316. *Ultracentrifugal and Diffusion Characteristics of Methylated Glycogen.*

By Basil R. Record.

Ultracentrifuge and diffusion studies have been made on methylated glycogen from five different sources. The average molecular weights calculated from these data range from 1 to $4{\cdot}4 \times 10^6$, in approximate agreement with osmotic-pressure estimates. All the specimens show considerable polydispersity, there being no particular molecular-weight species but a continuous range distributed more or less symmetrically about a mean value. No significant difference was observed between the methylated liver glycogen obtained from different sources or between methylated muscle and liver glycogen. The frictional ratio of 1·7—2·0 points either to appreciable hydration of the particles or to particles of a somewhat elongated form, or to a combination of both these effects.

Osmotic-pressure measurements on methylated and acetylated polysaccharides (Carter and Record, *J. Soc. Chem. Ind.*, 1936, **55**, 218; *J.*, 1939, 660) have shown that in general their particle weights are many times greater than the weights of the basal chain units as determined by Haworth's end-group assay methods. Glycogen, both as the polysaccharide, and in the form of its methylated and acetylated derivatives (Oakley and Young, *Biochem. J.*, 1936, **30**, 868; Carter and Record, *loc. cit.*) appears to be outstanding in this respect, the estimated particle weights being upwards of 250 times the minimum molecular weights deduced from the end-group assay.

The methods developed by Svedberg based on sedimentation and diffusion measurements, besides providing an independent means of determining particle weights, contribute information on the degree of polydispersity, the presence of any well-defined molecular entities, and the extent to which the particles deviate from the spherical form.

The present work, which was carried out in Uppsala in 1938, is concerned with the application of such methods to the examination of methylated glycogen from a variety of sources.

Materials Examined.—Specimens of methylated glycogen prepared from the liver of the rabbit, hake, haddock, and dog-fish, and also a specimen from dog-fish muscle were available for investigation. The specimen of methylated rabbit-liver glycogen was prepared by Haworth, Hirst, and Isherwood (*J.*, 1937, 577). The Haworth end-group assay pointed to basal chain units of 18 glucose units. The remaining specimens were produced in a similar manner by Haworth, Hirst, and Smith (*J.*, 1939, 1914); they had similar methoxyl contents and consisted of " minimum molecules " of 12 glucose units. The specimens from rabbit liver and the livers of dog-fish, hake, and haddock have already been examined osmometrically (Carter and Record, *loc. cit.*).

The specimens were stored in a vacuum over phosphoric oxide.

Methods and Results.—The technique developed for the determination of sedimentation constants and molecular weights has been described in numerous publications by Svedberg (see, *e.g.*, *Ind. Eng. Chem.*, 1938, **10**, 113), and is dealt with in detail by Svedberg and Pedersen, (" The Ultracentrifuge ", Clarendon Press, 1940).

Concentration Gradients.—Throughout the present investigation these have been determined as refractive-index gradients by the method of Lamm (*Z. physikal. Chem.*, 1928, *A*, **138**, 313; 1929, *A*, **143**, 177; *Nova Acta Soc. Sci.*, 1937, Ser IV., **10**, No. 6) in which a scale is photographed through the solution. The deviation, Z, of the scale lines from their normal position on a reference scale is related to the refractive index gradient dn/dx by :

$$Z = G.\ a.\ b.\ dn/dx$$

where G = camera magnification of the scale; a = cell thickness;
b = scale distance from cell.

A mercury-vapour lamp was used as a light source and the scale photographs were recorded on ordinary process plates in conjunction with a suitable light filter, so that all measurements refer to a wave length $\lambda = 436$ mμ.

Sedimentation Velocity.—Sedimentation constants were determined in the oil-turbine centrifuge as described by Svedberg and Pedersen (*op. cit.*). The solutions were made up in 0·2M-sodium chloride to approximately 1% the day previously. The runs were made at a rotor speed of 25,000 r.p.m. (centrifugal force = 30,000 × gravity). The sedimentation diagrams, *i.e.*, concentration gradient plotted against x, the distance from the centre of rotation, showed only a single sedimenting boundary in each case. A considerable broadening of the boundary as the run progressed points to a high degree of polydispersity, since in view of the low diffusion constants (see below), almost all the spreading observed must be attributed to polydispersity in the specimens. Fig. 1 shows a sedimentation diagram typical of all the specimens examined.

Sedimentation Constants.—These were calculated from the rate of movement of the maxima of the concentration gradient curves, and corrected for the density and viscosity of the solvent to sedimentation in water at 20°.

The sedimentation constant $s_{20} = \frac{dx}{dt} \cdot \frac{1}{\omega^2 x} \cdot \frac{\eta}{\eta_0} \cdot \frac{1 - V\rho_0}{1 - V\rho}$ where $\frac{dx}{dt}$ = the sedimentation velocity observed, ω = the angular velocity, V = partial specific volume of the solute, η and ρ are the viscosity and density of the solution, η_0 and ρ_0 those of water at 20°.

The values obtained for the five specimens examined are given in Table I.

Diffusion.—In order to calculate molecular weight from sedimentation-velocity determinations, it is necessary to have an independent measurement of the diffusion constant. For this purpose a micro-diffusion cell of the Lamm type was used, with plane parallel windows and a bakelite slide for forming the boundary. Exposures were taken at suitable intervals after forming the boundary between solution and solvent, and a reference scale was obtained at the end of each experiment after mixing the contents of the cell.

In the case of substances with marked heterogeneity, the calculation of the diffusion constant at different points on the concentration-gradient curve gives a set of values which increase with distance from the original boundary. Therefore, in the present work the standard deviation,

σ, was calculated for the whole curve from the first and second moments about the centroidal vertical (Lamm and Polson, *Biochem. J.*, 1936, **30**, 528). The diffusion constant is given by

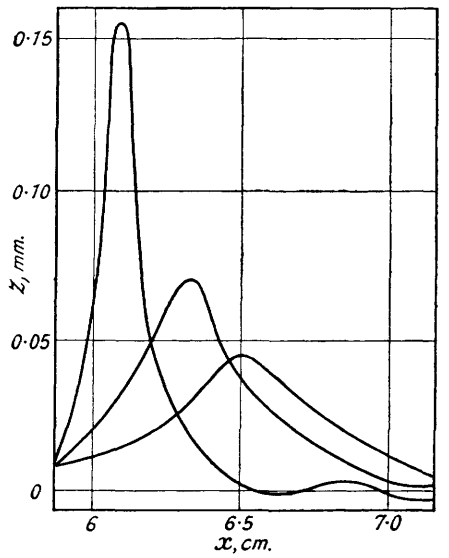
$$D = \frac{\left(\frac{1}{G}\right)^2 \sigma^2}{2t} \cdot \frac{(l - b)^2}{l^2}$$

where G = photographic enlargement factor of scale; t = time from formation of boundary; l = optical distance from scale to camera objective; b = optical distance from scale to centre of diffusion cell.

The solutions were made up to approximately 0·5% in 0·2M-sodium chloride and allowed to diffuse against 0·2M-sodium chloride. All measurements were made at 20·0° and corrected for the viscosity of the salt solution to diffusion in pure water. Fig. 2 shows a concentration gradient typical for the specimen examined.

FIG. 1.

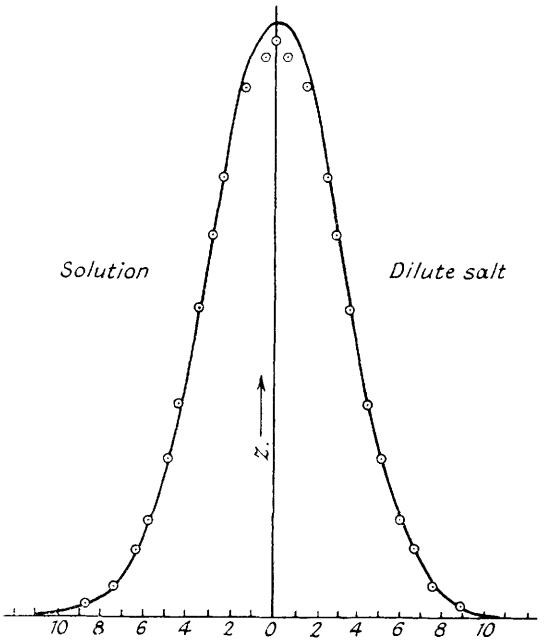
Sedimentation diagram of methylated haddock-liver glycogen (B).



c = 1·02%: *average speed* = 24,900 *r.p.m.*: *cell thickness* = 12 *mm.*: *scale distance* = 2·00 *cm.*: *interval between exposures* = 20 *minutes.*

FIG. 2.

Diffusion of methylated haddock-liver glycogen (B).



c = 0·99% *in* 0·2M-*sodium chloride. Exposure taken* 105½ *hours after forming boundary. Abscissæ represent vertical distances in the cell; ordinates are refractive-index gradients. The points marked refer to an ideal distribution curve having the same* σ *value as the experimental curve.*

In every case the curves were very nearly symmetrical, suggesting that the diffusion is independent of concentration in the range examined. This is in contrast to the abnormal behaviour found in the case of certain methyl and acetyl celluloses (Polson, *Kolloid Z.*, 1938, **83**, 172). The divergence from the ideal distribution curve having the same standard deviation is due to the polydisperse nature of the specimens. The apparent decrease in the diffusion constant with time (see Table I) may also be attributed to the presence of smaller more rapidly diffusing particles.

The Molecular Weight—The molecular weight of a substance can be calculated from its sedimentation and diffusion constants by means of the Svedberg formula (*Kolloid Z.*, Zsigmondy Festschrift, 1925, 37)

$$M = \frac{\boldsymbol{R}Ts}{D(1 - V\rho)}$$

where $\boldsymbol{R}$ = gas constant, T = absolute temperature, V = the partial specific volume of the solute, and ρ = the density of the solution.

The partial specific volume, V, was determined pyknometrically at 20° for an aqueous, electrolyte-free solution of methylated rabbit-liver glycogen which had been stored in an evacuated desiccator over phosphoric oxide for some months. The values 0·750 and 0·751 were obtained for a 2·12% and 1·05% solution respectively. The value 0·750 was assumed for the other specimens.

The molecular weights thus calculated for the five different specimens vary from $1{\cdot}0 \times 10^6$ to $3{\cdot}6 \times 10^6$ (see Table I).

TABLE I.

Methylated Glycogen. Collected Data.

Specimen.	$s_{20} \times 10^{13}$.	$D_{20} \times 10^7$. Time, hours.	D_{20}.	Mean.	$M_S \times 10^{-6}$.	f/f_0.	$M_E \times 10^{-6}$.
Rabbit liver	25·9	48	1·39	1·33	1·90	1·95	2·57
		72	1·27				
Dog-fish liver	41·2	55¾	1·14	1·12	3·58	1·87	4·40
		74¾	1·10				
Hake fish liver	20·7	59¼	1·78	1·78	1·13	1·72	2·51
		36	1·86				
		49	1·67				
Haddock liver (*A*)	15·7	59	1·61	1·57	0·97	2·05	1·71
		85½	1·52				
Dog-fish muscle	34·6	48	1·47	1·35	2·50	1·75	—
		72	1·33				
		95	1·26				

The Frictional Ratio.—The molar frictional constant of a substance may be calculated from its sedimentation constant and molecular weight to be

$$f = \frac{M(1 - V\rho)}{s}$$

(Svedberg and Sjogren, *J. Amer. Chem. Soc.*, 1929, **51**, 3594). If particles of the same molecular weight and partial specific volume were spherical, then, from Stokes's formula

$$f_0 = 6\pi\eta\boldsymbol{N}\left(\frac{3MV}{4\pi\boldsymbol{N}}\right)^{1/3}$$

where η is the viscosity of the solvent and $\boldsymbol{N}$ the Avogadro constant. Thus, if the particles of a substance are non-solvated and spherical, the ratio f/f_0 should be unity. A value exceeding unity may be due either to solvation of the particles, considered spherical, or to deviation from the spherical shape. The nearly constant values (1·7—2·0) obtained for the five methylated glycogen specimens examined (Table I) suggest a similarity in general structure and are probably due to the combined effect of hydration and molecular asymmetry.

The Effect of Concentration on Sedimentation and Diffusion.—The molecular-weight data presented in Table I do not take into account the possibility of errors in the s and D values arising from deviations from the ideal solution laws. The s values quoted in Table I are those obtained for 1% solutions. 0·5% Solutions were used in the diffusion experiments, and there was no evidence, in the form of skewness in the distribution on either side of the boundary, of any concentration effect on diffusion in 0·5% solutions. The limited quantity of several of the specimens available precluded extended investigations of the effect of concentration in sedimentation and diffusion. Detailed measurements have, however, been made on an independent specimen of methylated haddock-liver glycogen (preparation *B*) which was available in larger quantity.

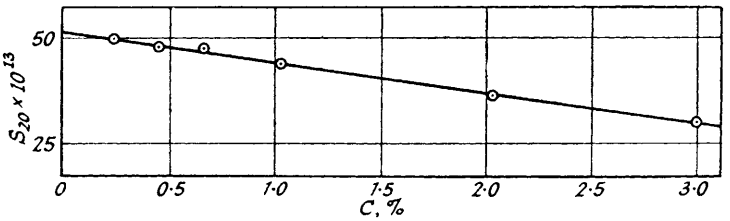
The variation in the sedimentation constant with concentration for this specimen in 0·2M-sodium chloride is shown in Fig. 3. A value obtained for a 2% solution in 0·5M-sodium chloride lies closely on the curve, showing that 0·2M-sodium chloride is sufficient to suppress any ionic effects. The value of s_{20} extrapolated to zero concentration is 51×10^{-13}. It is seen that the sedimentation constant decreases with increasing concentration, corresponding to an apparent decrease in the particle weight. A similar effect was observed in the osmotic pressure behaviour (Carter and Record, *loc. cit.*), the π/c values increasing with increasing concentration. The magnitude of the effect appears to depend on the extent to which

the particles deviate from the spherical shape. It is much greater in the polystyrenes (Signer and Gross, *Helv. Chim. Acta*, 1934, **17**, 59, 335, 726), various cellulose derivatives (see the recent comprehensive studies by Gralen, Dissertation, Uppsala 1944, and Jullander, *Arkiv Kemi, Min. Geol.*, 1945, **21***A*, No. 8), and the pneumococcus polysaccharides (this vol., p. 1561).

The effect of concentration on diffusion is shown in Table II. The diffusion constant for each exposure was calculated as before from the σ value obtained from the 1st and 2nd moments. The mean values obtained at three different concentrations show only variations inside the experimental error. The concentration-gradient curves were symmetrical in all cases (see, *e.g.*, Fig. 2). A fourth experiment in which a 1·17% solution was allowed to diffuse against one of 0·83% instead of 0·2M-sodium chloride gave a value in excellent agreement. In substances showing appreciable deviations from the van't Hoff law, asymmetrical curves are obtained, owing to the rate of diffusion on the solvent side of the boundary being different from that on the solution side. Such anomalies were observed in methylated cellulose (Polson, 1938; see also Graten, *loc. cit.*, and Jullander, *loc. cit.*) and the pneumococcus polysaccharides (Record and Stacey, *loc. cit.*).

FIG. 3.

Methylated haddock-liver glycogen (B) *: variation of sedimentation with concentration.*



The Error in calculating the Molecular Weight.—Taking a value of $1·03 \times 10^{-7}$ for D at zero concentration, we obtain for M_S a value of $4·82 \times 10^6$ and $f/f_0 = 1·84$. If the value of s_{20} for a 1% solution, *i.e.*, $43·0 \times 10^{-13}$, be taken instead of its extrapolated value, $M_S = 4·07 \times 10^6$ and $f/f_0 = 1·95$. Thus, owing to the variation in the sedimentation constant with concentration, the values of M_S given in Table I may be about 15% too low, and the true f/f_0 values will be slightly lower than those recorded.

TABLE II.

Effect of Concentration on Diffusion.

Methylated haddock-liver glycogen (*B*) in 0·2M-sodium chloride.

Concn., g./100 g. of solvent.	Time from start (hours).	$D_{20} \times 10^7$.	Mean $D \times 10^7$.
0·99	48	1·07	
	75	1·08	1·10
	105½	1·16	
0·517	48	1·05	
	72	0·99	1·03
	94	1·00	
0·192	48	1·06	
	70½	1·02	1·04
1·165 } 1·00	40	1·11	
0·830 }	68½	1·06	1·06
	88½	1·01	

Sedimentation Equilibrium Measurements.—The molecular weight of a solute can be calculated from the concentration distribution at equilibrium in a centrifugal field from the Svedberg formula :

$$M = \frac{2\boldsymbol{R}T \log c_2/c_1}{(1 - V\rho) \cdot \omega^2(x_2^2 - x_1^2)}$$

where c_2 and c_1 are the concentrations of the dissolved substance at the distances x_2 and x_1 from the axis of rotation, and ω is the angular velocity. The solutions must be sufficiently dilute to avoid anomalies due to deviation from the van't Hoff law. The sedimentation and diffusion measurements on methylated haddock-liver glycogen above indicate that such deviations are of such a magnitude as not to involve serious errors in concentrations below 1%. Four of the

methylated glycogen specimens in concentrations of 0·5—1·0% in 0·2M-sodium chloride have been examined in the Svedberg equilibrium centrifuge. Equilibrium was reached after 7—10 days' centrifuging. The concentration distribution curve was deduced from the refractive index gradient curve by the procedure described by Pedersen (*Biochem. J.*, 1936, **30**, 948), and the molecular weight calculated over 0·05 cm. intervals down the cell. The specific refractive increment used in converting dn/dx to dc/dx was calculated from the area of the diffusion curves (see below), and was found to be fairly constant for all the specimens. A mean value of $13{\cdot}2 \times 10^{-4}$ ($\lambda = 436$ mμ) was used. The details of a typical equilibrium run are given here to show the variation in molecular weight with distance from the axis of rotation (Table III). The final result is expressed as a weight average.

TABLE III.

Equilibrium run on methylated dog-fish liver glycogen.

Concentration at start	= 0·767 g./100 g. of 0·2M-sodium chloride.
Thickness of cell used	= 6·03 mm.
Bottom of cell	= 5·92 cm. from axis of rotation.
Meniscus of solution	= 5·55 cm. ,, ,, ,, ,,
Optical scale distance	= 7·20 cm.
Scale magnification, G	= 1·047
Temperature	= 20·0°
Average speed	= 22·5 revs. per second.
Shape of cell	= Sector.

Equilibrium was reached after 9 days' centrifuging.
Exposures used in the calculation were taken 210½, 219, and 234 hours after the start.
The following analysis was obtained :

Distance from axis of rotation (cm.).	Concentration gradient, dc/dx.	Concn., g./100 g. of salt solution.	$M \times 10^{-6}$.
5·55	0·650	0·368	1·59
5·60	1·036	0·410	2·25
5·65	1·450	0·472	2·72
5·70	1·967	0·557	3·10
5·75	2·801	0·675	3·61
5·80	4·284	0·849	4·35
5·85	6·968	1·125	5·29
5·90	—	—	—

The weight average $M = 4{\cdot}40 \times 10^6$.

The molecular-weight analysis thus obtained does not necessarily represent the molecular-weight distribution of the specimen, and is to be regarded only as an indication of the range of particle weights present. The smooth concentration gradients obtained in the sedimentation-velocity experiments, however, point to the absence of any definite molecular species and to the existence of a continuous range of particle sizes.

The weight average values for the four specimens examined are given in Table I (M_E). These values tend to be somewhat higher than those obtained from sedimentation and diffusion measurements. The discrepancy may arise partly from the error in extrapolating the limits of the refractive index gradient curve in the sedimentation equilibrium experiments. The appropriate value for the refractive index increment to be used in converting the refractive index gradient curve into concentrations throughout the cell to obtain values comparable with the sedimentation–diffusion results is also open to some doubt. Pedersen (*Biochem. J.*, 1936, **30**, 948) has suggested that this increment is not that obtained by direct measurement or from the area of the diffusion curves, but more correctly that determined from the area of the curve in a sedimentation-velocity run, which in the present investigation is some 20—30% lower in the different specimens, suggesting the presence of material of higher or lower molecular weight than shown by the curves. The diffusion constants, however, refer to an average value for the total dissolved material, so that perfect agreement between the M_S and M_E values cannot be expected. Considerations of the question as to what kind of average values are being determined in the sedimentation and diffusion experiments may throw further light on the problem. This subject is dealt with at length in the recent treatises by Gralen and Jullander (*locc. cit.*).

The Degree of Polydispersity.—In substances with a high sedimentation, a rapid spread of the sedimentation boundary during a run is a fairly certain indication of considerable polydispersity, since the true diffusion constant of high molecular weight substances is correspondingly small.

A comparison of the apparent diffusion constant determined from the sedimentation–concentration gradient curves with the true diffusion constant is a useful indication of the presence and extent of the polydispersity. The apparent diffusion constants, or boundary spreading coefficients (MacFarlane, *Biochem. J.*, **1935**, **29**, 407), have been calculated from the concentration gradients in sedimentation velocity runs on methylated haddock-liver glycogen (*B*) from the formula :

$$K = \frac{1}{4\pi(t_a - t_b)}\left[\frac{A_a^2}{Z_a^2} - \frac{A_b^2}{Z_b^2}\right]$$

where A_a, A_b are the areas and Z_a Z_b the maximum heights of the curves at times t_a, t_b. The values of K appear to increase as the run progresses. K also decreases with increasing concentration. Calculations from the first two exposures (20 minutes interval) of each run give values of K ranging from 30×10^{-7} for a 3% solution up to 90×10^{-7} for a 0·22% solution, in contrast to the value of $1{\cdot}0 \times 10^{-7}$ for simple diffusion. It is evident therefore that the specimens are very polydisperse. The polydispersity of aqueous solutions of glycogen extracted from rabbit liver was mentioned by Mystkowski (*Biochem. J.*, **1937**, **31**, 716), though in the absence of diffusion measurements there was no adequate evidence for this statement.

The Sedimentation Behaviour of Glycogen.—Sedimentation-velocity runs were carried out on two specimens of haddock-liver glycogen : (*a*) the crude polysaccharide once precipitated from water with alcohol; (*b*) the polysaccharide regenerated from glycogen acetate (see Haworth, Hirst, and Smith, *loc. cit.*). The solutions were made up to *ca.* 1% in 0·2M-sodium chloride. The concentration-gradient curves closely resembled those shown in Fig. 1, and the calculated sedimentation constants, 41·0 and 39·4 respectively, were of the same order. The partial specific volume of the crude glycogen specimen was 0·64, a value appreciably lower than that for methylated glycogen. However, the slower sedimentation consequent on the larger partial specific volume of the methylated polysaccharide is offset to some extent by the increase in the weight of the glycogen molecule due to the presence of methoxyl groups. This brief examination suggests that the average particle size of glycogen has a magnitude of the same order as its methylated derivative, and that acetylation and reconversion back to the polysaccharide has very little effect on the size of the physical units. This further substantiates the conclusions reached from the osmotic-pressure data (Carter and Record, *loc. cit.*) that the forces responsible for the aggregation of the basal chain units are of a more permanent nature than those occurring in ordinary physical aggregation, and lends support to the view expressed by Haworth, Hirst, and Smith (*loc. cit.*) that the union is effected by bonds of the primary valency type.

Discussion.—Sedimentation and diffusion measurements on methylated glycogen from five different sources point to average particle weights of $1{\cdot}0$—$4{\cdot}8 \times 10^6$. The slightly higher values obtained from sedimentation equilibrium measurements are in fair agreement when allowance is made for the errors introduced by the polydisperse nature of the material. The polydispersity has been demonstrated by comparing the rate of spread of the boundary during sedimentation with the spread resulting from diffusion only; spreading coefficients nearly 100 times larger than the diffusion constant are found for dilute solutions. This high degree of polydispersity appears to be characteristic of the polysaccharides, and is in marked contrast to the homogeneous nature of the proteins.

The above estimates of the average molecular weight of methylated glycogen are of the same order as those deduced from osmotic-pressure measurements, where values of $0{\cdot}3$—$0{\cdot}8 \times 10^6$ were obtained for the same specimens (Carter and Record, *loc. cit.*). The osmotic-pressure values are number averages and would therefore be expected to be of a lower order than those obtained from the centrifuge data for substances of such a polydisperse nature. Sedimentation runs on glycogen itself and on glycogen regenerated from the acetate provided sedimentation diagrams which are indistinguishable from those for the methylated derivative, suggesting that glycogen may be acetylated, methylated, and regenerated without any gross change in its particle size distribution. From osmotic-pressure measurements, Oakley and Young (*loc. cit.*) obtained values of $1{\cdot}1$—$2{\cdot}3 \times 10^6$ for rabbit-liver and muscle glycogen preparations which had been dialysed free from traces of impurities of low molecular weight; they were also able to show that methylation of the glycogen had very little effect on the mean particle weight, and that this was but little affected by the solvent, whether N/10-calcium chloride or benzene. Meyer and Jeanloz (*Helv. Chim. Acta*, 1943, **26**, 1784) have recorded a value as high as 6×10^6 for acetylated muscle glycogen from the mollusc, on the basis of osmotic-pressure measurements in benzyl alcohol. It seems probable that the variations in the particle weight of glycogen and its methylated and acetylated derivatives do not represent any intrinsic differences in the glycogen from different sources but are rather the result of differences in its extraction, treatment.

purification, etc. The large differences found between two specimens of methylated haddock-liver glycogen in the present work strongly suggest that this is the case.

The f/f_0 values (1·7—2·0) are substantially constant for all the specimens examined. If the departure from unity is the result solely of deviation from the spherical shape, it represents only a moderate degree of asymmetry; it is more likely, however, due to the combined effect of hydration and a relatively small degree of asymmetry. The relatively small effect of concentration on sedimentation and diffusion is in conformity with this view. Much higher asymmetry coefficients, together with large concentration effects, have been observed with cellulose derivatives and the pneumococcus polysaccharides.

The estimates of the particle weight of glycogen and its derivatives by various physico-chemical methods all point to physical units several hundred times greater than those deduced by the end-group assay method of Haworth. There is evidence that the chemical molecules are firmly linked together by primary valencies to form the large physical units. Considerations of shape lead one to conclude that the linkages do not take place only in an end-to-end direction, as in the long-chain molecules of cellulose, but also in breadth.

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