OSMOTIC PRESSURE STUDY OF PROTEIN FRACTIONS IN NORMAL AND IN NEPHROTIC SUBJECTS

By JAQUES BOURDILLON, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

(Received for publication, March 16, 1939)

This paper presents studies of protein osmotic pressures aimed to yield evidence on two questions, namely: (1) Whether albumin and globulin in the serum of patients with nephrosis are identical with albumin and globulin in normal serum, and (2) whether the albumin and globulin in the urine of such patients are identical with the corresponding proteins in the serum, either of normal subjects, or of the patients themselves.

1. Differences between Serum Proteins of Normal Subjects and Serum Proteins of Patients with Nephrosis

Earlier studies, reviewed by Goettsch and Reeves (6), and Alving and Mirsky (4), failed to reveal essential differences, either chemical or physicochemical, between normal serum proteins and the proteins in Bright's disease.

More recently, however, evidences of differences have been obtained. Working with the ultracentrifuge, McFarlane (11) has observed an abnormal sedimentation rate with the serum and urine from several cases of proteinuria, including nephritis, which suggested the presence of abnormally polydisperse albumin. As concerns nephrosis in particular, Tuchman and Sobotka (15) have found that the serum albumin contains more tyrosine, the globulin less, than in normal cases; Alving and Mirsky (4) have presented evidence of the existence of an abnormal albumin fraction with a low cystine content; Goettsch and Reeves (6) have observed immunological differences consisting in the fact that nephrotic albumin and globulin fail to precipitate completely with antisera developed against normal albumin and globulin.

Methods

Conditions for Constancy of Protein Osmotic Pressure Measurements.—In protein solutions the specific protein osmotic pressure (pressure per unit weight of protein) is constant only at high dilution (1,3). At such dilution the law of van't Hoff relating osmotic pressure to molecular concentration appears to be valid for proteins, since the molecular weights calculated from the pressures agree with those by other physicochemical methods (13). At concentrations over 10 or 20 gm. per liter the specific osmotic pressure of the plasma proteins increases with increasing concentration. The cause of this phenomenon is not entirely certain. Adair and Robinson (3) conclude that Donnan's law does not explain it. The question has been reviewed recently (13). The essential fact is that to yield exact results pressure measurements must be made on solutions dilute enough to avoid the deviation from van't Hoff law.

If serum is diluted enough to make the proteins conform to van't Hoff's law, and if the albumin and globulin fractions have each the same mean molecular size in the serum as in preparations of these proteins separated by salting out methods, the osmotic pressure of the serum should accord with the equation:

$$P = 25\left(\frac{C_a}{W_a} + \frac{C_g}{W_g}\right) \quad (1)$$

P is the osmotic pressure of the diluted serum in centimeters of water; 25 is the osmotic pressure of a millimolar solution of a non-electrolyte in water at 20°; C_a and C_g represent the concentrations, in mg. per liter, of albumin and globulin, respectively, and W_a and W_g the molecular weights calculated from the specific pressures of separated albumin and globulin. Adair and Robinson (3) have shown the basic validity of this equation by finding good agreement between the osmotic pressures of unfractionated serum at infinite dilution and those calculated from the partial pressures of albumin and globulin, using the molecular weights which they had found with preparations of separated albumin and globulin.

Pressure Measurements.—An apparatus has been recently described (5) which makes the accurate determination of low osmotic pressures a rapid and easy procedure. The amount of protein necessary for

one determination is only 0.2 cc. and its concentration need not be higher than 0.2 per cent. At such low concentrations, the van't Hoff law can be applied without corrections; therefore the results have been expressed, for simplicity's sake, directly as molecular weights.

The procedure previously indicated has been closely followed (5). Except in Table I, nearly all determinations were carried out in duplicate, sometimes triplicate or quadruplicate. There were practically no erratic results. Duplicate determinations usually checked within less than 5 per cent, the mean deviation for all the determinations being less than 2 per cent. The molecular weights given in the tables were calculated with the help of Table I in the preceding publication (5). The lower the concentration, the more closely (excluding experimental errors) should the figures given represent the true molecular weights. Actually, in the range of small concentrations used here, the differences are usually unappreciable.

Preparation of Material.—For the preparation of dilute serum samples (Table II), the blood was simply allowed to clot at room temperature, the cells were discarded after centrifugation, and serum and diluting fluid were mixed in the proportions indicated in Table II. Albumin and globulin concentrations were determined by Howe's precipitation and Van Slyke's manometric micro Kjeldahl method (12).

For the preparation of the protein fractions, it was felt that the simplest procedure would be the best, since it was the least likely to interfere with the state of aggregation of the proteins. In this instance there seemed to be no point in subjecting the material to such drastic treatments as have been applied by some investigators (17), especially since even the most elementary procedure of precipitation has been shown by the ultracentrifuge to cause definite irreversible changes (10), and since our aim for the moment was to find whether there were differences between normal and pathological sera, rather than to isolate more or less artificial products with apparently constant properties.

The procedure, which was entirely conducted at room temperature, was generally as follows:

The blood was allowed to clot and the cells discarded after centrifugation. To about 5 cc. of serum was added an equal volume of saturated ammonium sulfate

solution, and the precipitate formed was filtered off after a few hours. The globulin precipitate was washed several times on the filter with half saturated ammonium sulfate, then scraped from it and dissolved in a little water. Since with nephrotic globulins the solution remained as opaque as milk, it was then in some cases shaken twice with an equal volume of ether, which was syphoned off after centrifugation. This procedure left a practically clear solution, which was transferred into a cellophane bag for dialysis. The albumin was precipitated from the filtrate with an excess of solid ammonium sulfate, filtered off, and then transferred. An alternative procedure, which was successful with nephrotic serum only, was to centrifuge the precipitated albumin. It would then collect rapidly at the top of the tube like a thick yellow paste, and could be scooped up with a spatula, the remaining fluid being water-clear. Apparently the high lipid content of the albumin fraction in nephrosis is responsible for this behavior, since in normal cases centrifugation at usual speed is quite ineffective.

In some cases the procedure was slightly varied. In one case, the serum albumin was caused to crystallize at room temperature by adding M/1 acetic acid to the filtrate from globulin. The crystals were kept in the ice box over 2 months in the mixture recommended by Adair and Robinson (2) before they were dialyzed. In another case, normal globulin scraped from the filter was dissolved in 15 cc. of 0.15 M NaCl, then reprecipitated at half saturation before dialysis. Still in another, normal plasma instead of serum was used for a globulin determination. Details of technique are briefly indicated in the tables.

Dialysis was performed with small sections of cellophane tubing clamped flat against a piece of hard rubber plate and rocked in a trough for a few hours; the outer fluid, 0.15 M NaCl, was often renewed and the gradual decrease in it of ammonia concentration could be easily traced with Nessler's reagent. For the preservation of globulin solutions, a higher concentration was found preferable, therefore concentrated NaCl was added after dialysis so as to make the final salt concentration about 0.9 molar.

It should be clearly understood that the terms albumin and globulin used here mean nothing more than two rather easily separable fractions, and that no claim is made as to their individuality or homogeneity. By definition, the two fractions obtained in this way from normal serum represent what is usually understood as albumin and globulin, the characters of both of which are now, from the physicochemical standpoint, fairly well established; but in connection with nephrotic serum these two words are used here only in their restricted original sense, namely, to designate, respectively, that part of serum (or urine) protein which precipitates at complete saturation, and that part which precipitates at half saturation, with ammonium sulfate.

When normal serum is used, the separation of the two fractions in this way is quite sharp, the filtration can be carried out immediately, and the filtrate containing the albumin fraction remains clear indefinitely. The procedure was found to be less satisfactory when dealing with nephrotic serum, though filtration could be performed more rapidly than with Howe's method (9); the filtrate obtained was usually quite transparent after an hour or two, but would not remain so more than 12 or 24 hours.

The protein solutions obtained after dialysis sometimes contained a very slight precipitate which could be filtered off easily; in case of normal albumin and urine proteins, the filtrates were water-clear; with pathological sera and normal globulin, a slight milkiness usually persisted, even after treatment with ether. Treatment with ether did not appreciably affect the osmotic pressures measured.

The nitrogen content was determined by Van Slyke's gasometric Kjeldahl method (12). For the protein:nitrogen ratio, the factors 6.41 for albumin and 6.61 for globulin, obtained by Adair and Robinson (3) for horse serum, were provisionally used.

The nephrotic subjects from whom the serum was obtained were as follows:

G. B., female, 24 years, typical nephrotic syndrome of 1 year's duration. About 35 liters of edema in November, reduced to 15 liters 2 months later. Proteinuria: 30 gm. per day. Subnormal urea clearance.

S. G., male, 9 years, typical nephrotic syndrome of 1 year's duration. Considerable ascites and edema. Proteinuria: 6 gm. per day. Normal urea clearance.

P. F., male, 3 years, and W. H., female, 33 years; both cases of nephrotic syndrome with low urea clearances.

Results of Serum Protein Studies

Table I gives the molecular weights calculated from the osmotic pressures of normal human albumin and globulin. For albumin, the figure of 72,000 may probably be taken as a reasonable average, and it appears that the mode of precipitation had no effect on the results. For globulin, the figures given for Jan. 11 should probably be chosen as the most reliable; each one of them is the average of four determinations (in each case two osmometers were used and the determinations repeated on the same sample of serum). The most trustworthy osmotic pressure measurements obtained from

animal serum yield molecular weights of about 72,000 for albumin and 170,000 or 175,000 for globulin (13); it appears that the values

TABLE I

*Molecular Weights of Normal Human Albumin and Globulin Calculated from Observed Colloidal Osmotic Pressures**

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		
	Concentration	Molecular weight	Remarks
	per cent		
H. F. Oct. 1	0.342	71,600	After one crystallization
J. B. Nov. 28	0.215	71,800	Direct dialysis of albumin filtrate after globulin precipitation
	0.430	72,000	
	0.645	69,600	
	0.320	71,400	Albumin precipitated from filtrate before dialysis
	0.374	71,600	
	Globulin		
Dec. 31	2.08	162,000	One precipitation. Shaken with ether
	1.04	164,000	
	0.502	157,000	
Jan. 11	1.52	165,000	Two precipitations. Not shaken with ether
	0.760	163,000	
Dec. 9	0.615	183,000	From oxalated plasma

* Calculated by the formula

$$\text{Mol. wt.} = \frac{p_s}{p} \times c \times 10^5$$

p_s = pressure of a 0.1 mM solution at the T° of the experiment.
p = pressure of the unknown protein solution.
c = concentration of the protein solution in grams per cent.
(*Cf.* preceding publication (5) Tables I and II.)

for man are not appreciably different. Ether extraction of lipids had no effect on the results. In one case plasma instead of serum

was used, and the mean molecular weight found for the globulins was significantly higher. If one assumes that fibrinogen represents one-tenth of the total globulins, a rough calculation indicates that its molecular weight would have to be about twice that of the other globulins to cause the observed difference between plasma and serum; the point is left open for further investigation.

Table II gives the osmotic pressures developed by diluted serum both in normal and in nephrotic cases, and the theoretical pressures calculated with the help of equation 1 by taking 28.8 cm. of toluene as the pressure of a millimolar solution at 21°, assuming all the sera investigated to be a mixture of albumin with a molecular weight of 72,000 and of globulin with a molecular weight of 164,000, and taking for the concentrations the values indicated in the second column. Various diluting fluids were used, and the uniformity of the results shows that neither the salt content nor the pH of the fluid had any significant effect on the pressures developed. It appears that, whereas in the normal cases observed osmotic pressures agreed closely with those calculated from equation 1 with the molecular weights found for normal proteins, such agreement was not found for the nephrotic sera. The mean deviation of +4 per cent found with normal serum may be easily accounted for by the possibility that the albumin concentration is actually a little greater than the values obtained by Howe's method (9). The mean deviation of −35 per cent in the nephrotic cases indicates the presence of proteins with abnormally high molecular weights.

To ascertain whether the nephrotic proteins were in fact of abnormally high molecular weight, the osmotic pressures of albumin and globulin isolated from nephrotic sera were measured. The results, expressed as mean molecular weights, are given in Table III. The weights found for albumin are about 50 per cent higher, for globulin 100 per cent higher or more, than in normal cases. Above 2 per cent concentrations, the deviation from the van't Hoff law already becomes apparent, the molecular weights obtained being appreciably smaller than in more diluted solutions.

If we take the case of G. B., Jan. 10, (Table II) and substitute in equation 1 the values obtained on that day for albumin and globulin concentrations and those of Dec. 31 for the molecular weights (Table

TABLE II

Osmotic Pressure of Highly Diluted Human Serum

Temperature = 21°C.

Subject	Original concentration	Diluting fluid	Dilution	Pressure observed mm. toluene	Pressure calculated mm. toluene	Deviation
	per cent					*per cent*
Normal						
J. B.						
Nov. 12	A = 5.31	0.15 M NaCl	1:11	24.2	22.8	+6
	G = 2.19		1:22	12.3	11.4	+8
			1:22	12.0	11.4	+5
		M/15 Sørensen's phosphate;	1:11	23.0	22.8	+1
		pH = 7.65*	1:11	24.3	22.8	+7
		M/15 Sørensen's phosphate;	1:11	23.5	22.8	+3
		pH = 5.88*	1:11	23.5	22.8	+3
		0.1 M Na acetate	1:16	15.8	15.7	+1
		0.1 M acetic acid; pH = 4.64*	1:16	16.0	15.7	+2
Nov. 23	A = 5.13	0.12 N NaCl	1:11	24.3	23.1	+5
	G = 2.77	0.03 N $NaHCO_3$ + CO_2†	1:22	11.7	11.5	+2
Nephrotic						
G. B.						
Nov. 16	A = 1.37	0.15 M NaCl	1:11	5.2	8.0	−35
	G = 1.89		1:11	5.1	8.0	−36
		M/15 Sørensen's phosphate;	1:11	5.3	8.0	−34
		pH = 7.7‡	1:11	5.3	8.0	−34
		0.1 M Na acetate	1:11	4.9	8.0	−39
		0.1 M acetic acid	1:11	5.1	8.0	−36
Nov. 22	A = 1.61	0.12 N NaCl	1:11	5.3	9.2	−42
	G = 2.09	0.03 N $NaHCO_3$ + CO_2†	1:4	17.2	25.2	−32
Jan. 10	A = 1.57	0.15 M NaCl	1:4	16.5	25.2	−35
	G = 2.15		1:8	8.5	12.6	−33
			1.8	8.4	12.6	−33
			1.8	8.2	12.6	−35
S. G.						
Nov. 30	A = 0.82	0.15 M NaCl	2:7	15.0	24.4	−39
	G = 2.98		2:7	15.5	24.4	−36

* Determined with the glass electrode after dilution.

† This mixture was made by bubbling expiratory air through the solution.

‡ Calculated value before dilution.

III), the theoretical pressure for a serum diluted four times would be (taking 28.8 cm. of toluene as the pressure of a mM solution at 21°)

$$\left(\frac{15{,}700}{102{,}000} + \frac{21{,}500}{240{,}000}\right) \times 28.8 \times 1/4 = 17.6 \text{ mm. of toluene,}$$

instead of the 16.5 mm. observed. For a dilution of 1/8 the calculated values would be 8.8 mm. instead of the 8.2 to 8.5 observed.

TABLE III

Molecular Weights of Serum Albumin and Globulin in Nephrotic Subjects Calculated from Colloidal Osmotic Pressures

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin.

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	per cent		*per cent*	
G. B.				
Nov. 27	0.166	105,000		
	0.196	106,000		
Dec. 31	0.442	104,000	2.13	217,000
	0.628*	104,000	1.07	240,000
	0.314*	99,000		
S. G.				
Nov. 29	0.196	122,000		
Dec. 16			1.34	298,000
Dec. 31			2.17	314,000
			1.09	353,000
			0.504	346,000

* In these two samples the lipids were extracted with ether after dialysis, after which the sample was redialyzed for 2 hours.

More accurate calculations are probably not warranted, since the proteins were precipitated with ammonium sulfate for the molecular weight determinations, and with sodium sulfate for the determinations of concentration. The two salts have been found to precipitate approximately the same amounts of globulin in normal serum, but whether the same holds for nephrotic serum has not been investigated.

2. *Nature of the Proteins in the Urine of Nephrotic Patients*

The earlier literature about proteinuria has been reviewed by Hiller *et al.* (8), who have found that in nephrosis the albumin:globulin ratio is usually above 10, whereas it is usually lower in glomerulonephritis. It has been thus far generally assumed that the albumin and globulin of urine in Bright's disease were identical with the albumin and globulin of normal serum. McFarlane (11), however,

TABLE IV

Molecular Weights of Urine Albumin and Globulin in Nephrotic Subjects Calculated from Colloidal Osmotic Pressures

Outer fluid: 0.15 M NaCl for albumin; 0.9 M NaCl for globulin

Subject	Albumin		Globulin	
	Concentration	Molecular weight	Concentration	Molecular weight
	per cent		*per cent*	
S. G.				
Dec. 1	0.560	61,700		
	0.187	62,000		
G. B.				
Dec. 5	0.439	61,600	1.24	114,000
			0.620	120,000
Jan. 23			0.712	107,000
P. F.				
Dec. 5	0.555	66,700		
W. H.				
Dec. 12	0.403	57,200	1.13	120,000

has observed that the urine albumin of patients with Bright's disease is less homogeneous in the ultracentrifuge than is normal albumin.

Methods

For the preparation of albumin and globulin fractions urine was treated like serum, except that treatment with ether was omitted. The albumin-containing filtrate, after globulin precipitation, was always perfectly clear, as that of normal serum. Osmotic pressures were measured as indicated above.

Results with Urine Proteins

Table IV gives the results. The molecular weights calculated from the osmotic pressures of the urine albumin and globulin are definitely below the weights obtained for the albumin and globulin of normal serum; the difference is more marked for globulin than for albumin.

DISCUSSION

It goes without saying that the molecular weights given for albumin and globulin fractions in the tables should be assumed to indicate only the average sizes of the molecules in each fraction. The globulin fraction, from its salting-out curves, electrophoretic behavior (14) etc., is in general believed to include more than one distinct protein, and the albumin fraction of nephrotic serum appears also to be heterogeneous. Of the fractions here studied, the albumin of normal serum is the only one that, from its physicochemical behavior and its crystallizability, may perhaps be homogeneous. We have not been able to crystallize the albumin of nephrotic patients, either from serum or from urine.

McFarlane (11) has studied the sedimentation rates in the ultracentrifuge of the serum and urine proteins of five cases of proteinuria. One of these was apparently a case of nephrosis, another a case of nephritis. In all cases the serum showed the presence of polydisperse albumin. Our conclusions are therefore in accord with his.

The data of Table II indicate that the pressure of highly diluted serum can be expressed as the sum of the partial pressures of albumin and globulin, if correct figures for the specific pressure, or molecular weight, of each fraction are used in the calculation. This appears to be true not only for normal serum, but also for nephrotic serum, in which the molecular weights of both albumin and globulin, as separated with ammonium sulfate, were found to be from 50 to 100 per cent higher than in normal serum.

Since we are still in ignorance as to the laws which govern deviation from the van't Hoff law, it is probably not warranted to compare the results presented here with those obtained from osmotic pressure measurements on higher concentrations, and without attempt to estimate specific pressures at infinite dilution. This statement applies to the formulas, calculated by Govaerts and his followers (7, 16, 18),

relating the osmotic pressures of undiluted serum to its albumin and globulin concentration, and also to Widdowson's results (17).

The results of Table IV show that the urine proteins of the nephrotic patients differ from serum proteins of the same patients even more markedly than from the serum proteins of normal subjects. Roughly, the mean molecular weight of urine albumin is one-half that of serum albumin, that of globulin one-third the molecular weight of serum globulin, from the same patient.

These results support the idea that in proteinuria the kidney is more permeable to the proteins of smaller molecular size. It has long been known that albumin passes more abundantly than globulin into the urine (8). Our results indicate that, from the heterogeneous albumin fraction of nephrotic serum, the subfractions of lower molecular size pass more abundantly into the urine; and similarly for the subfractions of the globulins.

SUMMARY

In serum of patients with nephrosis both albumin and globulin showed by osmotic pressure nearly double the molecular weights of normal albumin and globulin.

In the urines of such patients, on the other hand, both proteins showed molecular weights lower even than in normal serum.

The colloidal osmotic pressures were measured by the author's method at such dilutions that the van't Hoff law relating pressures to molecular concentrations could be directly applied. For the albumin and globulin of normal serum the molecular weights found were 72,000 and 164,000 respectively, in agreement with the weights obtained by other methods.

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