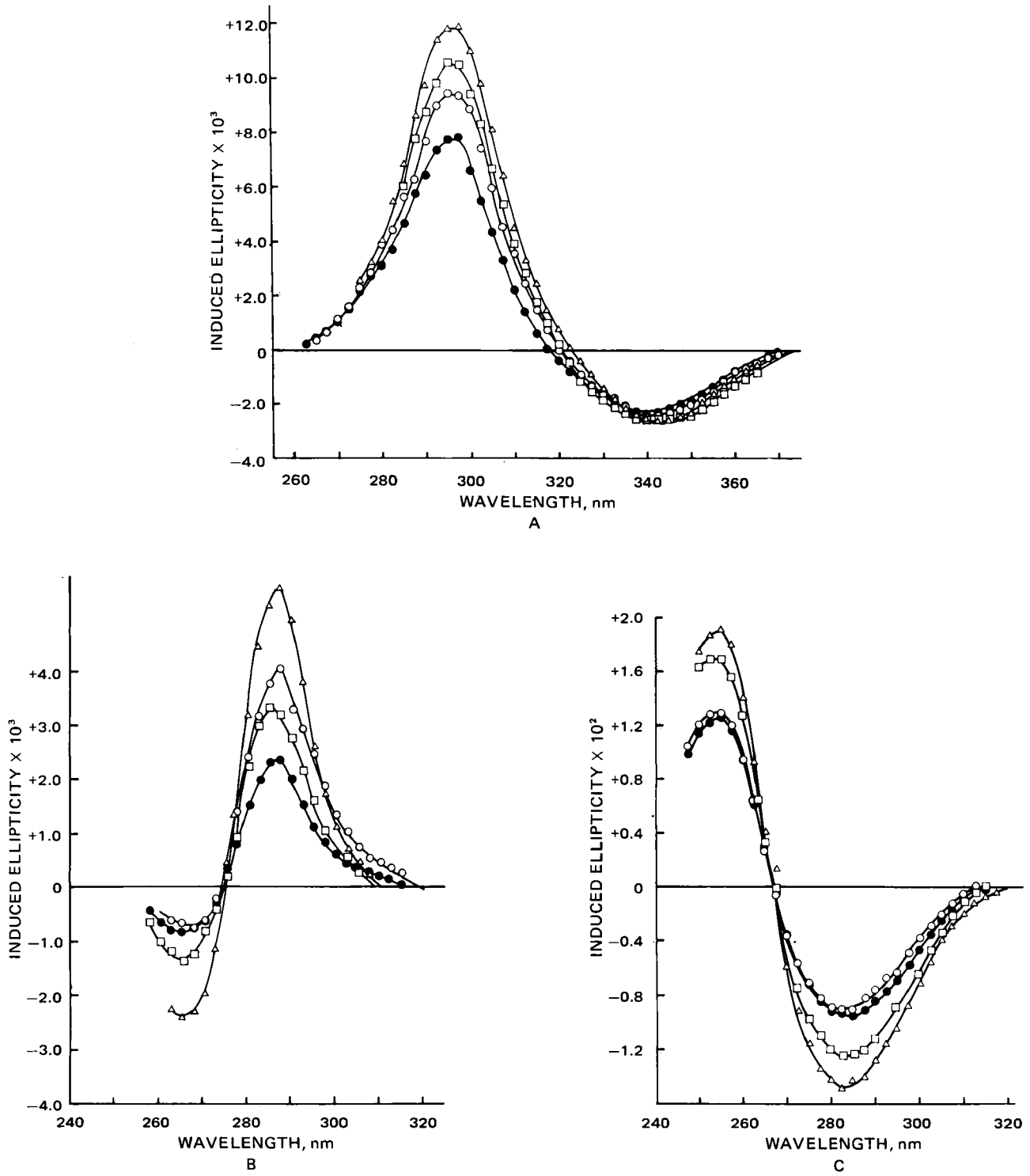

Figure 4—*Induced CD of flufenamic acid (A, 5 × 10^{-5} M), phenylbutazone (B, 2.59 × 10^{-5} M), and sulfaethidole (C, 1.27 × 10^{-5} M) with the four albumin samples. Human serum albumin concentration is 1.45 × 10^{-5} M in 10-mm cells at pH 7.4. Key: ●, albumin A; ○, albumin B; △, albumin C; and □, albumin D.*

poly-L-lysine as a model, is given by:

$$\% \ \alpha\text{-helix} = \frac{-[\theta]_{208} - 4000}{33{,}000 - 4000} \times 100 \qquad \text{(Eq. 1)}$$

Using a mean residue molecular weight of 113 for human serum albumin and substituting in this formula give the data shown in Table III. Samples A, B, and C had essentially the same helical content. The shape of the CD spectra was consistent with the remainder being primarily in the random coil conformation rather than being the β-structure (7). Sample D had the same shape CD curve, but it was of greatly reduced intensity due to the presence of nonabsorbing and nonoptically active impurities. This finding suggests that there had been no macrodenaturation of the albumins during their preparation.

Induced CD—Figures 3 and 4 show the induced CD of the four drugs following their binding to the various albumin samples. The resultant binding curves were apparently sensitive to impurities or a localized denaturation of the albumin. The four sets of dicumarol–albumin curves showed no new peaks; however, the shape of

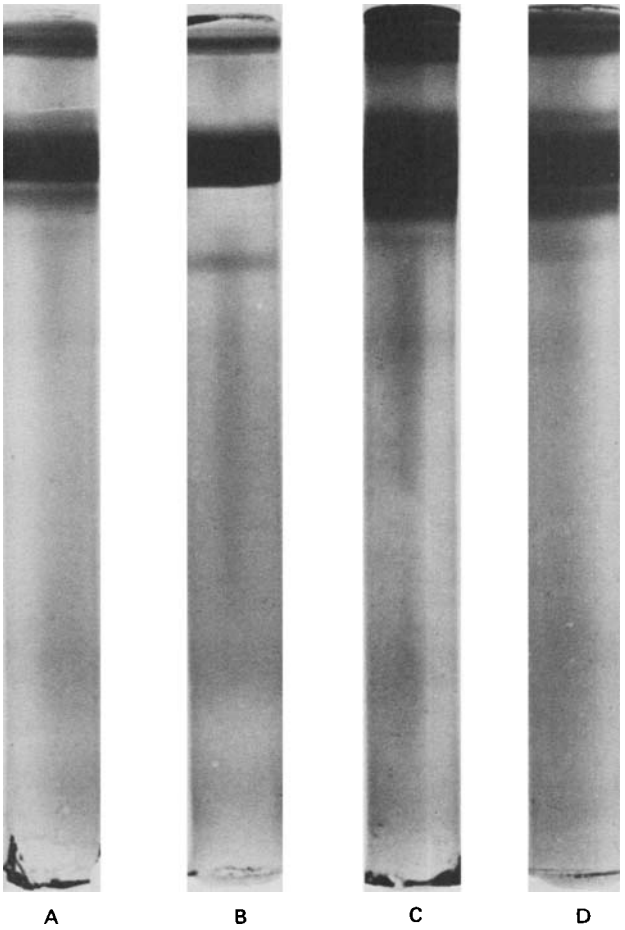

Figure 5—*Sodium lauryl sulfate gel electrophoresis. The letters refer to the albumin samples.*

the induced curves was different, as were their concentration dependencies. The curves of albumin Sample A–dicumarol gave characteristics similar to the fraction V sample used in earlier work (2). The widely different concentration dependence of the peak heights is immediately apparent on comparison of Samples A and B. The two crystalline samples (C and D) gave similar shaped curves when corrections were made for the impurity present in Sample D.

Figure 4 shows the induced CD of flufenamic acid, sulfaethidole, and phenylbutazone when bound to the various albumin samples. Curves obtained with Sample D were continually lower in peak height, and this problem appears to be entirely a concentration effect. The curves obtained with sulfaethidole (Fig. 4C) were similar for both fraction V samples, but the small differences between the two were reproducible; however, both curves were much smaller than the curves seen with the crystalline albumins.

The shape of the sulfaethidole-induced CD was concentration independent, and only one class of site was capable of inducing optical activity[8]. When sulfaethidole was bound to crystalline and fraction V samples of bovine serum albumin, the induced curves were similar in size and the binding parameters were of about equal value (9). The binding curves of flufenamic acid and phenylbutazone, which were very concentration dependent, emphasize the differences in the fraction V samples. Some small conformational change may, by slightly altering the nature of the binding process, account for the observed differences in the curves.

Gel Electrophoresis—The result of the sodium lauryl sulfate gel electrophoresis examination is shown in Fig. 5. The protein became negatively charged by the sodium lauryl sulfate and migrated toward the bottom. The gels were overloaded with protein to make apparent obvious inconsistencies between samples. It was immediately apparent that Sample B, a fraction V albumin, was of higher quality than the others tested. Samples A, C, and D had a small amount of low molecular weight material, probably about 50,000, and all samples contained a higher molecular weight material of molecular weight around 150,000. Since mercaptoethanol treatment in the presence of sodium lauryl sulfate destroys disulfide bonds (10), the dimer probably was eliminated; therefore, this high molecular weight material must have been another protein impurity.

[8] J. H. Perrin, unpublished observations.

Table III—Percentage of α-Helical Content of the Four Albumin Samples[a]

Sample	Observed Ellipticity at 208 nm × 10^2	Calculated α-Helix, %
A	3.51	55
B	3.49	54
C	3.50	54
D	2.41	33

[a] All solutions were 1.45×10^{-5} *M*; measurements were made in 20-mm cells using physiological phosphate buffer of pH 7.4.

Ultracentrifugation—Schlieren patterns developed during ultracentrifugation of the albumin samples are shown in Fig. 6. It can be seen from the areas of the peaks that Sample D contained less total protein than the other samples. Sample B showed a negligible amount of high molecular weight impurity; however, Samples A, C, and D all showed a small peak due to a high molecular weight material, possibly some albumin dimer or γ-globulin (γ-immunoglobulin G). The percentages of this high molecular weight material in the samples are given in Table IV.

CONCLUSION

The samples of albumin were assumed to be essentially free of globulins due to their method of preparation. Nevertheless, gel electrophoresis suggests the presence of a very small quantity of high molecular weight material which should not be albumin polymers. Globulins contain more aromatic residues than albumins,

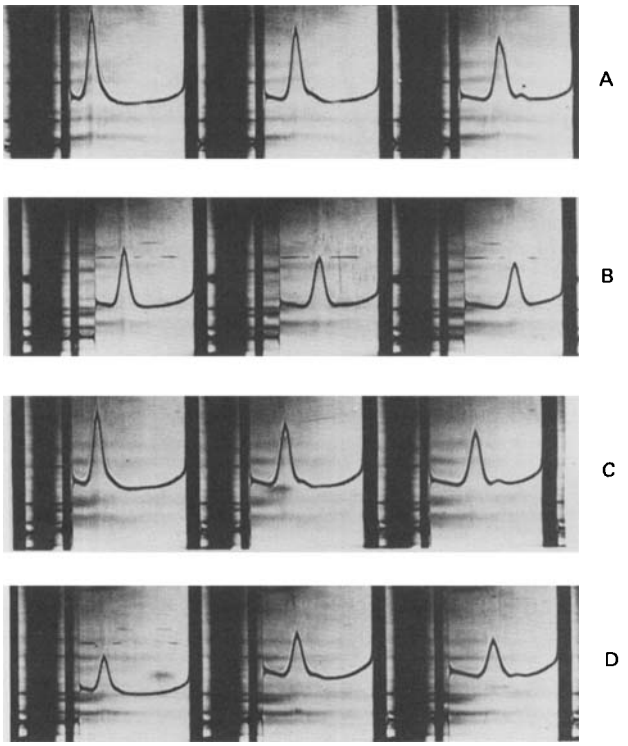

Figure 6—*Results of analytical ultracentrifugation analysis at 68,000 rpm for 2 hr. The letters correspond to the albumin samples.*

Table IV—Analysis of Impurity in Albumin Samples by Ultracentrifugation for Approximately 100 min at 68,000 rpm

Sample	Impurity, %
A	7.6
B	<1.0
C	6.5
D	7.7

and their presence would complicate the UV absorbances, particularly at 280 nm. Aromatic impurities can also arise from stabilizers and fractionating aids or as impurities in extracting solvents. The absorbance at 210 nm, although more difficult to measure, is a better analytical tool for total protein content because the main contribution at this wavelength is from the peptide bond.

There does not appear to be extensive literature on the binding of anionic drugs to other components of human serum such as globulins or lipoproteins. The anionic dye, methyl orange, does not appear to bind to γ-globulin (4, 11), but it has been reported to bind to β-lipoprotein as strongly as it does to albumin (12). It has also been reported that molecular aggregation of albumin in solution has no effect on its methyl orange binding capacity (5) whereas heat denaturation does (13).

The induced CD curves seem to be very sensitive to either impurities (perhaps lipoproteins, globulins, or albumin dimers) or minor changes in conformation. The effect of a high molecular weight impurity can best be seen when curves B and C are compared (Fig. 3). These albumins gave the largest induced CD with dicumarol, but C contained much more impurity than B. Therefore, the difference was probably not due to the impurity. Significant differences in shape and concentration dependence are evident in all CD curves of the dicumarol–albumin complexes. With the other drugs investigated, all albumins gave similarly shaped curves, but they exhibited variations in the magnitude of the induced signals.

CD curves of Samples A, B, and C in the 250–300-nm region were similar and showed considerable fine structure due to aromatic moiety contributions. These findings suggest that these albumin samples are of similar total albumin concentration. In the lower wavelength region, where the induced CD is primarily from secondary structure, the curves were again similar and suggest a helical content of about 55%; however, the small and reproducible differences shown may well be significant.

Small but locally significant conformational changes cannot be easily examined by CD since the technique measures the average contribution over the whole albumin molecule. Such small changes in the binding site region could well explain the differences observed and could be the results of variations in solvent or possibly heat treatment during the albumin extraction process. It is apparent that during any binding study, particularly if of a quantitative nature, a single lot of albumin should be used. Moreover, established analytical techniques such as reaction with biuret (14) or phosphotungstophosphomolybdate (15) or spectrophotometric color determination with bromcresol green (16), bilirubin (5), or methyl orange (4) should be used to check for gross contamination as was found with Sample D.

CD of albumins alone or in conjunction with a molecule giving extrinsic Cotton effects may be an extremely sensitive technique for assessing the quantity and quality of albumin samples. In any quantitative investigations using crystalline or fraction V albumins, an investigator should use a single lot of albumin.

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