XXXI. OPTICAL ROTATORY POWER AND DISPERSION OF PROTEINS.

By LESLIE FRANK HEWITT.

From the Hale and Dunn Clinical Laboratories, London Hospital, E 1.

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THE importance of optical rotatory power for the characterisation of proteins has received attention since 1880 [Fredericq, 1880; Starke, 1881; Sebilin, 1885].

There is however a wide divergence in the results obtained; *e.g.* in the case of serum albumin values for the specific rotatory power for the D-line vary from -33° [Reiss, 1904] to -63° [Young, 1922]; other values being -47.8° [Maximovitsch, 1901], -42° to -49° [Gürber, 1894], -48° [Michel, 1895], -51° to -66° [Lewis, 1922]. Similarly in the case of serum globulins, values varying from -42° [Porges and Spiro, 1902] to -51.2° [Seng, 1899] and -52° [Lewis, 1922] have been recorded. This divergence alone renders it desirable that the data be critically examined and certain of the determinations repeated. Also, until a recent publication of Hafner [1925] which appeared after the present work had commenced, no attention has been paid to the rotatory power of the proteins for light of different wave-lengths, *i.e.* their optical rotatory dispersion.

From many points of view we consider this work of Hafner to be unsatisfactory. Like all other workers in this field except Hardy and Gardiner [1910] and Young [1922] lipins were not removed from the proteins although these considerably modify their properties. In addition Hafner made no attempt to separate euglobulin and pseudoglobulin although they have different rotations; and the use of very dilute solutions for optical measurements and the lack of duplicate determinations make the results even less dependable. As will be shown later, the results are not acceptable on other experimental and theoretical grounds.

The optical rotation and dispersion of lipin-free serum and other proteins have therefore been determined.

EXPERIMENTAL METHODS.

Serum proteins.

Lipins were extracted from serum by a modification of Hardy and Gardiner's method [1910] and that of Hartley [1925]. Plasma, or preferably serum (100 cc.), at 0° is poured into a mixture (600 cc.) of alcohol (7 parts) and ether (3 parts) cooled to -15° . The mixture is vigorously stirred and the

temperature not allowed to rise above -5° . After allowing to stand at this temperature in an ice-chest for 2 hours the precipitated proteins are filtered off on a Büchner funnel in the ice-chest, washed several times with portions (40 cc.) of the alcohol-ether mixture at 0° , and with several portions (50 cc.) of ether at 0° . The filtration accelerates markedly when the ether washing is commenced. The proteins are then rapidly transferred to extraction thimbles and extracted for 2 hours in a Soxhlet apparatus with ether, metallic sodium being placed in the extraction flask to remove alcohol extracted from the proteins. After 2 hours' extraction the ether and sodium in the apparatus are renewed and the extraction is continued for 20 hours. It is found that if the proteins are dried *in vacuo* before ether extraction, an appreciable amount of alcohol remains in the precipitate and considerable denaturation occurs. After completion of the ether extraction the proteins are transferred to a desiccator and dried *in vacuo* for 48 hours. The dried proteins are not denatured by standing in contact with a trace of ether as they are by alcohol. When freed from ether the proteins are allowed to stand for a while in contact with laboratory air to become slightly moist, otherwise, when completely dry, they are difficult to work with, easily electrified and adhere to surfaces. Obtained in this manner the extracted proteins are white, powdery solids readily dissolving in water giving clear solutions. Reconstituted plasma can still be caused to clot and albumins may be crystallised.

The extracted serum proteins are dissolved in water and the volume made up to that of the original serum, and the euglobulin and pseudoglobulin removed in turn by additions of saturated ammonium sulphate solution to render the mixture one-third and one-half saturated with ammonium sulphate. The albumins are then either caused to crystallise or precipitated by addition of small amounts of 10 % acetic acid and saturated ammonium sulphate solution, the reaction being adjusted to p_H about 4.9. Precipitation of albumin by adjusting the reaction and addition of ammonium sulphate solution is preferable to addition of solid ammonium sulphate. The protein fractions are filtered off on Büchner funnels using Schleicher and Schüll hardened papers (No. 575), redissolved in water and reprecipitated twice more in a similar manner. This fractionation is sufficient for the present purpose. The proteins are then dissolved in water and dialysed in collodion membranes against distilled water in an ice-chest, the outer liquid being changed hourly for the first day and then daily for at least a week. The membranes used were tested for permeability to salts and dyes and were all impermeable to serum proteins. In the case of albumin and pseudoglobulin the dialysed solutions were used directly for the determination of rotatory power whilst the euglobulin was precipitated as a white solid on dialysis and was dissolved in 0.9 % sodium chloride solution. The solutions used were clear and colourless except the albumins which have a slight yellow colour in thick layers.

A Schmidt and Haentzsch polarimeter with a Hilger direct vision spectroscopic eyepiece was used for the measurement of rotations and a mercury

vapour lamp provided a light source for wave-lengths, 4358 (blue), 5461 (green), and 5780 (yellow) Ångström units, and a Nernst lamp with a crystal-violet filter, red light (6600 Å.U.). For the determination of proteins the author is indebted to Dr J. R. Marrack who used the micro-Kjeldahl method, using the factor 6.45 for the conversion of nitrogen content to protein concentration.

Serum albumin.

In this section all rotatory powers are given for the mercury green line at 20° ($[\alpha]_{5461}^{20^\circ}$), rotations for this light being most accurately determined.

Table I. $[\alpha]_{5461}^{20^\circ}$ of serum albumins.

	Crystalline			Precipitated				Mean
	Horse 1	Horse 2	Horse 3	Horse 4	Ox 5	Human 6	Human 7	
p_H 4.9	-78.4°	-81.0°	-78.7°	-76°	-79.6°	-79.3°	-77.7°	-78.1°
p_H 7.2	-78.4°	-81.5°	-81.8°	—	—	—	—	—

It is to be noted that:

(1) Precipitated and crystallised extracted serum albumins have approximately equal rotatory powers.

(2) Alteration of the p_H from 4.9 to 7.2 has but little effect on the rotation.

(3) Horse, ox, and human serum albumins have about the same rotatory powers.

(4) The results are in agreement with those of Young [1922], *i.e.* -78.3° in place of -78.1°, in contradistinction to those of Lewis [1922] and other workers who worked with proteins contaminated with lipins. Lewis used only the D-line for his determinations of rotatory power, but, on extrapolating his results on the basis of the curves obtained by us, his value for horse albumin is -70°. Using unextracted serum albumin we have obtained similar results, but, as Lewis found, it is difficult to obtain concordant results with such material.

If the albumins are allowed to remain in contact with alcohol at the ordinary temperature, or with acidified ammonium sulphate solution for long periods, or if the protein solutions are heated, a certain amount of denaturation occurs and the rotatory power is increased. Samples 8, 9 and 10 in Table II and possibly 2 in Table I were partly denatured.

Table II. *Denatured albumins (horse serum).*

$[\alpha]_{5461}^{20^\circ}$	8	9	10
	Crystalline -90.0°	Precipitated -89.0° -84.3°	

Serum pseudoglobulin.

Table III. $[\alpha]_{5461}^{20^\circ}$ of serum pseudoglobulin in water.

Horse 1	Horse 2	Horse 3	Human 4	Ox 5	Mean value
-68.8°	-66.2°	-69°	-70.7°	-69°	-68.7°

Good agreement is obtained between the values of $[\alpha]_{5461}^{20^\circ}$ obtained for extracted horse, human and ox serum pseudoglobulin. The mean value of -68.7° is higher than that calculated from Lewis's results for unextracted horse serum pseudoglobulin (-62.1°).

Serum euglobulin.

Table IV. $[\alpha]_{5461}^{20^\circ}$ of extracted serum euglobulin.

Horse		Ox	Mean
1	2	3	
-60.1°	-61.3°	-62.1°	-61.2°

Good agreement is obtained between the values of $[\alpha]_{5461}^{20^\circ}$ for horse and ox extracted serum euglobulin and the values are higher than those calculated from Lewis's figure (-51.0°) for unextracted horse euglobulin.

Egg-albumin.

To the whipped white of fresh eggs is added an equal volume of saturated ammonium sulphate, and after standing over night the precipitate is filtered off and the albumin in the filtrate precipitated by acidification and addition of ammonium sulphate. The precipitated albumins are filtered off, dissolved in water and extracted first with alcohol and ether in the cold, and then with ether at the ordinary temperature, and then dried, as described in the case of serum proteins. The extracted egg-albumin thus obtained gives a clear solution in water even at the isoelectric point and crystallises, but with some difficulty, in the ordinary way. Sørensen [1926] expresses the opinion that egg-albumin cannot be extracted with alcohol and ether in the cold without denaturation, and takes as criteria of undenatured egg-albumin, solubility in water and crystallisability. The extracted protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate is added, the solution filtered and the egg-albumin twice precipitated by additions of appropriate amounts of 10 % acid and saturated ammonium sulphate solution. The egg-albumin thus obtained is dissolved in water and dialysed until free from salts in the usual way. The albumin contained 7.7 mg. of phosphorus per 1 g. of nitrogen which corresponds to a molecular weight for egg-albumin of about 26,000, assuming there is 1 atom of phosphorus per molecule of protein. The generally accepted values for the molecular weight lie between 27,000 and 34,000 [cf. Cohn, Prentiss and Hendry, 1925]. The specific rotatory power, $[\alpha]_{5461}^{20^\circ} - 44.5^\circ$, is higher than that obtained by Young [1922] for unextracted egg-albumin (-37.5°).

Lactalbumin.

The caseinogen is removed from fresh skimmed milk either by addition of dilute acetic acid under the surface with vigorous stirring or by treatment at 37° with rennin for 45 minutes. The precipitated casein is removed by

filtration, and the lactalbumin is precipitated by addition of dilute acetic acid until the solution has p_H about 4.9, and of saturated ammonium sulphate solution. After standing, the precipitated proteins are filtered off, dissolved in water and extracted with a mixture of alcohol and ether in the cold and with ether in a Soxhlet apparatus, and dried *in vacuo* in the usual way. The white powdery protein is dissolved in water, the solution made neutral, an equal volume of saturated ammonium sulphate added, and the solution filtered. The lactalbumin is then twice precipitated by addition of dilute acetic acid and saturated ammonium sulphate solution, and filtered on Büchner funnels. The purified lactalbumin is then dissolved in water and dialysed until free from salts. The rotatory power is $[\alpha]_{5461}^{20} - 47.5^\circ$. The phosphorus content is 5.4 mg. per 1 g. of nitrogen in the protein. The molecular weight of lactalbumin, assuming that there is 1 atom of phosphorus in each molecule of lactalbumin, is thus about 37,000. For the phosphorus determinations the author is indebted to Dr Kay.

Caseinogen.

Caseinogen was prepared from milk by the method of Van Slyke and Baker [1918] as modified by Cohn [1922].

Gelatin.

Isoelectric gelatin was prepared from Coignet's "Gold Label" gelatin by the method of Loeb [1922].

Table V. *Specific rotatory powers of proteins and tyrosine for light of different wave-lengths.*

Protein	$[\alpha]_{\lambda}^{20}$			
	λ 4359	λ 5461	λ 5780	λ 6660 Å.U.
Serum albumin	-151.5°	-78.1°	-67.0°	-47.3°
Serum pseudoglobulin	-118.2	-68.7	-59.8	-43.1
Serum euglobulin	-105.2	-61.2	-54.0	-39.4
Egg-albumin	-83.9	-44.5	-38.3	-27.5
Lactalbumin	-88.9	-47.5	-41.1	-28.6
Caseinogen	-184.4	-105.1	-91.2	-66.0
Gelatin	-496.8	-282.8	-248.2	-181.1
Tyrosine in 20 % HCl	-7.8	-9.6	-9.3	-7.2
" 4 % HCl	-14.4	-12.5	-11.5	-8.9
" 4 % NaOH	-22.9	-15.1	-13.1	-9.8
" 20 % NaOH	-27.8	-17.4	-15.2	-11.2

In Table V are given the specific rotatory powers of the proteins examined for light of different wave-lengths. To confine attention for the moment to the rotation for the mercury green line ($\lambda = 5461$ Å.U.), which is probably most accurately determined, the following conclusions may be drawn.

(i) The considerably greater (10–15 %) rotation of lipin-free proteins, as pointed out in the experimental section.

(ii) The marked difference between the rotation of serum albumin and globulins, and between serum pseudoglobulin and euglobulin.

(iii) Caseinogen has a high laevo-rotation, $[\alpha]_{5461}^{20} - 105^\circ$, not a dextro-rotation as is inferred from the work of Zaykowsky [1923]. Tyrosine is included to show the effect of p_H on dispersion. This will be referred to later.

OPTICAL ROTATORY DISPERSION.

It is well known that, in general, optical rotation increases with decreasing wave-length of the light used for the determination; but Drude in a treatise on optics in 1907 was the first to obtain a satisfactory mathematical expression connecting rotatory power and wave-length of the light.

Drude's equation is:

$$\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2} \pm \frac{k_1}{\lambda^2 - \lambda_1^2} \dots \text{etc.},$$

where α_λ = rotation for light of wave-length λ ,
 k_0, k_1 = constants,
 λ_0, λ_1 = constants.

The rotation may be controlled by one, two, or more terms in the Drude equation. λ_0, λ_1 etc., have a further significance in that they are the characteristic wave-lengths of oscillating electrons in the molecule and as such give rise to bands in the absorption spectrum of the substance, so that a direct relationship exists between the rotatory dispersion and the absorption spectrum of the substance. Lowry [1913] was among the first to apply the Drude equation to chemical substances. He showed that if the rotatory powers of a substance can be expressed, as those of most substances can, by a single term Drude equation, *i.e.* $\alpha_\lambda = \frac{k_0}{\lambda^2 - \lambda_0^2}$, then a linear relationship exists between the reciprocals of the rotatory powers ($\frac{1}{\alpha_\lambda}$) and the squares of the wave-lengths (λ^2). This follows from a rearrangement of the equation:

$$\frac{1}{\alpha_\lambda} = \frac{\lambda^2}{k_0} - \frac{\lambda_0^2}{k_0};$$

and if $\frac{1}{\alpha_\lambda} = 0$, then $\lambda = \lambda_0$. That is to say, if $\frac{1}{\alpha_\lambda}$ is plotted against λ^2 the point where the line cuts the zero axis will indicate the wave-length of the absorption band controlling the dispersion.

In Fig. 1 are plotted the dispersions of the proteins in the way described. No deviation from a linear relationship between $\frac{1}{\alpha_\lambda}$ and λ^2 in the visible region of the spectrum can be detected.

Serum albumin, egg-albumin and lactalbumin all have similar dispersions, the dispersion curve cutting the axis at a point at, or near to, $\lambda = 2760 \text{ \AA.}$, which is the wave-length of the head of the absorption band in serum proteins nearest to the visible portion of the spectrum. In serum pseudoglobulin, euglobulin and caseinogen, however, the line appears to strike the axis at a point nearer the region of shorter wave-length, although these proteins have similar absorption spectra to the albumins. It would thus seem that in the albumins the absorption band of longest wave-length dominates the dispersion, whilst in the other proteins other bands have a predominant effect.

The case of gelatin is interesting since, owing to its low content of amino-acids containing a benzene nucleus, it does not possess absorption bands as near the visible region of the spectrum as the other proteins, and it is found, as expected, that the dispersion is correspondingly modified.

Fig. 1. Dispersion curves of proteins. Relation between $\frac{1}{[\alpha]\lambda}$ and λ^2 .

Fig. 2. Rotatory dispersion of tyrosine at different p_H .

The optical rotatory dispersion of tyrosine is more complex than that of the proteins, that is to say, two or more terms of the Drude equation are required to express the rotations for different wave-lengths (see Fig. 2).

Hydrogen ion concentration has a marked effect on the dispersion. In tyrosine therefore several absorption bands have a great influence on the rotation. Tyrosine has a very similar absorption spectrum to that of serum proteins [Stenström and Reinhard, 1925], and it does not vary greatly with alteration of p_H . In two respects therefore the absorption spectra and rotatory dispersion vary. The absorption spectra of serum proteins and of tyrosine are very similar but the rotatory dispersion is different; and the absorption spectrum of tyrosine is not greatly affected by hydrogen ion concentration whilst the rotatory dispersion is altered by change of p_H .

Fig. 3. Dispersion of serum proteins showing Hafner's [1925] results.

Hafner's results [1925] differ markedly from ours. First he finds the rotations of globulins higher than those of albumins, which is not supported by the work of other authors. In Fig. 3 are plotted Hafner's dispersion of serum albumin and our results on serum proteins. Hafner's figures (which are plotted inaccurately in his paper) lead him to the conclusion that the characteristic wave-length of an oscillating electron in the albumin molecule (*i.e.* that of an absorption band) is 4170 Å.u.; this would involve an absorption band in the visible region of the spectrum, an entirely unconfirmed hypothesis. Also, by carrying the determinations of rotatory power further into the blue region of the spectrum, we have shown Hafner's results to be inaccurate. Deviations of Hafner's curve from the straight line obtained by us are readily explicable on the basis of experimental errors, due to lack of duplicate determinations, dilution of solutions, etc. on Hafner's part.

PHYSICAL PROPERTIES OF THE GLOBULINS.

Chick [1914] and other workers express the opinion that euglobulin is a mechanical complex of pseudoglobulin and lipins which can be dissociated by extraction of the lipins with alcohol and ether. We have been unable to obtain evidence of such separation. After extraction with alcohol and ether

at -4° and with ether at laboratory temperature for 24 hours, euglobulin and pseudoglobulin retain their characteristic individual properties.

	Extracted euglobulin	Extracted pseudoglobulin
Solubility in saline	Soluble	Soluble
Solubility in distilled water	Insoluble	Soluble
When dialysed against distilled water	Precipitated	Remains in solution
$[\alpha]_{5461}^{20^{\circ}}$	-61.2°	-68.7°

SUMMARY.

1. The rotatory powers of lipin-free serum albumin, pseudoglobulin and euglobulin, lactalbumin, egg-albumin, caseinogen and gelatin for different wave-lengths have been determined.
2. Extracted serum albumin, pseudoglobulin and euglobulin have different and distinct rotatory powers. Euglobulin is not a mechanical complex of pseudoglobulin and lipin.
3. Serum albumin, egg-albumin and lactalbumin have rotatory dispersions dominated by an absorption band at about 2760 Ångström units. Serum globulins, caseinogen, and gelatin have different rotatory dispersions from the albumins.
4. Extraction of lipins from proteins results in a considerable increase of rotatory power, but crystallised and non-crystalline serum albumins have practically identical properties.

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