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An Electronic Colloid Osmometer and an Assessment of its Accuracy. The Molecular Weight of Bovine Plasma Albumin

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This osmometer was designed to study the changes occurring in serum and allied proteins in disease. An instrument was required to measure colloid osmotic pressure accurately and rapidly on small volumes of solution. Although the osmometers of Adair (e.g. Adair, 1925) were accurate they took 24 hr. or more to come to equilibrium, and required several millilitres of solution. The osmometer of Smithies (1953) yielded results of high accuracy on small volumes of solution, but also took many hours to reach equilibrium. A form of electronic osmometer has already been described briefly (Rowe, 1953), and in this paper an assessment of the developed form of the instrument is presented. The results obtained with bovine plasma albumin showed that measurements of high accuracy could be obtained in 30 min. on small volumes of solution.

EXPERIMENTAL

Principle. The osmometer is in the form of a U-tube, with the semi-permeable membrane inserted into one limb (Fig. 1). The membrane separates protein solution from solvent, and the flow of solvent through the membrane due to colloid osmotic pressure is opposed by an air pressure applied to the solution. When the tap is closed any flow through the membrane produces a rapid pressure change in the compartment between the membrane and the tap. This causes movements of a platinum foil in the wall of this

compartment, which are measured by the deflexion of a galvanometer in circuit with a mechano-electronic transducer attached to the foil. If the temperature of the system is constant, the absence of galvanometer movement after closing the tap represents pressure equilibrium across the membrane, so that

$$\text{Colloid osmotic pressure} = \pi = P + h_c\rho_c - h_s\rho_s - h_t,$$

where h_t is the effect due to the difference of surface tension between solution and solvent, the remaining symbols being as displayed in Fig. 1.

Membranes. Membranes were prepared from a batch of HL 120/170 nitrocellulose (Imperial Chemical Industries Ltd.) by the method of Wells (1932). The thickness of these membranes averaged 0·08 mm. Ultrafiltrates of albumin solutions made through them gave no precipitate with 3% sulphosalicylic acid.

Design of osmometer. Two osmometers of essentially similar design and dimensions were used in this work. The one shown in Fig. 2 has several features incorporated by Messrs Nash and Thompson, Oakcroft Road, Chessington, Surrey, who now manufacture the instrument. The membrane is clamped between a lower stainless-steel and an upper Perspex block, and supported on a grooved dome which holds it rigid without impairing its semi-permeability. The diameter of the colloid and solvent compartments in the Perspex block was 1·7 cm., and 0·5 ml. of solution is required to provide a meniscus above the membrane. The volume of solvent between the membrane and the tap was small (about 0·2 ml.), minimizing the effects of change of temperature on the readings. The tap is formed by a polytetrafluoroethylene (PTFE) bar sliding in a channel cut in the steel block. PTFE is self-lubricating and requires but a thin film of silicone grease to form an adequate seal; it is also slightly pliable and has the advantage over a rigid metal bar that its movements do not cause unwanted galvanometer deflexions due to distortion of the main steel block.

For providing the steady pressure, variable at will, to be applied to the solution, air from a cylinder fitted with a reducing valve is fed slowly into a 10 l. aspirator bottle, which forms a reservoir. The pressure in this reservoir is held at a constant level above room pressure by connexion to a rigid tube which can be immersed to a variable depth in a tank of water. There is also a common connexion from the reservoir to a U-tube water manometer and to the osmometer. The pressure applied to the solution is varied by altering the depth of immersion of the tube in the water tank. This controlled leak arrangement eliminates differential pressure changes due to variations in room pressure and to temperature variations of the reservoir. With the air flow correctly adjusted no fluctuations were observed in the U-tube water manometer.

Determination of equilibrium. The osmometers were assembled with phosphate buffer in all compartments 24 hr. before measurements were made, this time interval being necessary for the membranes to bed down on to their supports. A series of readings of the changes of galvanometer deflexion in 60 sec. were then obtained for different fluid levels of buffer in the colloid and solvent compartments. The readings were taken as the rapid change of deflexion which occurred on re-opening the tap after 60 sec., rather than the slow change while the tap was closed, so as to minimize errors due to electrical drift. The difference in

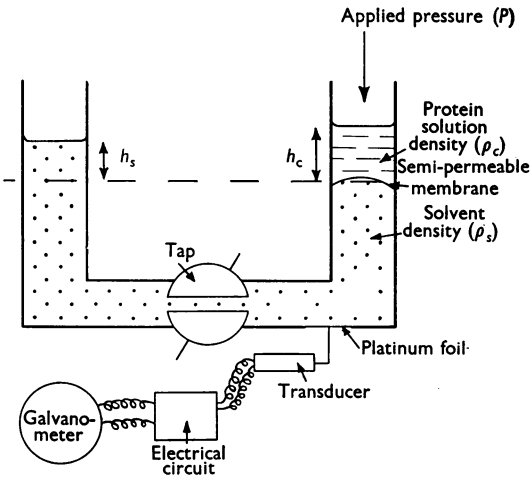

Fig. 1. A diagrammatic cross-section of the colloid osmometer.

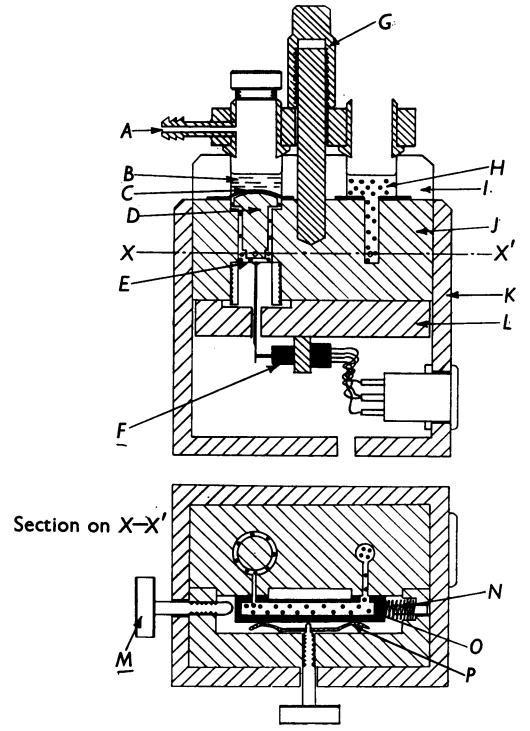

Fig. 2. Cross-sections of the osmometer to show the general construction and the design of the tap. *A*, Pressure connexion; *B*, protein solution; *C*, semi-permeable membrane; *D*, membrane support; *E*, platinum foil; *F*, transducer; *G*, clamping nut; *H*, solvent; *I*, Perspex block; *J*, stainless-steel block; *K*, Tufnol (Tufnol Ltd., Perry Barr, Birmingham) case; *L*, Tufnol transducer plate; *M*, tap-operating knob; *N*, return spring; *O*, PTFE bar; *P*, leaf spring.

fluid levels was then plotted against the change of galvanometer deflexion. The coincidence of zero deflexion with zero pressure difference provided a check on the working of the instrument. Ten values of the pressure required for zero deflexion, obtained in this way during the course of these experiments, are included in Table 2.

The phosphate buffer in the colloid compartment was removed and the compartment dried with a piece of soft filter paper. An albumin solution was inserted and a pressure approximately equal to the anticipated osmotic pressure was applied to the solution with the constant-pressure device. The tap was closed to prevent significant solvent transfer across the membrane. After 30 min. the equilibrium pressure was determined from the changes in galvanometer deflexion corresponding to small changes of applied pressure. The temperature as recorded by a thermocouple inserted into the steel block was noted. The 30 min. interval was considered adequate for the attainment of equilibrium after experiments of the type illustrated in Fig. 3, which showed that the equilibrium pressure rose during the first 15–20 min. after introduction of the solution and the application of pressure, due presumably to thermal effects and bedding down of the membrane. After this time the equilibrium pressure in the typical experiment illustrated showed a rise at the rate of 0·2% in 30 min. This was attributed to evaporation, since after standing overnight condensed water droplets were observed in the colloid compartment above the solution. It was not possible, however, to detect any concentration changes in the albumin solutions during a 30 min. period in the osmometers with the micro-Kjeldahl method; it was therefore concluded that the equilibrium pressures obtained at 30 min. represented the osmotic pressures of the solution actually introduced into the osmometers to at least as great a degree of accuracy as the method of estimating the protein concentration.

During all the measurements the osmometers were standing on the bench, protected from draughts during readings. The absence of significant zero error, the stability of the equilibrium pressure with time and the reproducibility of the results indicated that temperature fluctuations in the instrument under these conditions did not cause significant errors in measurement.

Protein estimations. Nitrogen concentrations were measured by a micro-Kjeldahl method (Ma & Zuazaga, 1942). The results, which were taken as the mean of three estimations which themselves had a scatter of less than 1%, were converted into protein concentrations by use of a factor of 6·25.

Calculation of results. As shown above,

$$\pi = P + (h_c\rho_c - h_s\rho_s - h_t).$$

The value of the correction due to the difference between ρ_c and ρ_s and due to h_t was estimated in the following way. An osmometer was assembled with phosphate buffer in all compartments, a small perforation was made in the membrane, and the difference between the fluid levels in each compartment was measured. The buffer was then removed from the colloid compartment and albumin solutions of known concentration were introduced. Care was taken to bring the solutions nearly to the same level as the phosphate buffer in the solvent compartment to prevent significant passage of albumin through the perforated membrane. Because of their greater density and owing to the effect of surface tension the levels of the albumin solutions were lower than that of the buffer. It was found that the difference in levels remained steady after 15 min. for at least 2 hr. Table 1 shows the values obtained for a height of column of 0·5 cm. above the membrane, which was greater than the maximum height ever used in the osmotic-pressure determinations. These values are 0·1% of the osmotic pressures of the solutions. This correction was therefore considered negligible for albumin solutions, so that in effect $\pi = P + \rho_s(h_c - h_s)$. The value of ρ_s was found to be 1·009. During the measurements of osmotic pressure $(h_c - h_s)$ did not exceed $\pm 0{\cdot}3$ cm. This maximum difference would require a correction for the difference between ρ_s and

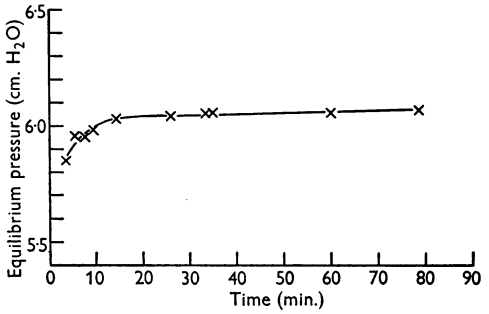

Fig. 3. Relationship of the equilibrium pressure reading of the osmometer to the time from introduction of an albumin solution.

Table 1. *Estimations of the corrections due to difference in surface tension and density between albumin solutions and phosphate buffer, made in an osmometer with a small perforation in the membrane*

Albumin concn. (g./100 ml.)	$h_s - h_c$ (in cm. H_2O)	Correction as % of π
0	0·001	—
0·489	0·002	0·1
1·468	0·006	0·1
2·389	0·011	0·1
2·997	0·012	0·1

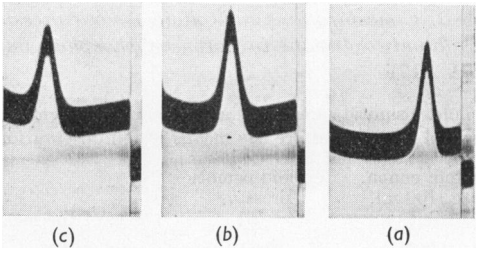

Fig. 4. Sedimentation diagrams of bovine serum albumin (Armour Batch no. 22049) in pH 7·17 Sørensen phosphate buffer + 0·2 M-NaCl in a Spinco Model E ultracentrifuge. The speed was 59 780 rev./min., and the protein concentration approx. 1 g./100 ml. (*a*) 68 min.; (*b*) 116 min.; (*c*) 150 min. Sedimentation occurs from right to left.

unity of 0·0027 cm. This correction was therefore also ignored and osmotic pressures were calculated from the expression $\pi = P + (h_c - h_s)$.

Preparation of solutions. Crystallized bovine plasma albumin (Armour, Batch no. 22049) was dissolved in pH 7·17 Sørensen buffer (0·047 M-Na_2HPO_4 + 0·020 M-KH_2PO_4) and dialysed for 24 hr. against several litres of the same buffer. Several concentrations were prepared by diluting the parent solution with the dialysing buffer. The osmotic pressures of 10 portions of each solution were measured, five in each of the two osmometers. The protein concentrations of the solutions were determined before osmometry. As a check that no change in protein concentration had occurred in the osmometers the concentration was also estimated after osmometry on several specimens. Fig. 4 shows the results of an ultracentrifugal analysis, which showed no obvious heterogeneity.

RESULTS AND DISCUSSION

The results are given in Table 2, where the osmotic pressures have been corrected to 24° assuming their proportionality to the absolute temperature. The temperature of measurement ranged from 23·5 to 25·7°. It will be seen that the standard errors expressed as pressures show no systematic correlation with the magnitude of the osmotic pressures, the measurements at higher pressures having therefore a higher percentage accuracy.

The molecular weight has been derived in the following ways:

(i) A polynomial regression of π against concentration (c) was fitted by the method of least squares. This yielded the expression

$$\pi = 0{\cdot}024 + 3{\cdot}665\ (\pm 0{\cdot}0026)\ c + 0{\cdot}339\ (\pm 0{\cdot}001)\ c^2 - 0{\cdot}0141\ (\pm 0{\cdot}00037)\ c^3. \quad (1)$$

Mol.wt. = 68 766 ± 48.

The cubic term was significant at the 5 % level of probability.

(ii) A regression was fitted to the plot of π/c against c, a conventional method to obtain the value of π/c when $c \to 0$. This regression did not depart significantly from linearity.

$$\pi/c = 3{\cdot}734\ (\pm 0{\cdot}037) + 0{\cdot}282\ (\pm 0{\cdot}0049)\ c. \quad (2)$$

Mol.wt. = 67 500 ± 670.

(iii) A regression was fitted to the plot of c/π against c, after Adair & Robinson (1930). This method often yields best fits to the linear relationship for human serum, and in this case did not depart significantly from linearity.

$$c/\pi \times 10 = 2{\cdot}652\ (\pm 0{\cdot}033) - 0{\cdot}159\ (\pm 0{\cdot}003)\ c. \quad (3)$$

Mol.wt. = 66 580 ± 840.

The molecular weights have been calculated by substitution in the van't Hoff equation $\pi = (c/M)\,\boldsymbol{RT}$, the linear coefficient from (1) and the limiting values for $c \to 0$ in (2) and (3) being used. $\boldsymbol{RT}$ in the units used was $2{\cdot}520 \times 10^5$.

The different values for the molecular weight yielded by the three methods of calculation indicate that the standard errors assigned to the molecular-weight values must be interpreted with caution. The standard errors are derived from those of regression coefficients calculated from specific equations for the assumed relationship between π and c. The results show that alterations in the form of the assumed equation produces changes in the calculated values of the molecular weight which exceed the apparent standard error. There is no reason to assume that the equation which yields the smallest standard error conforms most closely to reality, so that it is desirable to use several alternative forms of calculation when attempting to estimate molecular weights to the highest degree of accuracy.

Account must also be taken of systematic errors in assessing the accuracy of the determination. The error due to the uncorrected surface tension and density differences would result in the measurements of osmotic pressure being too low by approx. 0·1 %, whilst that due to evaporation during the 30 min. equilibration period would make the measurements too high by approx. 0·2 %. These errors therefore tend to be self-cancelling. The error of estimation of a single protein concentration does not exceed 0·5 %, but is only partially taken into account in the statistical analysis.

The range of molecular weights from 66 600 to 68 800, which has been derived from the results, agrees well with the value of 67 500 recently obtained by Harrington, Johnson & Ottewill (1956). The calculated values of the standard errors of the molecular weights are considerably less than those of any previous determinations by the osmotic-pressure method (Smithies, 1953).

Table 2. *Concentrations and colloid osmotic pressures of albumin solutions in Sørensen phosphate buffer* (pH 7·17)

Colloid osmotic pressures are recorded in cm. H_2O (corrected to 24°) and are the means of ten observations.

Protein concn. in g./100 ml. of solution	Colloid osmotic pressure (π)	S.E. of the mean
2·997	13·712	0·0485
2·389	10·578	0·0326
1·958	8·383	0·0177
1·468	6·051	0·0167
0·979	3·934	0·0207
0·734	2·881	0·0289
0·489	1·903	0·0313
0·00	0·014	0·0190

SUMMARY

1. The design, theory and operation of an electronic colloid osmometer is described.

2. Measurements could be made 30 min. after placing a solution in the instrument.

3. Ten observations on each of a series of seven solutions of bovine plasma albumin were made, in two osmometers. The S.E. of the means of the observations ranged from 0·017 to 0·048 cm. H_2O with osmotic pressures ranging from 1·9 to 13·7 cm. H_2O.

4. The value for molecular weight of bovine plasma albumin derived by various methods from these results ranged from 66 600 to 68 800.

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The Molecular Weights of the Proteins of Normal and Nephrotic Sera and Nephrotic Urine, and a Comparison of Selective Ultrafiltrates of Serum Proteins with Urine Proteins

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The outstanding functional defect in the nephrotic syndrome is the severe urinary loss of protein, and the characterization of the serum and urine proteins has been recognized to be of fundamental importance in the investigation of this condition. The recent immunological demonstration of the identity of the urine proteins with some of the serum proteins (Burtin & Grabar, 1954; Gell, 1955) is cogent evidence of their derivation from that source. Electrophoretic studies have shown that these proteins have a distribution of characteristic and complementary type, the urine containing chiefly albumin with smaller quantities of α_1-, β- and γ-globulins, whereas the serum may be deficient in the albumin, α_1- and γ-globulin fractions and show an increased concentration of α_2-globulin (Hardwicke, 1954*a*). Consequent upon these protein changes, especially the fall in the albumin concentration, the colloid osmotic pressure of the plasma is greatly reduced. It has been suggested, following the Starling hypothesis, that this reduction is the cause of the oedema which is the presenting feature of the condition.

The studies of Bayliss, Kerridge & Russell (1933) on the excretion of protein by normal animal kidneys indicated a specific molecular weight, that of haemoglobin, demarcating larger protein molecules which were retained in the circulation from smaller ones which passed into the urine. More recent studies, such as those of Brewer (1951) and Wallenius (1954), on animals infused with dextran molecules of graded molecular weights, showed a progressive reduction in clearance as the molecular weight increased. Wallenius demonstrated that dextrans with average molecular weights of 10 000 and 20 000 had a clearance of about 90 and 30 % respectively relative to creatinine, whereas the preparation with an average molecular weight of 50 000 scarcely appeared in the urine. Although these results cannot be compared directly with those obtained with proteins because of the effects of charge and the differences in degree of hydration and in shape of the molecules, they do imply that normal kidneys may act as selective molecular filters over this range of molecular weights.

It is pertinent to inquire to what extent the kidney retains this selective function in conditions of heavy proteinuria. The preponderance of albumin in the urine protein, even in the face of an inverted serum albumin:globulin ratio, has suggested that some selectivity is maintained, but it seemed likely that more precise information could be obtained by comparison of the average molecular weights of the serum and urine proteins. The results presented here, which were obtained by colloid osmotic-pressure measurements, confirm the highly selective nature of the urinary loss of protein in conditions of moderate and heavy proteinuria. In addition, it has been possible to reproduce the selective protein filtration, characteristic of the nephrotic kidney, by filtering normal and nephrotic sera through suitably prepared nitrocellulose membranes.