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Sedimentation and Diffusion of Human Albumins

4. LOW CONCENTRATION BEHAVIOUR OF SERUM ALBUMINS IN CASES OF LIVER DISEASE

By P. A. CHARLWOOD*

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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It has long been realized that the liver, even if not directly responsible for the synthesis of serum albumin, is at least intimately involved in its metabolism. The possible role of the liver in albumin formation was discussed by Madden & Whipple (1940), who concluded that it is highly likely that the liver constitutes a site of production. Further evidence in favour of this view is forthcoming from the work of Roberts & White (1949) and the radio-active studies by Peters & Anfinsen (1950).

The very close connexion between the liver and albumin formation suggests the possibility of alterations in the protein when that organ is diseased. Quantitative changes in the serum proteins have been reported by a number of authors who used the electrophoretic technique. Thus cases of cirrhosis have been studied by Gray & Barron (1943), Thorn, Armstrong & Davenport (1946), Martin (1949*a*), Rafsky, Weingarten, Krieger, Stern & Newman (1950), and Franklin *et al.* (1951). Sera from patients with infective hepatitis formed the basis of work by Gray & Barron (1943), Martin (1946), Cohen & Thompson (1947), Rafsky *et al.* (1950) & Franklin *et al.* (1951). A common feature of the patterns is the reduction in the relative concentration of albumin, although this decrease is by no means specific to diseases of the liver (Gutman, 1948).

Qualitative alterations in the serum albumin are indicated by two types of evidence. First, Luetscher (1939) and Hoch-Ligeti & Hoch (1948) found alterations in the electrophoretic characteristics of the albumin peaks when examined under special conditions. Secondly, albumin separated by electrophoresis of sera from patients with liver disease was

shown to behave abnormally in its ability to inhibit the cephalin cholesterol flocculation of γ -globulin, and in its effect on the thymol flocculation test, etc. (Kabat, Hanger, Moore & Landow, 1943; Moore, Pierson, Hanger & Moore, 1945; Hanger, 1947; Martin, 1949*b*). Unfortunately, the conditions used to isolate the albumin were not designed to remove all the α -globulin, so that the conclusions which can be drawn are not quite so definite as might be desired.

The abnormal molecular weights found by sedimentation and diffusion measurements for serum and urinary albumins in nephrosis (Charlwood 1952*b*), and the above considerations, have prompted a similar investigation in cases of liver disease, with the object of obtaining evidence of any abnormalities in albumin synthesis.

The main complication likely to be caused by the conditions prevailing in liver diseases lay in the generally raised bilirubin content of the sera. This could affect the actual molecular weight of the albumin, to which bilirubin is largely bound (Martin, 1949*c*). The colour, too, might affect optical conditions, more especially in the diffusometer, and give rise to anomalous results. Both these points are discussed later.

EXPERIMENTAL

Preparation of materials. Blood was collected from patients selected on the basis of a preliminary diagnosis of primary liver disease. It was processed as previously described (Charlwood, 1952*a*), the buffer in all cases being veronal (pH 8.6, $I=0.1$). Separations were controlled by the photoelectric arrangement described by Warren & Charlwood (1953), as in other similar work (Charlwood, 1954). Samples were diluted and redialysed, prior to sedimentation and diffusion, against a solution containing 0.05 I -NaCl + 0.02 I phosphate buffer, pH 8.0.

* Present address: Division of Applied Biology, National Research Council Laboratories, Ottawa, Ontario, Canada.

To examine the possible effects of bilirubin, two solutions were made up, according to the technique of Martin (1949*b*), to give albumin concentrations of about 0.2 and 0.6%, respectively, and to contain sufficient bilirubin for maximum binding. To a weighed amount of bilirubin dissolved in cold 0.01 *N*-NaOH was added 20 times its weight of ascorbic acid dissolved in phosphate buffer, followed by the protein in the same buffer. The solution was diluted to the requisite volume for dialysis. The albumin was the Harvard preparation A used in earlier work (Charlwood, 1954). The bilirubin to which it was added was approximately double the maximum amount bound under these conditions, as given by the results of Martin (1949*b*). After dialysis for 3 days, the precipitate, containing excess of bilirubin, was removed by centrifugation and the deeply coloured solution dialysed for a further 2 days against fresh buffer. In the lower-concentration experiment the buffer used was the same as that in which the sedimentation and diffusion of the pathological albumins took place (see above). At the higher protein concentration the buffer mixture was double strength, i.e. 0.1 *I*-NaCl + 0.04 *I* sodium phosphate buffer, pH 8.0.

Sedimentation and diffusion experiments. These were made in accordance with previous practice (Charlwood, 1952*a, b*). As usual, a single ultracentrifuge run and two pairs of diffusion runs were carried out on each solution, except that it was felt that a single pair of diffusion runs would suffice for the albumin/bilirubin mixture at 0.6% concentration.

The work of Waugh & Yphantis (1952) and Shulman (1953) on rotor temperatures in the Spinco Ultracentrifuge have been briefly discussed by Charlwood (1954). It was again decided to record values of $S_{20, w}$ based on the mean of initial and final rotor temperatures, for reasons given in that discussion.

RESULTS

All sedimentation constants, corrected to water at 20°, are shown in Svedberg units, whilst diffusion constants, similarly corrected, are in units of 10^{-7} cm.² sec.⁻¹. Concentrations (v) refer to wavelengths of retardation in the diffusimeter, a protein concentration of 1% corresponding to $v \sim 38$.

Diffusion

The results obtained with the albumin/bilirubin mixtures are shown in Table 1. Comparison with the figures given for the same albumin, free from added bilirubin (Charlwood, 1954), shows agreement within experimental error.

The figures shown in Table 2 for the pathological albumins refer only to those cases in which the preliminary diagnosis was subsequently confirmed. The mean value for the diffusion constants is 6.31, which is virtually identical with the mean value (6.32) found for normal human albumins at similar low concentrations (Charlwood, 1952*a*). The mean values for the cases of cirrhosis (6.30) and infective hepatitis (6.33) are also not significantly different from normal nor from each other. The single instance of carcinoma is well within the normal range. Indeed, the only individual value lying outside this range is the first, which is just below the lower limit of 6.11 (Charlwood, 1952*b*).

Table 1. *Sedimentation and diffusion of albumin-bilirubin complex*

(v = Concentrations of protein referred to wavelengths of retardation in the diffusimeter at 1% protein conc. $v \sim 38$.)

Buffer	v	$S_{20, w}$	$D_{20, w}$
0.05 <i>I</i> -NaCl + 0.02 <i>I</i> sodium phosphate, pH 8.0	8.46	4.25	6.13
0.1 <i>I</i> -NaCl + 0.04 <i>I</i> sodium phosphate, pH 8.0	21.82	4.05	6.02

Table 2. *Sedimentation and diffusion of serum albumins in various liver diseases*

(Phosphate buffer, pH 8.0, $I = 0.02$, and NaCl, $I = 0.05$.)

Case	Diagnosis	v	$S_{20, w}$	$D_{20, w}$
1	Cirrhosis	8.69	4.21	6.09
2	Cirrhosis	8.72	4.26	6.47
3	Cirrhosis	8.36	4.23	6.14
4	Infective hepatitis	9.27	4.18	6.31
5	Infective hepatitis	7.99	4.26	6.29
6	Infective hepatitis	7.75	4.27	6.26
7	Carcinoma	8.46	4.18†	6.24
8*	Cirrhosis	6.58	4.23	6.51
9	Infective hepatitis	9.27	4.16	6.42
10	Cirrhosis	8.04	4.19	6.26
11	Infective hepatitis	8.23	4.17	6.35
12	Infective hepatitis	8.59	4.12	6.24
13	Cirrhosis	7.52	4.10	6.30
14	Infective hepatitis	7.64	4.10	6.46

* Patient had been treated with bromsulphalein.

† Mean of two determinations.

Sedimentation

The results for the albumin-bilirubin complex, shown in Table 1, are again indistinguishable from those for the same albumin alone at corresponding concentrations (Charlwood, 1954).

In Table 2 are the results given by the pathological albumins in the chronological order in which they were obtained. There is a small, but significant difference ($0.01 > P > 0.001$) between their mean (4.19) and the mean (4.25) for normal albumins isolated under the same conditions (Charlwood, 1952*a*). For reasons discussed below, only the first six sedimentation constants in Table 2 are considered reliable to the usual degree of accuracy. These show no appreciable differences between the albumins from patients with infective hepatitis and those with cirrhosis, nor between either of these groups and normal. The last eight values in Table 2 (mean 4.16), taken together, are most definitely below normal ($P < 0.001$).

DISCUSSION

Before this work was begun, the possibilities of interference by bilirubin were considered. If there were much absorption of light by the solution in the

diffusiometer cell, the theory of the instrument would no longer be strictly applicable (Cecil & Ogston, 1951). However, examination of the light absorption curve of bilirubin in presence of human albumin (Martin, 1949*b*), and under buffer conditions similar to those it was proposed to use, showed relatively little absorption at the wavelength generally used in the diffusiometer ($\lambda = 5461 \text{ \AA}$). This high transparency was confirmed both visually, and by the density of the photographic record. Moreover, the degree of pigmentation of the serum albumins did not, except in a few instances, approach the maximum indicated by Martin (1949*b*). This could be seen by visual comparison of the colour intensity of the pathological albumin solutions with the albumin/bilirubin mixture made up at the lower concentration.

The other, more direct effect produced by bound bilirubin would be an increase in the molecular weight of the albumin. A simplified calculation was made to gain an idea of the possible magnitude of such an alteration. If the molecular weight of electrophoretically isolated human albumin be taken as 61500 (Charlwood, 1952*a*), and it be assumed that three molecules of bilirubin are bound to each one of albumin (Martin, 1949*b*), the molecular weight of the complex is about 62070. If the combination occurs without drastic change of molecular shape (i.e. the frictional ratio is unaltered), the diffusion constant of the complex should be about 0.3 % lower, and the sedimentation constant about 0.6 % higher than that of the original albumin. (This argument, of course, ignores the small amount of bilirubin originally attached to the normal albumin, the effect of which would be to decrease slightly the changes calculated.) Differences of this magnitude would not be detectable through small numbers of observations at the present degrees of accuracy (Table 1). Indeed, even on the whole set of measurements shown in Table 2, it would be necessary to show a difference from the normal average for $D_{20,w}$ of 0.9 % to register a value of $P < 0.05$. The actual difference of about 0.16 % can, therefore, be attributed purely to experimental error. The fact that it is in the right direction, and of the order to be expected from incomplete binding of bilirubin, is purely a coincidence.

The pH of 8.0 was chosen for this work as it came within the scope of the observations of Martin (1949*b*) on the interaction of bilirubin and human-serum albumin. Comparison of measurements made under these conditions with those made on normal albumins in unbuffered sodium chloride solution (Charlwood, 1952*a*) is fully justified by the experiments of Charlwood (1954). Values obtained in different solutions (particularly in unbuffered sodium chloride, and in sodium chloride solution containing phosphate buffer, pH 8.0) gave virtually

the same figure on extrapolation to zero concentration, and also at the low concentrations used in this work.

It has already been pointed out that the lower average value of the sedimentation constants in Table 2 is due to the preponderance of low figures in the group formed by the last eight. There is no correlation between these low figures and the relative numbers of cases of cirrhosis or infective hepatitis in this group and the first group of six. However, it was noticed that the drop in $S_{20,w}$ coincided fairly closely with the installation of a new drive-unit in the ultracentrifuge. This alteration was made between measurements on cases 6 and 7. Careful comparison of plates taken before and after the change showed that the quality of definition of the edge of the index mark was poorer and the apparent width of the meniscus had increased. The focus of each lens was checked, confirming that these effects were not due to maladjustment of the optical system. Other possible sources of error were also examined (Charlwood, 1952*a*). The conclusion was then reached that there must be sufficient vibration set up by the new drive-unit to account for the lowered values and other phenomena. A statistical calculation carried out on the last eight sedimentation constants in Table 2 gave value of P slightly greater than 0.05. Thus the evidence does not imply any progressive deterioration in the behaviour of the machine.

Since the above sedimentation constants refer to pathological materials, it was felt desirable to strengthen the opinion about the decreased reliability by means of runs on more normal proteins. To this end, β -lactoglobulin and bovine plasma albumin were used. The β -lactoglobulin solution (1.2 % protein in a mixture of 0.1N sodium chloride, 0.1N sodium acetate, 0.04N acetic acid) had previously given a value of 2.78 in the Spinco machine (Charlwood, 1952*a*). On this occasion 2.73 was recorded. The difference, although not significant taken alone, is in the direction and of the size to be expected from Table 2. Bovine plasma albumin has been examined by several authors in the Spinco ultracentrifuge. Values of $S_{20,w}^0$, all lying between 4.27 and 4.32, have been obtained by Kegeles & Gutter (1951), Creeth (1952), Miller & Golder (1952), Taylor (1952) and Shulman (1953). From the figures given by these various authors it appears that $S_{20,w}$ for bovine plasma albumin at 0.46 % is about 4.17. A run in phosphate buffer (pH 7.0, $I = 0.1$) at this concentration gave a value of 3.96. The difference of 5 % in $S_{20,w}$ is again consistent with a general lowering of 2.5 % superimposed on the random errors. The conclusion is, therefore, that the last eight sedimentation constants in Table 2 are probably about 2.5 % lower than they would otherwise be, merely on account of a slight defect in

the machine, and not because of the pathological nature of the albumins.

It has been shown that no measurable difference is detectable between the sedimentation and diffusion constants of serum albumins from normal persons and those suffering from the liver diseases represented in this survey. If it is assumed that measurements at a concentration of about 0.2% protein can be used here for calculations of the molecular weight (Charlwood, 1952*a, b*; 1954), and that the partial specific volumes of normal and pathological albumins are the same, it follows that the molecular weights are also indistinguishable. Now, Luetscher (1939) found that, in cases of cirrhosis of the liver, the ratio of the areas of the two peaks which separate during electrophoresis of human-serum albumin at pH 4.0 in 0.2 *I* acetate buffer differs from normal. However, since these components have essentially the same sedimentation and diffusion constants (Charlwood, 1954), there is no reason, on the basis of Luetscher's discovery, to anticipate abnormal molecular weights for serum albumins in cirrhosis of the liver. The data presented in this paper give no indication of a significant qualitative difference between albumin separated from normal serum and that from persons suffering from liver disease.

SUMMARY

1. The albumin fraction has been isolated electrophoretically from the sera of patients with cirrhosis of the liver, infective hepatitis, and one case of carcinoma of the liver.

2. Sedimentation measurements in the Spinco Ultracentrifuge, and also diffusion measurements in the Gouy diffusimeter, have been carried out on these albumins at a protein concentration of the order of 0.2%.

3. Possible interference of bilirubin with the determinations has been studied.

4. There is evidence that the albumins extracted from the sera of patients with liver diseases are normal as regards molecular weight, and the data presented give no indication of qualitative differences between the albumins isolated.

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