782. *Physicochemical Studies on Starches. Part XVI.* The Molecular Weight and Apparent Molecular-weight Distribution of Rabbit-liver Glycogen.*

By W. A. J. Bryce, C. T. Greenwood, and I. G. Jones.

The effects of extraction with hot alkali and cold trichloroacetic acid on the molecular weight of rabbit-liver glycogen have been examined. Molecular weights have been obtained by both sedimentation–diffusion and light-scattering measurements. Apparent distribution curves of sedimentation coefficients for various glycogen samples have been calculated. Glycogen extracted by cold trichloroacetic acid appears to be more representative of native glycogen than that isolated by hot alkali. Difficulties in the sub-fractionation of glycogen are discussed.

Our recent physicochemical studies [1] of various glycogen samples have indicated that polydispersity † is quite common, and that good agreement between the molecular weights derived from sedimentation–diffusion and light-scattering measurements is unusual. Some of the factors involved for rabbit-liver glycogen are described here, as a preliminary to use of the results as standards for the hydrodynamic behaviour of branched glucosans. The molecular weight of this glycogen from sedimentation measurements has been reported [1,2] to be about 6×10^6, but in recent light-scattering work by Stetten, Katzen, and Stetten [3] molecular weights of $11—80 \times 10^6$ were obtained when extraction was with cold trichloroacetic acid, whilst alkaline extraction gave products of molecular weights $2—6 \times 10^6$. However, determination of the molecular weight of glycogen by only one physicochemical method is inadequate.[1] In the present work, we isolated the glycogen by different methods and investigated the products by (1) sedimentation-velocity measurements, to give an apparent molecular-weight distribution, and (2) turbidimetric measurements, to give the weight-average molecular weight ($\overline{M}_w$).

For use of glycogen as a standard for hydrodynamic behaviour, fractions with a narrow molecular-weight distribution are preferable. The sub-fractionation of glycogen has therefore also been examined.

Experimental

Isolation.—Livers from freshly killed rabbits were minced and divided into two portions. The glycogen in one portion was isolated by the classical Pflüger method of extraction with 30% sodium hydroxide solution and subsequent reprecipitation with ethanol and 80% acetic acid.[1] Glycogen isolated by this method is termed *OH*-glycogen. {Typical analytical figures were: glucose, 99% (on hydrolysis and estimation of the reducing power with alkaline potassium ferricyanide [4]); $[\alpha]_D^{16}$ +194° (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 41%.} The other portion was extracted with trichloroacetic acid at 2° and the glycogen-product purified as described by Stetten, Katzen, and Stetten.[3] Glycogen isolated by this method is termed *TCA*-glycogen. {Typical analytical figures were: glucose, 98%; $[\alpha]_D^{16}$ +190 (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 45%.}

Sedimentation–Diffusion and Light-scattering.—These were carried out as described in Part XII of this series,[1] the solvent for the glycogen samples being 0·1M-sodium chloride.

Diffusion. These measurements were carried out in the Antweiler microelectrophoresis and diffusion apparatus at 20°. The concentration at a point *x* in a diffusion column is a function [5]

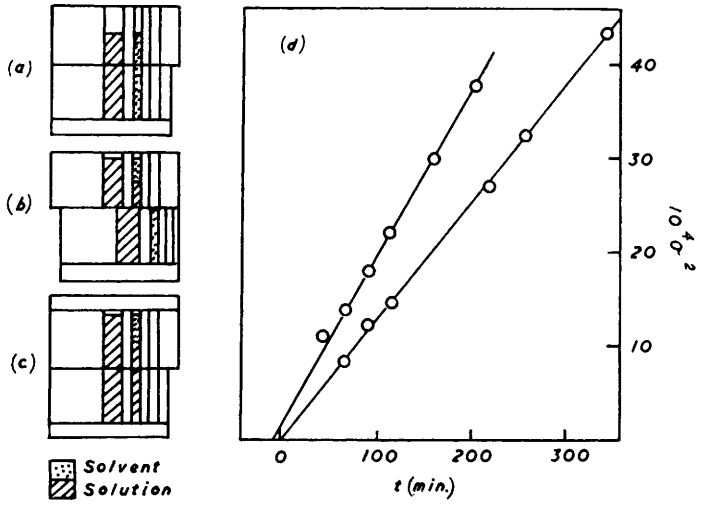
* Part XV, *J.*, 1958, 3558.

† The term "polydisperse" is used to describe a polymer system containing more than one component, whilst "polymolecular" denotes a chemically homogeneous polymer having a variation in molecular weight.

[1] Bryce, Greenwood, Jones, and Manners, *J.*, 1958, 711.
[2] Greenwood, *Adv. Carbohydrate Chem.*, 1952, **7**, 289; 1956, **11**, 335.
[3] Stetten, Katzen, and Stetten, *J. Biol. Chem.*, 1956, **222**, 587.
[4] Lampitt, Fuller, and Coton, *J. Sci. Food Agric.*, 1955, **6**, 656.
[5] Longsworth, *Ann. N.Y. Acad. Sci.*, 1945, **46**, 211.

of $x/\sqrt{t}$, and hence, if x is decreased by 10, the time required to reach a given concentration is reduced by 100. The duration of the experiment can therefore be considerably decreased if dn/dx can be measured at very small values of x. In practice, as there is usually an upper limit of dn/dx which can be measured, this can be achieved only by employing a cell with a short optical path, dn/dx for given values of x and t being then proportionately decreased. Hence, with a microcell a much shorter time is required for diffusion experiments. When solvent–solution boundaries are formed in the Antweiler all-glass diffusion cell by simply sliding one compartment over the other, the position of the initial boundary is obscured and readings of refractive index gradient at this point have to be interpolated throughout the measurements. This difficulty can be avoided by filling the cell as in Fig. 1*a*. The upper compartment is then moved to the position shown in Fig. 1*b*. Careful addition of more solution to the comparison compartment (by means of a micrometer syringe) will raise the

FIG. 1. (*a*), (*b*), *and* (*c*), *Antweiler diffusion cell* (*see text*). (*d*) *Typical graphs of* σ^2 *against t for* (1) OH-*glycogen* 1, (2) TCA-*glycogen* 1.



boundary from its interfacial position. The cell is then moved into position 1*c*, after removal of the residual solvent and its replacement by solution. In this manner, extremely sharp boundaries were formed. The refractive index gradient (dn/dx) in the liquid column was obtained either by arithmetical differentiation of the results from manually scanning the column with the Jamin interferometer, or by photography of the gradient obtained directly by the Schlieren optical-system attachment. For high concentrations of glycogen, the boundaries were too sharp to be measured satisfactorily by the interference method; in other respects the results from both methods of observation were the same.

Diffusion coefficients were evaluated by either the area–maximum ordinate method (D_a) or the second-moment method (D_m), where [6]

$$D_a = \left[\int_{-\infty}^{+\infty} y \,.\, dx\right]^2 \Big/ 4\pi t(y_{max.})^2 = A^2/4\pi t(y_{max.})^2$$

and

$$D_m = \left(\int_{-\infty}^{+\infty} x^2 y \,.\, dx\right) \Big/ \left(2t\int_{-\infty}^{+\infty} y \,.\, dx\right) = \sigma^2/2t$$

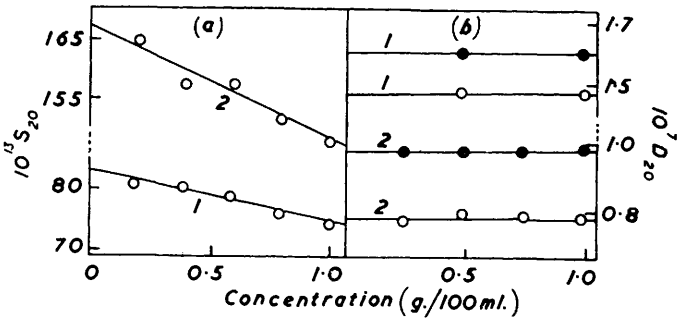
Here, x = the distance perpendicular to the boundary; $y = dn/dx$, the gradient of the refractive index; t = the time in seconds; σ^2 = the second moment of the curve; and A = the area. In all cases, the graph of σ^2 against t was linear and intercepted the t-axis at, or close (at a negative value) to, the origin (see Fig. 1). The time values used in calculations of diffusion coefficient were corrected for any apparent displacement of the zero time. Values are thought to be accurate to $\pm 3\%$ at any given concentration.

[6] Neurath, *Chem. Rev.*, 1942, **30**, 357.

Ultracentrifugation. These measurements were made with a Spinco ultracentrifuge. Runs were usually carried out at 20,000 r.p.m. and at concentrations of 2·5—8·0 g./l. A speed of 8000 r.p.m. was necessary in studying the apparent molecular-weight distribution of *TCA*-glycogen. Sedimentation coefficients (S_{20}) were evaluated from measurements of the movement of the mode of the sedimentation diagram [*i.e.*, $(dn/dx)_{max.}$] in the usual manner. S_{20} values therefore represent the sedimentation coefficient of the molecular species apparently present in the largest amount.

Light-scattering. High-speed centrifugation could not be used to clarify the solutions before turbidity measurements, as rapid sedimentation of very large particles occurred (see below). Clarification was again achieved by filtration of concentrated solution through sintered glass (G4) under gravity. (Millipore filters and G5 filters were not satisfactory and tended to remove polysaccharide from solution.) For each sample, measurements were made at 4 or 5 concentrations in the range 1—10 × 10^{-5} g./ml. obtained by successive addition of the concentrated filtered solution to optically clear solvent. (The concentration of the original filtered solution was obtained by hydrolysis and estimation of the liberated glucose with

FIG. 2. (*a*) *Plot of* S_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1.
(*b*) *Plot of* D_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1; ● D_m *values*; ○ D_a *values*.



alkaline ferricyanide.[4]) This procedure gave reproducible turbidities and dissymmetries. Molecular weights were calculated from the equation: $Hc/\tau = 1/M(P_{90°}) + 2Bc/\boldsymbol{R}T$, where $H = 32\pi^3n^2(dn/dc)^2/3\lambda^4\boldsymbol{N}$; $(P_{90°})$ = a particle scattering factor, which was calculated on the assumption that the molecules were spherical; [1] B = the solute–solvent interaction parameter; dn/dc = the refractive index increment, which was taken [1] as 0·146 (c in g./ml.) for glycogen in 0·1M-sodium chloride at 546 mμ. Within experimental error, Hc/τ and I_{45}/I_{135} were found to be independent of c for the range of concentrations examined. The term $2B(P_{90°})c/\boldsymbol{R}T$ was therefore negligible.

The partial specific volume ($\bar{V}$) of glycogen was taken [1] as 0·62.

Subfractionation.—This was attempted by cooling a 0·1% solution in 15% (v/v) aqueous ethanol, and by differential centrifugation as described by Stetten, Katzen, and Stetten.[3] Stepwise addition of ethanol to aqueous solutions at room temperature was also tried.

RESULTS AND DISCUSSION

Concentration Dependence of S_{20} *and* D_{20}.—Sedimentation coefficients for rabbit-liver glycogen have been determined previously, but early investigations [7,8] were limited to only one concentration, Bridgman [7] stating that the concentration-dependence of S_{20} was less than the experimental error. However, recently we have confirmed Larner, Ray, and Crandall's results [9] that the concentration-dependence is real.[1] For the range of concentrations studied, $S_{20} = (S_{20})_0(1 - k_sc)$. Representative data are shown in Fig. 2*a*. By the method of least squares, values of k_s of 0·12 and 0·11 for *OH*- and *TCA*-glycogen respectively were obtained.

[7] Bridgman, *J. Amer. Chem. Soc.*, 1942, **64**, 2349.
[8] Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **42**, 405.
[9] Larner, Ray, and Crandall, *J. Amer. Chem. Soc.*, 1956, **78**, 5890.

For diffusion coefficients also, early data [7,8] were restricted to one concentration. Bridgman's values [7] of $D_m = 1{\cdot}1 \times 10^{-7}$ for the majority of his samples were limiting values for time-dependent measurements. Ogston and his co-workers [8] gave values of $1{\cdot}27$—$1{\cdot}21 \times 10^{-7}$, whilst Larner and his co-workers' results [9] for samples of comparable

TABLE 1. *Molecular-weight data for* OH- *and* TCA-*glycogen.*

Method	Sedimentation–diffusion		Light-scattering			
Sample	$10^{13}(S_{20})_0$	$10^{-6}\,\overline{M}_{SD}$ [a]	$10^{-6}(\tau/Hc)$ [b]	I_{45}/I_{135} [b]	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$
OH-Glycogen 1	84	3·1	13·6	1·70	19·0	6·1
			4·0 *	1·18 *	4·5 *	1·5
,, 2	86	3·3	—	—	—	—
,, 3 ‡	94	3·9	6·9	1·20	7·8	2·0
,, 4	95	3·9	7·1	1·20	8·0	2·1
TCA-Glycogen 1	168	9·4	38·5	2·00	62 †	6·6
			40·5	1·94	63 †	
,, 2	173	9·8	90	2·40	160 †	16·0
			91	2·40	162 †	
,, 3	163	9·1	—	—	—	—

[a] Calc. from data in Fig. 4. [b] Values at infinite dilution; I_{45}/I_{135} = dissymmetry ratio.
* Values after centrifugation at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge. Preparative rotor A.) † Independent determinations. ‡ Values from ref. 1.

S_{20} were larger ($1{\cdot}3$—$1{\cdot}5 \times 10^{-7}$) and possessed a definite, but variable, concentration-dependence. Our experimental results (see Fig. 2*b*) suggest that the dependence is negligible. In all instances, symmetrical diffusion curves were obtained, again indicating negligible concentration-dependence.[6,10]

Comparison of OH- *and* TCA-*Glycogen with regard to Molecular Weight and its Distribution.*—The sedimentation coefficients in Table 1 indicate a large difference between *OH*- and *TCA*-glycogen. A quantitative estimate of this difference can be obtained only from the distribution of sedimentation coefficients $g(S)$, where: [7,11]

$$g(S) = (\mathrm{d}c/\mathrm{d}x)\omega^2 t x^3/c_0 x_0^2$$

where ω = angular velocity (radians/sec.), t = time (sec.) from the start of the sedimentation, x = distance (cm.) of a point in the boundary from the axis of rotation, x_0 = distance (cm.) of the meniscus from the axis of rotation, and c_0 = total concentration of the solution. An absolute distribution results only if diffusion is negligible and S is independent of c. Corrections for these effects can be made.[11,12] Here, Baldwin's method [11] has been employed to correct for diffusion and obtain apparent distributions $g'(S)$ (the calculations necessary for this distribution are detailed in Part XV). If $g'(S)$ is obtained at identical concentrations and the sedimentation behaviour of the samples is the same, the resultant curves should be comparable, although corrections for the Johnston–Ogston effect [11,13] and the concentration dependence [11] of S should ideally be applied. The $g'(S)$ curves shown in Fig. 3 emphasise the radical difference between *OH*- and *TCA*-glycogen (*e.g.*, only 8% of *OH*-glycogen 4 has $S > 150$, whilst *TCA*-glycogen 1 has 61% and *TCA*-glycogen 2 has 72%). Table 2 shows calculated values of the standard deviation, mean (or weight-average) sedimentation coefficient, and skewness. The standard deviation for *OH*-glycogen 4 calculated from Baldwin's most recent work,[14] taking into

[10] Jullander, *Arkiv Kemi Min. Geol.*, 1945, *A*, **21**, 1; Beckmann and Rosenberg, *Ann. N.Y. Acad. Sci.*, 1946, **46**, 229; Bevilacqua, Bevilacqua, Blender, and Williams, *ibid.*, p. 309.
[11] Baldwin, *J. Amer. Chem. Soc.*, 1954, **76**, 402.
[12] See, *e.g.*, refs. in article by Kinell and Rånby in "Advances in Colloid Science," Vol. III, Interscience Publ. Inc., New York, 1950; Baldwin and Williams, *J. Amer. Chem. Soc.*, 1950, **72**, 4325; Gosting, *ibid.*, 1952, **74**, 1548; Williams, Baldwin, Saunders, and Squire, *ibid.*, p. 1542; Baldwin, *J. Phys. Chem.*, 1954, **58**, 811; Williams and Saunders, *ibid.*, p. 854; Williams, Saunders, and Cicirelli, *ibid.*, p. 774; Eriksson, *Acta Chem. Scand.*, 1956, **10**, 378.
[13] Johnston and Ogston, *Trans. Faraday Soc.*, 1946, **42**, 789.
[14] Baldwin, *Biochem. J.*, 1957, **65**, 490.

account the concentration dependence of S, is the same (*i.e.*, $36S$) as that calculated from the $g'(S)$ curve at 8·0 g./l. Both *TCA*-glycogens have a large positive skew; that for *OH*-glycogen is relatively small. The ratio [15] D_m/D_a (Table 2) from diffusion measurements indicated the increased polymolecularity of *TCA*-glycogen.

FIG. 3. *Plot of $g'(S)$ against S for* (1) OH-*glycogen* 4, (2) TCA-*glycogen* 1, (3) TCA-*glycogen* 2[$g'(S) = 0$ *at* $S = 1400$; *the amount of this sample with* S *between* 600 *and* 1400 *is about* 25%].

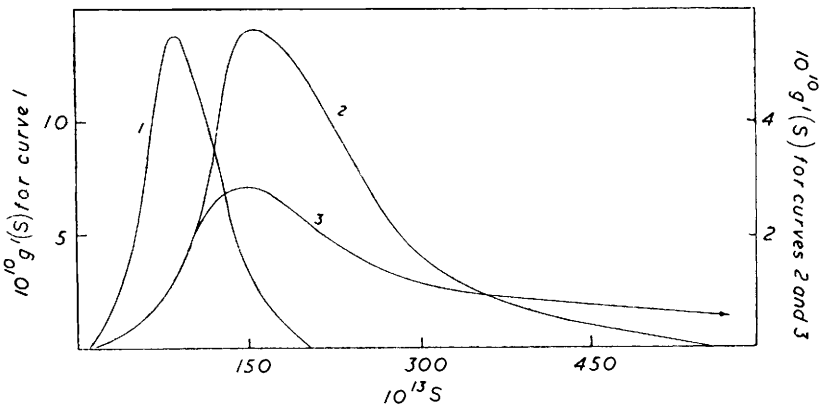
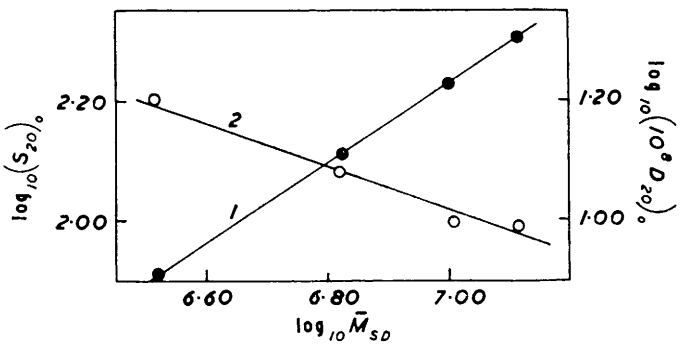


FIG. 4. *Plots of* (1) $\log_{10}(S_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$, *and* (2) $\log_{10}(D)_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$ *for glycogen samples.*



When molecular weights were calculated from S_{20} and D_m (to give $\overline{M}_{SD}$) for four samples, then $S_{20} \approx \overline{M}_{SD}^{0.63}$ (see Fig. 4). From Kuhn and Kuhn's results,[16] (1) $S \approx M^{\frac{1}{2}}$ for a matted coil, and (2) $S \approx M^{\frac{2}{3}}$ for a sphere, and hence glycogen may behave as essentially

TABLE 2. *Sedimentation coefficients and derived quantities from $g'(S)$ curves.*

Sample	$10^{13}S_{20}$ [a]	$10^{13}S_m$ [b]	σ [c]	Sk [d]	D_m/D_a	$10^{-6}\,\overline{M}_{SD}$ [e]	$10^{-6}\,M$ [f]
OH-Glycogen 4	87	95	34	+0·23	1·08	3·9	5·0
TCA-Glycogen 1 ...	150	220	99	+0·70	1·27	9·4	18
,, 2 ...	150	417	310	+0·87	—	9·8	45

[a] Sedimentation coefficient as measured from the mode. [b] Calc. mean sedimentation coefficient [c] Standard deviation. [d] Skewness = (mean − mode)/σ (see, *e.g.*, Yule and Kendall, " Introduction to the Theory of Statistics," Griffin, London, 1950. [e] Values from Table 1. [f] Molecular weight calc. from S_m by assuming $k_s = 0{\cdot}12$ and data in Fig. 4.

spherical particles. Similar conclusions can be drawn from the fact that $D_{20} \approx \overline{M}_{SD}^{0.37}$ (see Fig. 4), an exponent of 0·33 being expected for a sphere. It is realised these relations

[15] Gralén, Inaugural Diss., Uppsala, 1944.
[16] Kuhn and Kuhn, *Helv. Chim. Acta*, 1943, **26**, 1394.

are, at best, only approximate, as fractionated samples should have been used: this was not possible (see below). {The compact nature of the particles is confirmed by viscosity measurements. $[\eta]$ was 6·2 for *OH*-glycogen 1 and 7·1 for *TCA*-glycogen 1. (Units of concentration = g./ml.; solvent = 0·1M-sodium chloride; temperature = 22·5°.)}

Molecular weights from sedimentation–diffusion measurements on extremely polymolecular polymers are not simple averages, but depend [17] on the shape of the molecule and the methods of evaluating S and D, and hence no direct correlation is to be expected with the results of light-scattering measurements ($\overline{M}_\tau$ values) shown in Table 1.

$\overline{M}_\tau$ for *OH*-glycogen is much less than that for *TCA*-glycogen. Differences between $\overline{M}_\tau$ for *TCA*-glycogens 1 and 2 are explained by the differences in skewness of the $g'(S)$ curves. The ratio $\overline{M}_\tau/\overline{M}_{SD}$ appears to give a qualitative measure of skewness of the distribution and values are given in Table 1. Molecular weights calculated by using mean sedimentation coefficients from the $g'(S)$ curve are more comparable with $\overline{M}_\tau$ values (see Table 2).

Stability of TCA-*Glycogen.*—The effect of various reagents on the sedimentation behaviour of *TCA*-glycogen was examined to investigate whether physical aggregation was occurring. Ultrasonic experiments (which will be described in detail elsewhere) showed that under conditions which rapidly degrade amylopectin no appreciable change occurred in either S_{20} or the appearance of the leading edge of the sedimenting boundary. *TCA*-Glycogen also appeared to be stable to dilute acid and alkali at room temperature. After 72 hr., 0·5% solutions in 0·2M-potassium hydroxide and -acetic acid had the same S_{20} value as a control solution in 0·2M-sodium chloride, and there was no apparent change in the leading edge. When an aqueous solution (under air) was heated on a boiling-water bath, S_{20} was virtually the same after 1 hr. (158S ⟶ 151S), and even after 4 hours' heating, there was relatively little effect (131S). Changes in the leading edge were then apparent which did not appear to be reversible. Limited degradation or disaggregation must therefore have occurred.

The above experiments suggest that *TCA*-glycogen dissolves to form an essentially molecular dispersion. Further, $\overline{M}_\tau$ for the limit dextrin produced by the action of β-amylase had decreased by 50% compared with the 45% enzymic conversion into maltose. This is in agreement with Stetten, Katzen, and Stetten's results,[3] and suggests that aggregation was limited, as it appears unlikely that the extent of any aggregation, persisting after the 41% loss of weight on β-amylolysis, would be equivalent to that before treatment with enzyme.

However, when a 0·2% solution in 30% aqueous potassium hydroxide (under air) was heated on a boiling-water bath, S_{20} decreased rapidly and then remained constant

TABLE 3. *Sub-fractionation of* TCA-*glycogen.*

Sample:	1			2			
Method:	Cooling to 0° and centrifugation			Differential centrifugation			
Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$	Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$
45	10·3	66	6·4	38	—	250	—
30	7·0	55	7·9	42	12·7	90	7·1
20	8·6	50	5·8	16	8·6	13·3	1·5

* Calc. from $(S_{20})_0$ value and data in Fig. 4.

(168S ⟶ 100S in ½ hr. ⟶ 83S in 1 hr. ⟶ 86S in 4 hr.). The sedimentation diagram was then indistinguishable from that of *OH*-glycogen. A similar effect was found when heating was in a nitrogen atmosphere. *TCA*-Glycogen appears to be alkali-labile, but oxidative degradation appears to be insignificant. *OH*-Glycogen must be a degradation product relatively stable to alkali.

[17] Singer, *J. Polymer Sci.*, 1946, **1**, 445.

Effect of Isolation Procedure on Molecular Weight.—We support Stetten, Katzen, and Stetten's conclusions [3] that *TCA*-glycogen is more representative of native glycogen than *OH*-glycogen. Extraction with hot 30% potassium hydroxide solution causes obvious degradation, and molecular-weight values reported previously [1,2] for glycogens isolated from tissues by this method are undoubtedly those of degraded products. Since degradation may also have occurred during the isolation of *TCA*-glycogen,[3] the extremely high weight-average molecular weight of this material suggests that " native " glycogen may well *not be amenable to study by conventional physicochemical methods* (compare, for example, ref. 18). The difficulties involved in the study of the size of " native " glycogen are obviously very great. It should be noted, however, that for bacteria [19] and yeasts [20] *TCA*-glycogens are smaller than *OH*-glycogens, probably because the acid has only limited access and only material of low molecular weight is extracted without prior alkaline cytolysis.

The polydispersity of *OH*-glycogens apparent on sedimentation measurements varied; some were monodisperse, whilst others had both a large and a small component. Any large component could be removed by centrifugation, no significant amount (<5%) of material being lost (see sample 1, Table 1), and reprecipitation often removed the smaller component. In view of results with trichloroacetic acid, we regard polydispersity in *OH*-glycogen as due to an artefact. It is of interest that Bridgman [7] found evidence of components of low molecular weight in some of his samples.

Our previous results [1] indicated that S_{20} for *OH*-glycogen was comparable with that for glycogen isolated by boiling water. In view of the degradative effect of alkali, glycogen in the tissues which is accessible to the solvent action of hot water must be comparable in size with the degraded product.[21]

Sub-fractionation of TCA-*Glycogen.*—The results of our experiments are shown in Table 3. No significant fractionation occurred with successive addition of alcohol. The methods suggested by Stetten, Katzen, and Stetten [3] gave limited sub-fractionation, but changes in $\bar{M}_r$ are due almost entirely to changes in very large material. In no instance was there any real narrowing of the apparent molecular-weight distribution as shown by the sedimentation diagrams. Sub-fractionation of *TCA*-glycogen is obviously very difficult in view of the large molecular sizes involved.

[*Added, September 8th,* 1958.—Stetten *et al.*[22] have recently reached essentially the same conclusions as ours concerning *TCA*-glycogen.]

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THE UNIVERSITY, EDINBURGH, 9. [*Received, April 16th,* 1958.]

[18] Lazarow, *Arch. Biochem. Biophys.*, 1945, **7**, 337.
[19] Holme, Laurent, and Palmstierna, *Acta Chem. Scand.*, 1957, **11**, 757.
[20] Bryce and Greenwood, unpublished experiments.
[21] Cf. Orrell and Bueding, *J. Amer. Chem. Soc.*, 1958, **80**, 3800.
[22] Stetten, Katzen, and Stetten, *J. Biol. Chem.*, 1958, **232**, 475.