CXXVI. THE OSMOTIC PRESSURE OF GLYCOGEN SOLUTIONS.

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HAWORTH and his co-workers have recently developed a chemical method for the determination of the minimum mol. wt. of polysaccharides, which involves assay of the relative amounts of tetramethyl- and trimethyl-glucoses formed by the hydrolytic cleavage of the fully methylated polysaccharide. For glycogen this method originally gave a minimum mol. wt. of about 2500 [Haworth and Percival, 1932] while that for starch was about twice this value. Later experiments however [Haworth, 1934] showed that some specimens of glycogen had mol. wts. approaching that of starch. The figure for starch is not in agreement with that determined osmotically by Samec and his collaborators, who obtained values of 100,000–500,000 [Samec and Meyer, 1921; Samec, 1934] but this and other discrepancies can be explained on the basis that the large particles in solution are aggregates of the smaller chemical molecule [Haworth, 1934].

As the development of an osmometer for low pressures [Oakley, 1935] enabled an accurate determination of the osmotic pressure of glycogen solutions to be carried out, it became of interest to compare the value for the particle weight of glycogen given by this method with the chemically determined minimum mol. wt. Furthermore as the preparation of methyl-glycogen is an essential step in Haworth's method of mol. wt. determination, and as this method has been criticised [Irvine, 1932] on the basis that the methylation process might involve some degradation of the molecule (*cf.* however Bell and Kosterlitz [1935]), a comparison of the particle weights of glycogen and of methyl-glycogen, determined osmotically, was of some interest.

Methods.

Osmometer. The special form of low pressure osmometer used in this work has been described recently [Oakley, 1935], but since further experience has indicated several improvements in design and technique, the modified form is illustrated in Fig. 1. The most important alteration is that the tap connecting the levelling tube with the main vessel is diagonally bored to eliminate the risk of a slow leak, and is placed with its axis vertical, thus enabling control to be effected by a long handle projecting from the thermostat. The levelling tube has been brought to within 1 cm. of the main vessel to reduce the angle through which the cathetometer must be turned; the alcohol manometer connection is now led off from the side of the main vessel so as to allow an unobstructed view of the menisci; the manometer is connected by a ground glass instead of a rubber joint. The tap at the top of the main vessel is now eliminated.

The temperature control must be within $\pm 0.005^\circ$.

When tap 1 is closed the liquid enters the sac *C* by osmosis and the alcohol rises to an equilibrium position in *E*. This equilibrium position fluctuates

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regularly with the temperature fluctuation of the thermostat but should not exceed 0.2 mm. Errors of parallax can be avoided by fixing a celluloid scale on to the face of the thermostat just opposite to and parallel with the capillary tube of the alcohol manometer and viewing the meniscus in line with some arbitrary mark on the scale.

The alcohol manometer reading is converted into a figure expressed as mm. of colloid solution by opening tap 1 and adjusting the level of the liquid in tube *D*, with a capillary dropper, until the alcohol meniscus attains its previous equilibrium position. It has been found that the alcohol level falls 0.1–0.2 mm. in 5 min. after adjustment and a final levelling must then be made. The difference in levels of the menisci in *C* and *D* is then read off by the cathetometer. It has been found that this "single reading" method is quicker and more reliable than the method first described [Oakley, 1935] in which an interpolation curve was plotted connecting manometer with cathetometer readings. It is advisable that the menisci in *C* and *D* should be arranged to lie in the same focal plane, since cathetometers frequently show a focussing error. Successive readings are reproducible to within 0.1 mm.

Collodion sacs were made by Adair's method [Adair, 1925].

Fig. 1. *A* = cylindrical outer vessel. *G* = tube, 1 cm. in diameter, fitting into *A* by a ground glass joint. *C* = collodion sac, 10 cm. long, attached to *G*. *D* = levelling tube of the same diameter as *G*. *E* = capillary tube, 0.2 mm. in diameter, with scale attached. *F* = alcohol. *B* = meniscus and air space. Diameter of tube is 3 mm. 1 = diagonally bored tap with long handle. 2 = tap to facilitate adjustment and filling.

Preparation of glycogen. "Aqueous-extracted glycogen" and "alkali-extracted glycogen" were prepared from both liver and muscle tissue by the methods outlined by Bell and Young [1934]; in some cases further purification was effected by precipitation with 80% acetic acid as described by Bell and Young. The purity of the sample was determined in each case by the criteria laid down by these authors. All solutions were thoroughly dialysed for some days before determinations were made.

RESULTS.

Rabbit liver glycogen. Two rabbit livers from fed animals were each divided into halves and one-half of each used for the preparation of "alkali-extracted glycogen" (B), and the remainders were used for "aqueous-extracted glycogen" (A) preparation. A sample of the latter specimen was further purified by acetic acid precipitation (C).

Table I. A. *Aqueous-extracted, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	9.93	55.0	5.52
0.05 N CaCl ₂	9.93	28.1	2.83
0.1 N CaCl ₂	9.93	28.3	2.86
"	7.16	16.3	2.28
"	5.28	11.6	2.26
"	4.49	9.1	2.03
"	3.33	7.4	2.23
"	2.83	6.5	2.30
"	2.24	4.7	2.10
"	1.73	3.8	2.20

Average $P/C = 2.23$. $M = \text{mean mol. wt.} = 1.18 \pm 0.025 \times 10^6$.Table II. B. *Alkali-extracted, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	10.39	56.8	5.48
0.1 N CaCl ₂	10.39	17.4	1.68
"	8.39	11.7	1.40
"	6.02	7.15	1.19
"	4.53	5.3	1.17
"	3.42	3.95	1.16
"	2.33	2.7	1.16

Average $P/C = 1.17 \pm 0.01$. $M = \text{mean mol. wt.} = 2.16 \pm 0.02 \times 10^6$.Table III. C. *Aqueous-extracted, reprecipitated with acetic acid, dried at room temperature.*

Solution	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
0.1 N CaCl ₂	7.38	10.6	1.57
"	5.50	6.45	1.18
"	4.74	5.05	1.07
"	3.76	4.1	1.09

Average $P/C = 1.11 \pm 0.06$. $M = \text{mean mol. wt.} = 2.27 \pm 0.12 \times 10^6$.

The results are given in Tables I–III. All measurements were made at 25°. To eliminate possible effects of a Donnan membrane equilibrium resulting from the presence of any ionogenic groups in the colloid, experiments were carried out using $N/10$ CaCl₂ as solvent. That such a precaution is necessary is shown by the large drop in osmotic pressure when CaCl₂ solution is substituted for pure

water. Such a fall in osmotic pressure might be due to increased aggregation of the particles; if this were so, however, the average degree of aggregation would not be expected to remain independent of concentration but to decrease with decreasing concentration, thus giving increasing rather than constant values for P/C .

Fig. 2. Osmotic pressure of solutions of liver glycogen in 0.1 N CaCl_2 .

The apparent "mean mol. wt.", or average particle weight, was calculated from the equation used by Adair:

$$M = 10RT \cdot C/P = 2,520,000 \cdot C/P \text{ at } 25^\circ$$

where M = mol. wt., C = concentration of glycogen in 100 g. water, P = osmotic pressure in mm. water at 4° . The concentration was estimated by weighing the residue after evaporating the solution *in vacuo* at 100° , and subtracting the weight of CaCl_2 calculated to be present on the assumption that it was present in the same concentration relative to water on both sides of the membrane.

The data of Tables I and II are plotted in Fig. 2. A good straight line is obtained below a concentration of 6%.

Samples B and C both have "mean mol. wt." of about 2,000,000, while sample A has a value of about 1,000,000. It seems probable that this lower value is due to the presence of a small quantity of an impurity of much lower mol. wt., since precipitation with acetic acid considerably raised the value as shown by sample C.

Alkali-extracted rabbit muscle glycogen. The preparation was dialysed for 2 days and the osmotic pressure then measured against water and $N/50$ NaCl with the results given in Table IV and Fig. 3.

Table IV. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	3.18	121.4	38
$N/50$ NaCl	3.18	20.3	6.4
"	2.47	12.7	5.3
"	1.51	6.4	4.2
"	1.00	4.0	4.0
"	0.64	2.5	3.9
"	0.45	1.65	3.7

P/C (limit) = 3.6.

M = mean mol. wt. = 0.7×10^6 .

The results were much less satisfactory than those with liver glycogen and it appeared that the concentration of NaCl was too low completely to repress ionic pressure effects. For this reason the experiment was repeated with $N/10$ CaCl_2 as solvent with the results given in Table V and Fig. 3.

Table V. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
$N/10$ CaCl_2	4.25	12.3	2.9
"	2.29	5.3	2.3
"	1.47	3.3	2.2
"	1.13	2.7	2.4
"	0.72	1.9	2.6

P/C (limit) = 2.3.

M = mean mol. wt. = 1.1×10^6 .

Fig. 3. Osmotic pressures of muscle glycogen solutions.

The P/C curve is lower and flatter than in the previous experiment, showing that the effect of ionic pressure has been almost suppressed, although the last two points are high.

Acetic acid-precipitated muscle glycogen. An alkali-extracted specimen of rabbit muscle glycogen was precipitated with acetic acid and again treated with KOH followed by another acetic acid precipitation and dialysis. The results are given in Table VI and Fig. 3.

Table VI. *Rabbit muscle glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
$N/10$ CaCl_2	4.43	7.9	1.78
"	3.06	5.3	1.73
"	1.91	2.9	1.55
"	1.48	2.1	1.42

P/C = 1.40.

M = mean mol. wt. = 1.8×10^6 .

The curve for P/C is still lower and flatter than in the previous experiment giving a mean particle weight of nearly 2×10^6 and it seems probable that there is no significant difference between the apparent mol. wt. of the specimens of liver and muscle glycogen used, but that the latter is much more difficult to free from impurities and is admixed with substances of lower mol. wt. which can be removed only with difficulty.

Methyl-glycogen in water. We are greatly indebted to Dr D. J. Bell of the Department of Physiology, University of Aberdeen, for a sample of methyl-glycogen prepared according to the method of Haworth and Percival [1932] from rabbit liver glycogen [Bell, 1935]. This specimen had a methoxyl content of 45.1% and was readily soluble in cold water. The osmotic data are given in Table VII and Fig. 4.

Table VII. *Methyl-glycogen.*

Solvent	C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
Water	9.37	172	18.4
N/10 CaCl_2	9.37	51.1	5.47
"	6.44	24.7	3.83
"	4.23	12.0	2.83
"	2.7	6.0	2.22
"	1.76	3.5	1.99
"	1.14	2.15	1.89

P/C (limit) = 1.85.

M = mean mol. wt. = 1.36×10^6 .

As appears from Fig. 4, P/C does not reach its limiting value until a concentration of about 1% is reached. The mean particle weight is of the same order as that of glycogen itself, the value after correction for methyl content being 1.08×10^6 .

Methyl-glycogen in benzene. It might be expected that a change from water to an organic solvent would profoundly alter any micellar equilibrium should this exist, and the possibility that benzene might favour the dispersion of the highly methylated compound, owing to the affinity between the aliphatic methyl groups and the organic solvent, arose.

Fig. 4. Osmotic pressure of solutions of methyl-glycogen.

Collodion membranes were used, being transferred first to water, then to alcohol and finally to benzene by way of alcohol-benzene mixtures. As stopcock lubricant for the osmometer metaphosphoric acid was used, this being protected from the water of the thermostat by rings of ordinary stopcock grease in appropriate positions. Owing to the large coefficient of expansion of benzene the fluctuation of the alcohol meniscus in the manometer amounted to about 1 mm. so that it was hardly possible to take readings below 3 mm.; in spite of this the results lie on a good curve as shown in Fig. 4 and Table VIII. The particle weight corrected for methyl content is 2.7×10^6 and although it is about $2\frac{1}{2}$ times

that in water it is still of the same order of magnitude. Carter and Record [1936] have recently found that the osmotic pressure of methyl-glycogen in carbon tetrachloride or nitromethane indicates an average particle weight of the order of 1×10^6 , a value not greatly different from those which we have observed in aqueous or benzene solution.

Table VIII. *Methyl-glycogen in benzene.*

C = conc. in g./100 g. solvent	P = osmotic pressure in mm. of water	P/C
7.57	9.15	1.21
6.10	6.25	1.03
5.33	4.75	0.89
4.57	3.97	0.85
3.70	3.00	0.81

P/C (limit) = 0.75.

M = mean mol. wt. = 3.4×10^6 .

Apparent electrovalency. If we assume that the value of P/C associated with the osmotic pressure of the colloidal particles only is equal to the observed limiting value of P/C in CaCl_2 solution, *i.e.* the value of P/C when $C=0$, and is constant for all concentrations of glycogen, then the observed rise of the value of P/C in CaCl_2 solution with increasing concentration might be due to the influence of ions, the effect of which becomes appreciable only at colloid-ionic concentrations which are significant in comparison with the concentration of Ca^{++} and Cl^- ions.

The number of ions dissociated per glycogen particle at concentration c would then be given by

$$\frac{\left(\frac{P}{C}\right)_c \text{ water} - \left(\frac{P}{C}\right)_0 \text{ CaCl}_2}{\left(\frac{P}{C}\right)_0 \text{ CaCl}_2},$$

where $\left(\frac{P}{C}\right)_c \text{ water}$ = the value of $\frac{P}{C}$ in water at concentration c , and

$\left(\frac{P}{C}\right)_0 \text{ CaCl}_2$ = the value of $\frac{P}{C}$ in CaCl_2 at concentration equal to zero.

In Table IX are given the values thus calculated for the apparent electrovalency of glycogen, *i.e.* the number of ions dissociating from each particle of glycogen.

Table IX.

Substance	Concentration %	Apparent electro- valency
Liver glycogen A	9.93	1.5
Liver glycogen B	10.39	3.7
Muscle glycogen	3.18	16.0
Methyl-glycogen	9.37	9.0

It is impossible to say whether this charge is associated with the real structure of the molecule, for example the dissociation of a phosphoric acid group, or is due to an adsorbed or free impurity; it may merely illustrate the fact that even chemically neutral particles may show a charge in water, *e.g.* air or amyl alcohol. It must be remembered that a charge of a few units on a particle weighing some 2,000,000 represents an exceedingly small stoichiometrical quantity.

DISCUSSION.

The data obtained show that treatment with potassium hydroxide and precipitation with 80% acetic acid have little, if any, significant effect on the particle weight of rabbit liver glycogen in aqueous solution. The value of about 2×10^6 obtained here is significantly different from that obtained by Samec and Isajevic [1923], who found a value for the micellar magnitude of dog liver glycogen in water of about 114,000.

The value of about 2×10^6 for the mean particle weight of rabbit liver glycogen in aqueous solution is in agreement with that given in Table VI for highly purified rabbit muscle glycogen although a difference of the particle size might have been suspected on the grounds that the specimen of muscle glycogen used gave, as do many muscle glycogen preparations, a distinct purplish tint with iodine, quite different from the brown colour given by liver glycogen. This common, though not universal property of muscle glycogen preparations may depend on the length of the chain of the chemical molecule rather than on the particle size in solution. The difference in colour with iodine was the main distinction in properties between the specimens of muscle glycogen used in this investigation and liver glycogen, as determined by the criteria laid down by Bell and Young [1934].

Methylation of glycogen seems to have little effect on the size of the particles in aqueous solution, although one might expect that if the glycogen particle in water consists of an aggregate of large numbers of chemical molecules, the substitution of hydrophilic hydroxyl groups by hydrophobic methoxyl groups might have a marked effect on the state of aggregation in aqueous solution. Furthermore, the fact that the particle size of methyl-glycogen in benzene is of the same order of magnitude as that in water seems to indicate that the aggregating forces may be different from those concerned with known cases of physical aggregation, which are usually modified by a change from aqueous to organic solvents.

If fully methylated glycogen gives an aggregated particle of the same order of magnitude as that of non-methylated glycogen then those hydroxyl groups which are accessible to methylation cannot be responsible for the aggregation [Haworth, 1929]. Bell [1935], however, has recently shown that methyl-glycogen, with a methoxyl content of 45.5%, contains two free hydroxyl groups in each dodecasaccharide unit so that it seems possible that these unmethylated hydroxyl groups are concerned with the aggregating forces. Bell has, however, introduced nitro- and dichloroacetyl groups into the free hydroxyl groups of apparently fully methylated glycogen and found that the properties of the compound were not affected in any obvious manner, but the criteria which he used were not such as would easily indicate a change of aggregation. If these fully substituted compounds were shown to possess a much higher degree of dispersion, this would be strong evidence that the two non-methylated hydroxy-groups in ordinary methyl-glycogen are responsible for the aggregation, and a plausible hypothesis would be that they induce aggregation by engaging in co-ordinated oxygen bridges both in methylated and in ordinary glycogen. This would explain why the degree of aggregation of methyl-glycogen is not significantly affected by a change of solvent. Osmotic pressure determinations with solutions of methyl-glycogen in mixed solvents should be made, however, since greater dispersion is sometimes obtained in such solutions than with solutions in pure solvents.

The values for the "molecular weight" given in this paper are, of course, only mean values and we have no evidence whether glycogen in solution is

mono-disperse or not. Lamm [1934], using the ultracentrifuge, showed that certain preparations of starch were polydisperse; whether glycogen exhibits a similar behaviour can be shown only by a similar means.

SUMMARY.

1. Solutions of rabbit liver glycogen, rabbit muscle and methylated rabbit liver glycogen in $N/10$ CaCl_2 all give osmotic pressures indicating a mean particle weight of the order of 2×10^6 .
2. A solution of methyl-glycogen in benzene gave an osmotic pressure indicating a larger particle size but of the same order of magnitude (3.4×10^6).
3. The significance of these results is discussed.

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