

An Inverted Microosmometer and Its Use for the Determination of Molecular Weight of some Specimens of Potassium Hyaluronate

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A microosmometer of about 500 μ l capacity is described. A semi-permeable cap is on the upper end of an open capillary and both are completely immersed in the outer liquid.

The hydrostatic pressure-difference is produced by an air column in the capillary confined between the inner and the outer liquid respectively. The height of the air column is varied by varying the pressure on the outer liquid between 1 and 0.1 atmosphere. At each pressure-difference the flow through the membrane in 10 minutes is determined. The height for zero flow is determined graphically.

One advantage is that the capillary correction is nearly zero, another that pressure-differences of about 1 mm water column can still be measured.

In the course of time many osmometers have been constructed all of which, however, come within one of the two types: 1) the static-elevation cell in which the osmotic pressure of the solution is counterbalanced by the liquid column developed by the influx of solvent into the solution, and 2) the dynamic-equilibrium cell in which the osmotic pressure is counterbalanced by an externally applied pressure of known magnitude. As examples of more recent static-elevation cells we name in chronological order the osmometers of Krogh and Nakazawa², Schulz³, Herzog and Spurlin⁴ and Bull⁵. The dynamic-equilibrium principle which underlies the osmometer to be described here, was first used by Tammann⁶ and some years later by Berkeley and Hartley⁷ in their studies of sucrose solutions, and Sørensen⁸ determined the molecular weight of egg albumin by a dynamic-equilibrium osmometer constructed by one of the authors of the present article (described in the paper of Sørensen⁸). Many osmometers based upon the same principle have been described in the literature, a more recent one by Güntelberg and Linderstrøm-Lang⁹.

Description of the New Apparatus. The principal parts of the apparatus are outlined in Figs. 1 and 2. The osmometer consists of a testtube-shaped

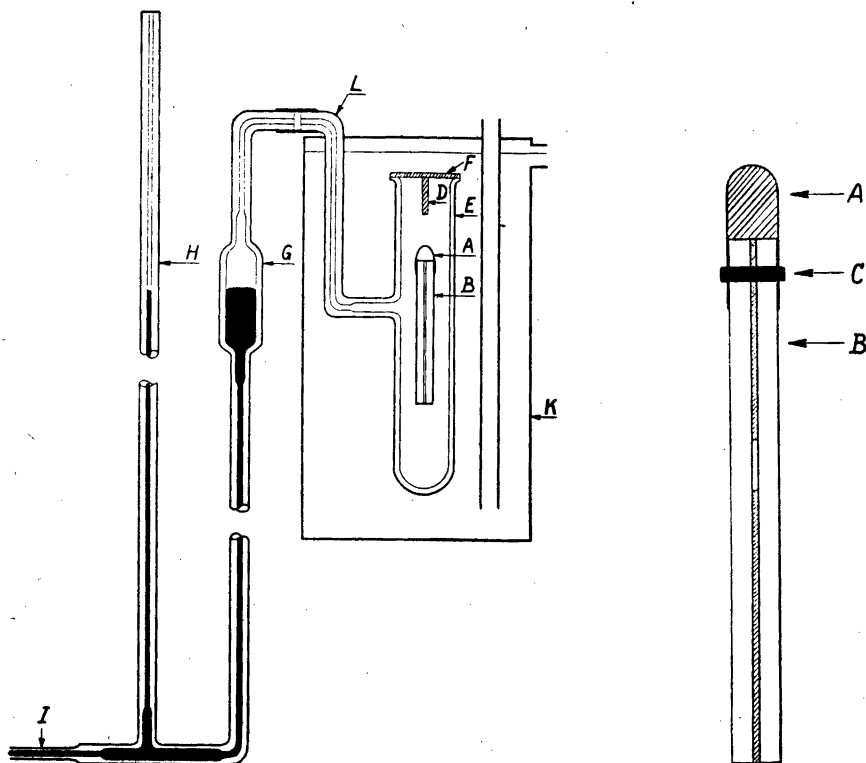


Fig. 1. The principal parts of the apparatus. For explanation see the text.

Fig. 2. The osmometer proper showing the air column.

semi-permeable membrane *A*, tightly connected to a glass capillary *B* of about 0.7 mm bore, outer diameter approximately 7 mm, length approximately 70 mm. The volume of the cap is about 400 μ l. A rubber ring, marked *C* in Fig. 2, ensures perfect tightness. A support arm *D*, provided with two clamps, holds the osmometer which is completely immersed in the outer liquid contained in the glass vessel *E*. *D* is cemented to a lid *F*, which covers the vessel *E*, whose edge is ground plane. The lid, which is also plane, the arm and the clamps are made of perspex. Only the upper part of the arm *D* is indicated in Fig. 1. By means of a clamping device mounted upon the upper part of *E*, (not shown in Fig. 1) the apparatus can be made water-tight. *E* is connected with a pressure device, *G H*, by means of rubber tubing. Part of the space of the pipette *G*, (white in Fig. 1) the connecting capillary *L* and the vessel *E* are completely filled with the outer liquid. The rest of pipette *G*, (black in Fig. 1) and the manometer tube *H*, contain mercury. Tube *I* is connected to a mercury levelling device. Actually we use a mercury-tight steel-piston pump operated by means of a screw. This device is exceedingly convenient, but we suppose that a mercury levelling device similar to those used in many gas-

analysis apparatuses, e.g. in A. Krogh's modification of the Haldane apparatus (described by Lindhardt¹⁰) may be sufficiently convenient, and it will certainly be much cheaper to manufacture.

The height of the mercury column in H is roughly measured on a meter stick divided in millimetres. By means of the arrangement the total pressure on the system can be varied from just above one atmosphere to about one tenth of that amount. Water from a thermostat is circulated through the perspex walled rectangular container K , thus keeping the temperature of the container constant at 20° C (Variations about 0.01° C).

The Semi-permeable Caps. For some measurements we used collodion caps prepared by ourselves essentially according to the method elaborated by one of us and described in Sørensen's paper¹¹ and for others cellophane caps kindly presented to us by the cellophane factory "Kapcello".

As we have modified the procedure for making collodion caps a little it may be useful to describe it in detail. As a mould we use a test tube provided with a small hole in the bottom. The outer diameter of the test tube is about one tenth of a millimeter less than that of the osmometer capillary. Before use it is rinsed thoroughly with soap and water and wiped dry with a towel. It is then attached to a slowly rotating shaft (40—60 r.p.m.) which is so mounted that it can be inclined from zero to at least about 30° relatively to the horizontal position. At first the shaft is inclined and the collodion solution is poured on the bottom end of the testtube. A few seconds after that the rotation is stopped and excess solution is allowed to dribble off. After further rotation for about 2 minutes the application of collodion solution is repeated but so that the desired length of the tube becomes coated. After a momentary stop to remove a drop of excess collodion by means of a spatula the shaft is turned into the horizontal position, and the rotation continued for about 4 minutes, after which time a second coating is applied in the same manner, but concluding with a rotation (shaft horizontal) for 20 minutes. Finally the coated tube is placed in water for at least two hours. Then a ring is cut in the coating with a razor blade so as to produce a cap of suitable length, usually between 10 and 20 mm.

The cap is easily slipped off the test tube by hand and is now ready for use. They should be kept in water saturated with toluene. Under these circumstances they seem to retain their permeability for water at least for months.

The collodion solution is prepared from "Collodion B.D.H." which is dried to constant weight. 15 g of the dry collodion is dissolved in 375 ml anhydrous alcohol and 125 ml anhydrous ether by repeated shaking during some days.

The Kapcello caps are furnished moist from the factory (Kapcello, Mosevej, Copenhagen) and are kept in the laboratory in water saturated with toluene at about 0—3° C. Before use they are placed in running tap-water for a couple of hours and rinsed a few times in distilled water. They are impermeable to the compounds investigated by us and are easily permeable to water and dissolved low-molecular substances. We have therefore omitted the drying procedure used by Rehberg¹². For further details regarding Kapcello caps see Rehberg¹² and Grandjean¹³. According to our experience they are somewhat less permeable to water than the collodion caps, which follows from Table 1, but both are convenient for practical use. In one case we have determined

the molecular weight of the same specimen both by collodion and Kapcello caps and found mutually agreeing figures, the slopes of the interpolation curve (mentioned later in this paper) being 3.2 and 1.4 respectively.

Anyhow the test-tube shape and the small dimensions tend to facilitate the attainment of partition equilibrium in respect of diffusible particles.

Preparation of the Osmometer. The osmometer capillary is ground in both ends with fine carborundum powder and thoroughly rinsed for grains of abrasive and for grease.

The cap, whose inner diameter must be just a trace smaller than the outer one of the capillary, is placed on one end of the capillary as shown in Fig. 2. To secure complete tightness a narrow rubber ring *C* is placed around the supported part of the cap.

To perform the latter operation we have constructed a special device, Fig. 3. It consists mainly of two pieces of brass tubing one sliding in the outer. The innermost is a little wider inside than the osmometer (the cap included). The relative movement of the tubes is limited by crosspin and slit as indicated. A spring causes the inner tube to protrude about 5 mm relatively to the outer one. In this position the rubber ring is placed on the inner tube. When now the osmometer is held as in Fig. 3 a withdrawal of the inner tube causes the rubber ring to slide onto the cap in the desired position, and the osmometer is now ready for filling.

Filling the Osmometer. To fill the osmometer we have found it necessary or at least very convenient to use a special apparatus. This consists of two parts, a manipulator table provided with a clamp for the osmometer, the table being movable in three directions, and a fixed pipette with a capillary tip which can easily pass through the capillary of the osmometer. The table is shown in Fig. 4, which is drawn to scale and diminished in the ratio (3 : 1).

As base we use the tripod of an ordinary laboratory stand provided with two adjustment screws to secure vertical position of the axis. Just above the base is a cross-table *a* enabling the upper part to be moved by screws in two mutually perpendicular horizontal directions.

The cross-table wears a vertical hollow column *b* which is connected, through a rack-and-pinion device, with a vertical bar carrying a horizontal

Table 1. The figures in the first column give the rate of movement of the upper meniscus of the air column for a pressure-difference of 1 mm water column when the caps quoted in the second column are used.

Slope of interpolation curve	Type of caps
2.3	Kapcello 1
3.0	Collodion 1
3.2	Collodion 1
4.2	Collodion 2
1.2	Kapcello 2
2.6	Kapcello 1
—	—
0.5	Kapcello 3
—	—
0.6	Kapcello 3

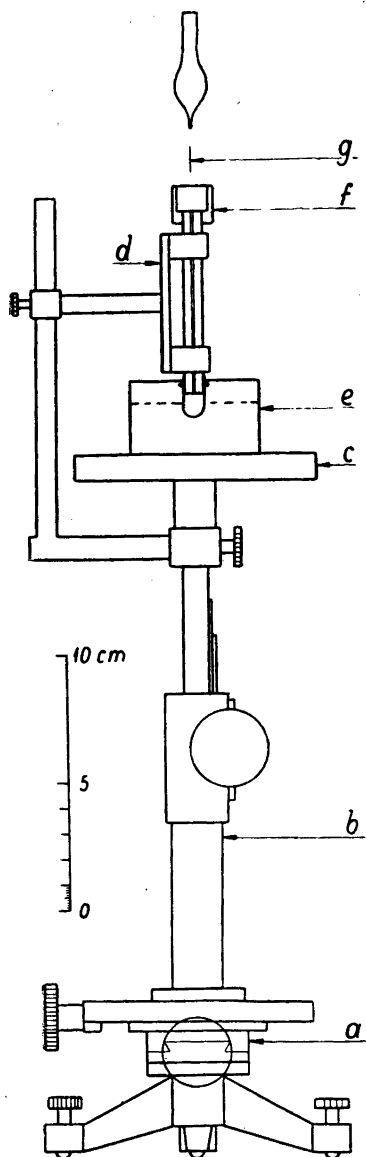


Fig. 4. The apparatus for filling the osmometer. Explanation in the text.

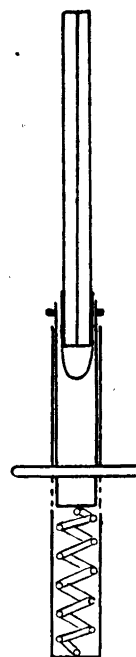


Fig. 3. The device for placing the rubber ring.

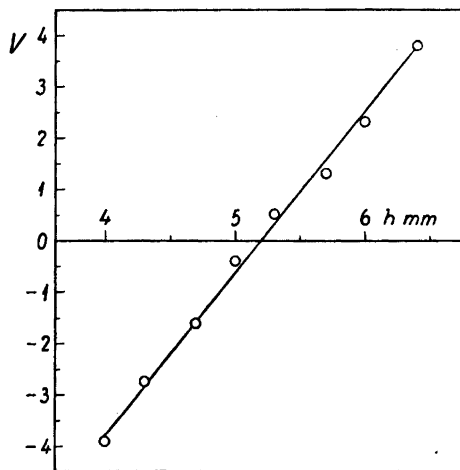


Fig. 5. The graph shows the relation between the height of the air column (abscissae), and the rate of movement of the upper meniscus of the air column (ordinates) in micrometer divisions in ten minutes.

wooden table *c* and a spring-clamp *d* for the osmometer. During the operations the cap of the osmometer can be immersed in a beaker *e* containing the outer liquid to avoid undue drying of the membrane. For filling the osmometer the upper end of the capillary is provided with a removable but tightly fitting perspex cup *f*. Above this is a fixed pipette ending in a capillary *g* so finely drawn that it can easily pass through the whole length of the osmometer capillary. The upper end of the pipette is connected to a syringe the air-tight piston of which can be moved by means of a screw.

The filling is now performed as follows: By means of the manipulator the center of the orifice of the osmometer capillary and that of the capillary tip of the pipette are made to coincide. Next the osmometer is raised vertically so much that the capillary tip is near the bottom of the semi-permeable cap. Now a suitable amount of the inner liquid is filled into the perspex cup. By means of the syringe air is slowly withdrawn from the osmometer which is simultaneously lowered so that the tip is always just above the level of the liquid. The osmometer is thus filled with inner liquid to about somewhat less than half of the capillary. After some practice we have had no difficulties in avoiding air bubbles in the inner liquid, which is essential.

Finally the osmometer is lowered so that the capillary tip is free, and the rest of the capillary is filled with the outer liquid by a similar procedure, care being taken that an air column of a suitable length separates the two liquids. After removal of the perspex cup the osmometer is placed upwards down in the apparatus Fig. 1, which is filled with the outer liquid.

The Microscope. The reading microscope is of the commercial type. It has a vertical stem with two adjustments. By means of the one the microscope is raised to a suitable height and fixed. By means of the other its vertical position can be varied by means of rack-and-pinion. The latter displacements are registered on a vertical scale with vernier (accuracy 0.1 mm). The magnification of the microscope is low and the object distance rather large. The eyepiece micrometer has a scale with 10×10 divisions, the whole length of the scale being 5.1 mm.

Calculation of the Pressure-difference. The pressure is calculated from the height of the air column by multiplication with the density of the outer solution and the gravitational constant at the place of the measurements. Strictly speaking three corrections should be applied: The first in regard to the fact that the density of the air column is not zero, the second should take account of the difference in densities of the inner and the outer solutions respectively, and the third should be calculated from a knowledge of the difference in surface tension of the two solutions. All of them are small, however, and we have neglected them in the present investigation, but in precision measurements their magnitude, and especially that arising from the difference in surface tensions must be evaluated.

Mode of Operation of the Osmometer. To begin with the vessel *E*, the connecting capillary *L*, and the bulb *G*, are filled with the outer solution. This can be accomplished by filling *E* and using the mercury levelling device as a pump. In this way all air can be removed. (Very small air bubbles are harmless.) Next the osmometer capillary is placed in the perspex clamp attached to the lid *F*. The latter is placed in its proper place, care being taken that no air

Table 2. Nitrogen and potassium content, viscosity and molecular weight of different preparations of potassium hyaluronate. The nitrogen is determined by a Kjeldahl procedure and the potassium as potassium sulphate. W is the weight in mg of hyaluronate dissolved in 1 ml of the outer solution (0.2 M KCl). h is the height in cm of the air column at equilibrium.

% N	% K	Relative Viscosity Conc. lg/l	W mg	h cm	Molecular weight $\times 10^{-3}$
3.36	11.36	11.80	13.00	0.62	517
3.35	10.82	11.91	11.00	0.51	530
3.31	8.05	12.19	10.88	0.52	516
3.34	7.12	12.12	10.26	0.48	527
2.90	10.02	5.46	19.35	1.30	367
2.88	11.00	68.46	9.91	0.14	1 750
2.82	13.20	5.29	—	—	—
1.16	17.01	5.75	28.16	2.20	316
5.78	6.56	4.62	—	—	—
2.99	7.86	76.39	27.61	1.23	554

bubbles remain, and fastened by means of the clamping device (not shown in Fig. 1). Finally the container K , is filled with water and the circulation of thermostated water is started.

The measurements proper can only begin after the lapse of a certain relaxation time, some 30 minutes or more, during which time diffusion equilibrium is established. To prevent undue dilution of the inner liquid (1 μ l corresponds to about 2.5 mm in the capillary), the length of the air column, which must be "too small" at atmosphere pressure, is adjusted as soon as possible by means of the mercury levelling device in such a way that its upper meniscus moves slowly or not at all during the relaxation time.

The measurements proper are performed as follows: The air column is set at a definite length which is measured either by means of the scale on the vertical stem of the microscope or by means of the eyepiece scale. The corresponding displacement of the upper meniscus in 10 minutes is measured on the eyepiece scale. When a number of such measurements have been made, the movements being partly upwards and partly downwards, the results are plotted and the equilibrium length found graphically. Besides this the slopes of the lines in the graphs which, apart from random errors, are straight give a rough measure of the permeability for the outer solution, rough because the surface of the caps are somewhat different in the different experiments.

As an example we give the graph in Fig. 5.

Measurements on Solutions of Potassium Hyaluronate. The outer liquid is in all the experiments reported here a 0.2 molar solution of potassium chloride. To avoid supersaturation with air, which sometimes causes trouble, the solution is boiled and cooled down before use. The inner solutions are prepared by weighing a suitable amount (10–30 mg) of the specimen in question and dissolving in 1.00 ml of the outer solution.

The results are in Table 2. The specimens are those described in a recent paper by one of us¹⁴, and they occur in Table 2 in the same order as in that paper. It must be added that owing to the preliminary nature of the experiments and to the fact that the Donnan effect according to the composition of

the liquids may be expected to be small, no correction for the latter was introduced.

The molecular weight M has been calculated from the expression

$$M = qw/h$$

where w is the amount of substance in grammes dissolved in 1 cm³, h is the equilibrium height of the air column in cm and q equals RT/gd , where $R = 8.314 \cdot 10^7$ erg/mole degree, $T = 293.16$, $g = 981.6$ cm/sec². (latitude of Copenhagen), $d = 1.0078$ gramme/cm³. Calculation yields $q = 246.8 \cdot 10^5$ cm⁴/mole.

The great majority of viscosity determinations of hyaluronic acid vary from 1 to 8 most of them being below 4 as seen in a table by Hadidian and Pirie¹⁵. On the basis of measurements of double refraction of flow and viscosity Mayer and Palmer¹⁶ and Blix and Snellman¹⁷ have arrived at estimates of the molecular weight of hyaluronic acid ranging from 2×10^5 to 5×10^5 . The smaller figures were found for synovial hyaluronic acid, the higher for human umbilical cord hyaluronic acid. As seen in Table 2 some of our preparations show a very high viscosity and the same is true regarding the molecular weights.

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REFERENCES

1. Christiansen, J. A., and Jensen, C. E. *Acta Chem. Scand.* **5** (1951) 849.
2. Krogh, A., and Nakazawa, F. *Biochem. Z.* **188** (1927) 241.
3. Schultz, G. V. *Z. physik. Chem. A* **176** (1936) 317.
4. Herzog, R. O., and Spurlin, H. M. *Z. physik. Chem. Bodenstein-Festband* (1931) 239.
5. Bull, H. B. *J. Biol. Chem.* **137** (1941) 143.
6. Tammann, G. *Z. physik. Chem. A* **9** (1892) 97.
7. Berkeley, E. J., and Hartley, E. G. *J. Trans. Roy. Soc. London, Ser. A.* **206** (1906) 481.
8. Sørensen, S. P. L., and Høyrup, M. *Compt. rend. trav. lab. Carlsberg.* **12** (1915-17) 296.
9. Güntelberg, A. V., and Linderstrøm-Lang, K. *Compt. rend. trav. lab. Carlsberg* **27** (1949) 1.
10. Lindhard, J. in *Aberhalden's Handbuch der biologischen Arbeitsmethoden* **5** (1927) 1584.
11. Sørensen, S. P. L., Christiansen, J. A., Høyrup, M., Goldschmidt, S., and Palitzsch, S. *Compt. rend. trav. lab. Carlsberg* **12** (1915-17) 28.
12. Rehberg, P. Brandt. *Acta Physiol. Scand.* **5** (1943) 305.
13. Grandjean, L. C. *Acta Physiol. Scand.* **24** (1951) 192.
14. Jensen, C. E. *Acta Chem. Scand.* **7** (1953) 603.
15. Hadidian, Z., and Pirie, N. W. *Biochem. J. (London)* **42** (1948) 260.
16. Meyer, K., and Palmer, J. W. *J. Biol. Chem.* **114** (1936) 689.
17. Blix, G., and Snellman, O. *Arkiv Kemí, Mineral. Geol. A* **19** (1945) No 32.

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