2. Ultracentrifugal analysis of the recrystallized enzyme has revealed only one protein component.

3. The optimum pH for polyol oxidation is about 10 and that for ketose reduction is about 7.

4. Michaelis constants have been determined for xylitol, sorbitol, ribitol and diphosphopyridine nucleotide. K_m for xylitol is about one-sixth of that for sorbitol and one-tenth of that for ribitol. The rates of oxidation of typical substrates have been compared under different conditions of pH and substrate concentration.

5. The substrate specificity of the enzyme conforms to the rule for L-iditol dehydrogenase except for L-arabitol; however, this anomalous oxidation occurs significantly only at high substrate concentration at a high pH.

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The Relation of the Rotatory Dispersion Behaviour of Human Serum Albumin to its Configuration

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Two of the methods used for studies of the secondary and tertiary configuration of proteins in aqueous solution are deuterium-hydrogen exchange (Linderstrøm-Lang, 1958) and optical rotatory dispersion (Schellman, 1958).

There is no systematic study in the literature of the optical rotatory properties of human serum albumin and, because of variations in procedure, it is impossible to compare all the available data. In the experiments presented here we aim to demon-

strate the type of changes in optical rotatory dispersion that take place in the albumin molecule on alteration of the normal environment and to relate these changes to the native structure of the protein.

EXPERIMENTAL

Materials

Two batches of human serum albumin were used, prepared by the ether-fractionation technique (Kekwick & Mackay, 1954). No 'stabilizer' was added to these specimens because it has been shown that these additives affect the configuration of the molecule (Mackay & Martin, 1957). The data on these two albumins are shown in Table 1. 2-Chloroethanol was twice fractionally distilled, b.p. 128–129°. Urea and all inorganic salts were A.R. grade.

Methods

Moving-boundary electrophoresis. Samples were dialysed to equilibrium at 2° against sodium phosphate buffer, pH 8.0, *I* 0.2, and diluted with buffer to a concentration of 1.5% of protein. Measurements were made in the Tiselius apparatus at 0° with a potential gradient of 6v/cm. Optical observations, by the diagonal-schlieren method (Philpot, 1938), were recorded photographically with light at 546 mμ.

Ultracentrifugal analysis. Dialysed samples were diluted to a 1% protein concentration so that the solution contained phosphate buffer, pH 8.0, *I* 0.2, in 0.15M-NaCl. The solutions were submitted to 250 000*g* in the Svedberg oil-turbine centrifuge, a 12 mm. cell being used. The optical-recording technique was the same as used for electrophoresis.

pH measurements. These were made with a glass electrode and calomel half-cell with liquid junction.

Immunoelectrophoresis. Samples were subjected to electrophoresis in agar (Schiedegger, 1955) and tested against antisera against normal human serum proteins prepared in rabbits (Weitz, 1952).

Chemical estimations. Total hexose was measured by the method of Sorensen & Haugaard (1933). Sialic acid was

measured by the method of Svennerholm (1957, 1958). Nitrogen was measured by the Kjeldahl method with a nitrogen-protein conversion factor of 6.25. Chloride was measured by the method of Van Slyke & Sendroy (1923).

Dispersion measurements. The optical-dispersion measurements were made on an assembly of instruments consisting of five main units. A schematic drawing of the apparatus is shown in Fig. 1.

There are two essential differences between this assembly and others in common use. A secondary monochromator is introduced to eliminate 'stray light' passed by the primary monochromator.

In the polarimeter the analysing prism is made to oscillate through a predetermined angle and the polarizing prism is rotated to balance the rotation of the sample. This is the reverse of the layout in earlier instruments. The oscillation of the analyser can be 2°, 5°, 10° or 15°. For the work described, 5° oscillations were used. Measurements were made in 10 cm. tubes at a temperature of 22 ± 0.5°. The vernier scales allow rotation readings down to 0.001° and reproducible readings are obtainable down to 0.002°, giving specific-rotation values for the materials used to an accuracy of ± 0.5°.

Solutions for optical measurements were prepared under standard conditions by dilution of a 5% stock solution of albumin in water. Each solution was kept at room temperature for at least 12 hr. Nitrogen was determined on this solution.

INTERPRETATION OF RESULTS

The early interpretations of dispersion data were based on the simple Drude equation:

$$[\alpha] = K/(\lambda^2 - \lambda_c^2).$$

Optical rotatory dispersion studies of synthetic polypeptides (Blout, 1960; Doty, 1959; Schellman, 1958), and of crystalline proteins, such as insulin and ribonuclease (Yang & Doty, 1957), showed that whereas randomly coiled forms gave dispersion plots of the simple Drude type, anomalous dis-

Fig. 1. Optical layout of the spectropolarimeter. A, Tungsten source; B, condenser; C, prism; D, collimator; E, slit; F, second monochromator; G, stop; H, polarizer; I, sample; J, oscillating analyser; K, photocell; L, galvanometer. Components C, D and E constitute the first monochromator, and components G, H, J and K the polarimeter.

person plots were obtained in the presence of α -helix.

Moffitt (1956), on theoretical grounds, had suggested modifications of the simple Drude equation that to some extent allowed for the complications introduced by the α -helix. Fitts & Kirkwood (1956*a, b*) ascribed the specific rotation to a helical form and found that it compared with the observed differences of specific rotation between a randomly distributed and a completely coiled model, provided that the helix was assumed to be the right-handed form. Independent crystallographic studies suggest that the right-handed helical form is the more probable in native proteins (Elliott & Malcolm, 1958).

In this paper we use the general equation, and a modification, which are both based on that of Moffitt (Imahori, Klemperer & Doty, 1957).

$$[m] = \frac{3}{n^2 + 2} \frac{M_0}{100} [\alpha] = (a_0^R + a_0^H) \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + f b_0^H \left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right)^2$$

n refers to refractive index of solvent; M_0 refers to mean residue weight (= 110 for albumin). For albumin in water the equation can be rearranged to give the following form:

$$[\alpha] = 1.39 \left[(\Sigma a_0^R + f a_0^H) \left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right) + f b_0^H \left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} \right)^2 \right]$$

$[\alpha]$ refers to specific rotation; λ_0 refers to a constant (212 $m\mu$); a_0^R refers to the contribution of the residues; a_0^H and b_0^H refer to the helical contribution; f denotes the fraction of residues in helical form.

The value of 212 $m\mu$ for λ_0 has been computed from observations on a number of synthetic polypeptides (Mitchell, Woodward & Doty, 1957; Applequist & Doty, 1958; Doty, Imahori & Klemperer, 1958), which indicate that the variation is not more than 2%.

By plotting $[\alpha](\lambda^2 - \lambda_0^2)$ against $1/(\lambda^2 - \lambda_0^2)$ a straight line is obtained, the values of $(\Sigma a_0^R + f a_0^H)$ and $f b_0^H$ being obtained from the intercept and slope respectively; Σa_0^R is directly obtainable when a_0^H and $b_0^H = 0$, i.e. the random coil configuration. Σa_0^R has the value of -620 for bovine albumin

(Imahori, 1960). Our own observations on human albumin give a similar value. With Σa_0^R known $f a_0^H$ may be calculated.

Dispersion measurements on synthetic polypeptides in the 100% helical configuration (Moffitt & Yang, 1956; Doty & Lundberg, 1957) have given values for a_0^H and b_0^H of +650 and -630 ($\pm 10\%$) respectively. With these limiting values of a_0^H and b_0^H known it is possible to obtain two independent estimates of f , the fraction of the residues in the helical form.

In this paper the results have been calculated with both the simple Drude and the extended equations. This allows direct comparison with published work and eliminates some of the ambiguity that has arisen from the use of only one of the equations.

RESULTS

The principal parameters used in assessing the purity of the two albumin samples studied are shown in Table 1.

Alterations in protein concentration over the range in which one could obtain satisfactory transmission of light and significant rotation (0.2 to 2.0 g./100 ml.) produced no significant change in rotatory dispersion, although the errors are greater at lower concentrations. It has therefore been found convenient to work with about 0.5% of protein. Readings were made at intervals from 320 to 600 $m\mu$. Variations in extrinsic ion concentration by the addition of sodium chloride at pH 6.0 from 0.05M to 1.5M showed that within these ranges no significant changes occurred and the readings did not differ significantly from solutions of albumin in water.

The effect of pH was studied between pH 1 and 12. The general effects observed were similar to those reported in earlier work (Jirgensons, 1958*a, b*) for bovine albumin, there being a slight fall in $[\alpha]_D$ from pH 1 to pH 4, after which the graph flattens up to pH 9. Thereafter there is a decrease, followed by a rapid increase in $[\alpha]_D$ up to pH 12 (Fig. 2) and corresponding changes in dispersion data (Table 2).

Table 1. *Analytical data on human albumins examined*

	Sample 1	Sample 2
Fraction	AP2	AP3
Electrophoresis analysis (ascending pattern)	Albumin, 97% β -Globulin, 3%	Albumin, 100%
Immunochemical inspection against antisera to normal human serum	Two components only: the faster corresponded to human albumin; the slower was a trace component in the area of β -globulin	One component (corresponding to human albumin)
Ultracentrifugal analysis $S_{20,w}^0$	4.20	4.22
Sialic acid content (mg./g.)	0.9	1.2
Total hexose content (mg./g.)	2.0	2.5
Chloride content (mg./g.)	1.25	0.94

Fig. 2. Variation in optical rotation of albumin with pH. Rotation at 500 mμ plotted against pH for albumin solution (0.5 g./100 ml.). ●, Sample 1; ○, sample 2.

Table 2. Variation of dispersion constants with pH

Measurements were made on albumin solution (0.5 g./100 ml. of aq. 0.15M buffer). Buffer systems were: pH 1-3.4, glycine-HCl; pH 3.6-5.6, acetic acid-sodium acetate; pH 8.0-9.5, boric acid-potassium chloride-NaOH; pH 10-12.2, glycine-NaOH; pH 5.5-6.5, unbuffered albumin solutions. K and λ_c were derived from the Drude equation, a_0^H and b_0^H were derived from extended equation.

pH	$-10^{-6}K$	λ_c (mμ)	$+a_0^H$	$-b_0^H$
(a) Sample 1				
2.25	20.1	260	239	272
3.13	19.6	260	253	281
3.88	18.9	265	258	281
5.70	17.6	263	297	281
8.23	17.9	263	297	261
9.30	17.4	264	292	260
10.33	17.1	264	300	268
10.96	19.1	257	259	242
12.20	23.1	240	157	177
(b) Sample 2				
1.00	19.1	257	262	260
2.14	18.0	263	286	290
3.12	18.2	263	272	251
4.14	18.1	261	274	238
6.33	17.2	262	292	260
8.01	17.2	262	292	260
9.05	16.3	266	315	260
10.10	16.3	266	311	264
11.10	17.1	263	292	260
12.10	20.2	251	229	173

The effects of urea and 2-chloroethanol were studied on albumin dissolved in water, the solution having an observed pH between 5.5 and 6.0.

The variations in specific rotation with urea concentration at 500 mμ for the two samples are shown graphically in Fig. 3. There is no effect in concentration below 2M-urea; at higher urea concentrations the rate of hydrogen-bond rupture, and hence unfolding of the molecule, is a linear progression. The minor differences between the two samples may be due to impurities in sample 1.

Fig. 3. Variation in optical rotation of albumin with increasing urea concentration. Rotation at 500 mμ plotted against molar concentration of urea for albumin solution (0.5 g./100 ml.). ●, Sample 1; ○, sample 2.

Table 3 shows the variation of calculated constants for the two samples, the last column being an estimate of the percentage of α -helix present in the molecule. This column shows that as the urea concentration is increased, so the percentage of α -helix decreases. Only when sample 1 in 9M-urea was heated for 1 hr. at 78° did the constants indicate complete loss of helix.

The results for albumin in 2-chloroethanol are shown, in Fig. 4 and Table 4, in the same form as for the urea experiments. There is an approximately linear decrease in rotation with increasing 2-chloroethanol, this being the exact opposite to the effect of urea. The constants in Table 4 indicate a progressive increase in α -helix until in pure 2-chloroethanol the molecule contains some 75% of α -helix, which is in agreement with work on bovine serum albumin (Doty, 1959). Fig. 5 shows a plot of the K values of sample 2 in urea and 2-chloroethanol. There is a progressive increase in K from the maximal values in pure 2-chloroethanol to those in 9M-urea, except in the range 3M-2-chloroethanol to 2M-urea, where the protein is in its stable native configuration. The plot suggests that decrease in 2-chloroethanol concentration and increase in urea concentration have the same effect, most probably the rupture of intramolecular hydrogen bonds.

Table 3. *Variations of dispersion constants with urea concentration*

Measurements were made on albumin solution (0.5 g./100 ml. of aq. urea). K and λ_c were derived from the Drude equation, a_0^H and b_0^H were derived from extended equation.

Concn. of urea (M)	$-10^{-6}K$	λ_c (m μ)	$+a_0^H$	$-b_0^H$	Helix (%)
(a) Sample 1					
0	17.5	264	286	291	40-50
2	17.5	264	286	291	40-50
3	19.9	253	253	253	37-42
4	21.8	250	214	231	30-38
6	24.6	245	140	182	20-28
8	27.3	240	64	88	9-15
9	31.7	223	39	62	5-12
9 (+ Heat)	32.7	220	0	0	0
(b) Sample 2					
0	18.2	265	282	302	40-50
2	18.2	265	282	302	40-50
4	20.0	258	244	261	35-45
6	23.5	247	182	244	27-37
8	29.2	226	75	157	12-22
9	29.3	226	47	70	5-12

Table 4. *Effect of 2-chloroethanol on the dispersion constants of albumin*

Measurements were made on albumin solution (0.5 g./100 ml. of aq. 2-chloroethanol). K and λ_c were derived from the Drude equation, a_0^H and b_0^H were derived from extended equation.

2-Chloro-ethanol (%)	$-10^{-6}K$	λ_c (m μ)	$+a_0^H$	$-b_0^H$	Helix (%)
(a) Sample 1					
0	17.4	266	291	273	40-50
20	17.2	270	302	325	42-52
30	16.9	273	310	334	45-55
40	16.0	277	323	344	48-58
50	15.5	282	330	351	50-60
60	13.5	288	375	363	55-62
70	12.8	295	392	409	58-66
80	11.0	298	425	425	62-70
90	10.2	305	449	431	66-72
(b) Sample 2					
0	18.3	265	271	307	40-50
10	18.3	265	272	307	40-50
20	18.0	265	285	323	42-52
30	16.9	276	304	334	45-56
40	15.8	282	323	350	48-58
50	15.3	284	340	362	50-60
60	13.8	290	370	376	54-62
70	12.8	296	395	392	58-65
80	12.0	298	412	405	60-68
90	10.0	304	455	415	66-70
100	8.3	314	495	443	70-75

Table 5. *Composite dispersion data on the effect of controlled denaturation of a solution of sample 1*

The solution of sample 1 contained 1 g./100 ml. K and λ_c were derived from the Drude equation, a_0^H and b_0^H were derived from extended equation.

Treatment	$-10^{-6}K$	λ_c (m μ)	$+a_0^H$	$-b_0^H$
Heated to 80°* and cooled	19.6	240	245	121
8M-Urea, then dilution to 2M-urea	20.3	243	233	163
pH 12, then adjustment to pH 4.5	15.4	254	326	169
4M-Urea	21.8	250	214	231

* Heated albumin was prepared by placing aq. albumin (2% solution) in a water bath at 80° for 6 hr. The solution was cooled to 2° and stored at this temperature.

DISCUSSION

Fluctuations in the optical rotation of albumin with pH show a range of relative stability between pH 4 and 8 in which we assume the molecular form approaches the native configuration.

The reproducible 'irregularity' in the curve at about pH 4 and the increase in laevorotation at lower pH values are in the range which, by titration techniques, Tanford (1952) showed changes that he ascribed to expansion of the molecule and in which marked changes in net surface charge were observed (Luetscher, 1939). Similar changes in specific rotation of bovine serum albumin have been observed and attributed to molecular expansion (Jirgensons, 1952; Yang & Foster, 1954). It is evident from these three parameters that this is a pH range in which structural and therefore, presumably, configurational instability occurs. At pH 10 and above it seems that simple unfolding is taking place with consequent decrease in α -helix.

In the urea and 2-chloroethanol studies it is assumed that neither of the two reagents materially disturbs bonds other than the H bond, although at urea concentrations above 6M some $\cdot S \cdot S \cdot$ exchange probably takes place (Hospelhorn, Cross & Jensen, 1954). The results suggest that with successive change in environmental conditions, from the highest concentrations of urea to the highest concentration of 2-chloroethanol, the albumin molecule is transformed from a random coil structure to one having a high helical content. Fig. 5 shows that there is a plateau from 2M-urea to 3M-2-chloroethanol, where, according to these results, the molecule has a relatively stable configuration corresponding to the native form.

The highly coiled structure in 2-chloroethanol is due to the greatly increased intramolecular H bonding, and the uncoiling on addition of water is a result of the destruction of both peptide and side-chain H bonding. Our data suggest a smooth transition from the rigid highly helical structure in 2-chloroethanol to the open random coil in 9M-urea and support the concept that albumin in the native form is a single folded polypeptide chain containing some 50% of α -helix. Even in pure 2-chloroethanol our values do not indicate the 100% helical form observed in synthetic polypeptides (Yang & Doty, 1957). This may be due to restraints imparted by the disulphide bridges between different parts of the molecule.

Our observations on the structure of albumin denatured by urea, heat and alkali appear consistent with the view that a partial loss of α -helix has taken place, with the introduction of an extended configuration, the β -form, resulting from intermolecular hydrogen bonding (Low, 1953; Imahori, 1960).

Fig. 4. Change in optical rotation of albumin with increasing 2-chloroethanol concentration. Rotation at 500 $m\mu$ plotted against molar concentration of 2-chloroethanol for albumin solution (0.5 g./100 ml.). ●, Sample 1; ○, sample 2.

Fig. 5. Change of rotation of albumin with solvent composition. Rotational constant K , plotted against molar concentration of urea to the left, and 2-chloroethanol to the right, of the central broken line for a solution (0.5 g./100 ml.) of sample 2.

Table 5 gives the dispersion constants for sample 1 heated to 80° and cooled, the reversal of urea and alkali denaturation, and compares these with the results in 4M-urea.

The constants for 4M-urea solution, typical of simple unfolding, differ considerably from the other three, suggesting that in them neither simple unfolding nor complete reversal to the native state has taken place.

Many workers, notably Warner & Levy (1958), Huggins, Tapley & Jensen (1950, 1951), Gorbacheva, Bresler & Frenkel (1957) and Steinrauf & Dandliker (1958) have put forward evidence that $\cdot S \cdot S \cdot$ exchange plays an important role in the irreversible process of heat denaturation. It seems probable that intermolecular aggregation takes place, involving the free SH group as well as intermolecular hydrogen bonding between independent molecules.

These views are strengthened by X-ray-scattering experiments (Echols & Anderegg, 1960) on the reversal of urea denaturation of bovine serum albumin, showing that the process is not completely reversible and that the resultant molecule consists of intermolecular aggregates involving the free SH group. By infrared-spectral studies on films of polypeptides thrown from alkaline solutions (Blout & Idelson, 1958) the presence of α -helix and of β -form in the same molecule has been demonstrated.

The results cited above and those in the present paper suggest that whereas low concentrations of urea cause reversible denaturation, high concentrations of urea, strong alkali and heat cause irreversible denaturation. This irreversibility is presumably due to the $\cdot S \cdot S \cdot$ exchange that occurs more readily in the presence of such denaturants.

The newly formed $\cdot S \cdot S \cdot$ bonds possibly offer restraints to the re-formation of certain intramolecular hydrogen bonds and the molecule is stabilized by the formation of intermolecular hydrogen bonds giving the proposed β -structure.

Thus the native molecule would seem to be a fairly stable hydrated macromolecule consisting of a single folded polypeptide chain with some 50 % of right-handed α -helix. The helical folding is maintained by intramolecular hydrogen bonds and is limited by the disulphide bridges. This folding can be modified by moderate changes in environmental conditions, such changes being reversible on removal of the agent. More drastic treatment, allowing $\cdot S \cdot S \cdot$ exchange, causes irreversible changes in the molecule. Under such conditions partial unfolding, followed by folding in a new form, takes place.

SUMMARY

1. Optical rotatory dispersion has been used as a means of studying structural changes in human serum albumin under various environmental conditions.

2. The spectropolarimeter is described and a summary of the theoretical basis for the interpretations included.

3. The effects of protein concentration, ionic strength, pH, urea and 2-chloroethanol are described.

4. Albumin is unaffected by pH in the range 4–9 but outside this range configurational changes occur. Urea is shown to decrease the α -helix content and 2-chloroethanol to increase it.

5. Conditions causing irreversible denaturation are discussed and the introduction of intermolecular hydrogen bonding into the albumin structure is suggested.

6. The albumin molecule is thought of as a single folded polypeptide chain with some 50 % of right-handed α -helix. It appears fairly stable and changes are reversible unless the conditions are sufficiently drastic to cause $\cdot S \cdot S \cdot$ exchange.

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Net Exchange of Certain Inorganic Ions and Water in the Alimentary Tract of the Milk-Fed Calf

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The present work was undertaken initially to study the effective sites of magnesium and calcium absorption in the alimentary tract of the milk-fed calf and to examine further the decrease previously observed (Smith, 1957, 1958*a*, 1959*c*) in the net absorption of these substances with increasing age.

Magnesium is of particular importance in cattle (and sheep) nutrition since, except in very young calves (Smith, 1957, 1958*a*, 1959*c*), it appears to be poorly absorbed by these animals from normal diets (Blaxter & McGill, 1956; Rook & Balch, 1958; Field, McCallum & Butler, 1958), and clinical deficiency sometimes occurs under normal management conditions. The little that is known of the mechanism of magnesium utilization in the ruminant, mainly from experiments with sheep, suggests that the small intestine is the principal site of absorption (Stewart & Moodie, 1956; Field, 1961). This was also found by Graham, Caesar & Burgen (1960) for the human. The rumen does not appear to be an important site when the rumen fluid contains magnesium in concentrations within normal limits (Storry, 1960; Smith, 1959*a*).

Knowledge of the sites of calcium absorption is in a similarly fragmentary state. The rumen again appears to be unimportant under normal conditions (Storry, 1960), and absorption in the small intestine, found for rats (e.g. Nicolaysen, 1951), has also been demonstrated in the sheep (Storry, 1960),

particularly from the duodenal end. However, several workers have reported calcium absorption in different animals from calcium-containing suppositories inserted in the large intestine (Braun, 1948; Marolda, 1954). Evidence from rat experiments suggests that the small intestine is an important site for the action of vitamin D (Nicolaysen, 1951), but Harrison & Harrison (1951) reported that it had its greatest effect in the more distal parts of the intestine where calcium solubility was low, and Marolda (1954) reported that calcium absorption from the human rectum was increased on vitamin D administration.

I have used suitably fistulated calves of different ages fed basically on milk with, in some experiments, supplementary vitamin D and, in others, different dietary levels of magnesium, to examine the amounts of magnesium and calcium appearing at different points in the alimentary tract relative to an inert marker (polyethylene glycol). A similar examination has been carried out for potassium, sodium, chloride, water and, in a more limited way, inorganic phosphate. This was done both for its intrinsic interest and in the hope of finding changes related to the changes which occur in magnesium absorption with age. Suggestions that other ions affect magnesium absorption, based either on direct experiment or interpretations of secondary effects, have been made for potassium (Kunkel, Burns & Camp, 1953), sodium (Ross, 1961),