714. *Physicochemical Studies on Starches. Part XV.* The Action of β-Amylase on Glycogen as shown by Molecular-weight Distribution.*

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The changes in molecular-weight distribution occurring during the action of β-amylase on glycogen have been investigated by analysing the sedimentation diagrams obtained on ultracentrifugation of the original glycogen and two dextrins. The resultant distribution for the β-limit dextrin suggests that all the glycogen molecules in the sample, independently of molecular size, are hydrolysed to the same relative extent. Examination of an intermediate dextrin showed that during β-amylolysis, a mechanism involving degradation of all the polysaccharide molecules to the same extent appeared to be the most probable. The significance of these results is discussed.

Methods are available for converting sedimentation-velocity diagrams obtained on the ultracentrifugation of a polymer solution into molecular-weight distributions.[1-6] Little work of this type has been carried out on the components of starch. These studies are extremely valuable, however, as the pattern of action of any degradative agent can be followed *on a molecular basis*. As a preliminary to such studies on the starch components, we have analysed the changes in molecular-size distribution occurring during the action of β-amylase on glycogen in order to investigate the action-pattern of the enzyme and determine whether it has any degree of specificity with regard to the molecular size of the substrate. (This enzyme attacks the outer chains of glycogen and hydrolyses $45 \pm 5\%$ of the polysaccharide into maltose.) No previous work on this problem has been reported, although a similar study of phosphorylase action on glycogen has been made recently by Larner, Ray, and Crandall.[7] Glycogen has the advantage for this work that it possesses the most ideal sedimentation behaviour of all the starch-type materials; the concentration dependence of its sedimentation coefficient is small,[8] and this simplifies the calculations involved.

Our recent work [9] has shown the observed boundary gradient curve of glycogen to be very wide and dependent on the method of isolation. For an analysis of the distribution of sedimentation coefficients $g(S)$, a relatively narrow molecular fraction is preferable. This is not easily obtained from material of high molecular weight,[9] and hence a subfraction of alkali-extracted glycogen was used for these studies.

If the diffusion coefficient is negligible and the sedimentation coefficient (S) is independent of the concentration (c), the refractive-index gradient curve can be converted directly into a distribution of sedimentation coefficients $g(S)$ by the expression (cf. Bridgeman [1] and Baldwin [3]):

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

Here, ω = angular velocity (in radians/sec.); t = time (in sec.) from the start of the sedimentation; x = distance (in cm.) of a point in the boundary from the axis of rotation;

* Part XIV, *J.*, 1958, 2629.

[1] Signer and Gross, *Helv. Chim. Acta*, 1934, **17**, 726; Bridgeman, *J. Amer. Chem. Soc.*, 1942, **64**, 2349; see also references given by Kinell and Rånby in "Advances in Colloid Science," Vol. III, Interscience Publ. Inc., New York, 1950.

[2] Baldwin and Williams, *J. Amer. Chem. Soc.*, 1950, **72**, 4325; Gosting, *ibid.*, 1952, **74**, 1548; Williams, Baldwin, Saunders, and Squire, *ibid.*, p. 1542.

[3] Baldwin, *ibid.*, 1954, **76**, 402.

[4] Williams and Saunders, *J. Phys. Chem.*, 1954, **58**, 854.

[5] Williams, Saunders, and Cicirelli, *ibid.*, p. 774.

[6] Baldwin, *ibid.*, p. 1081; *Biochem. J.*, 1957, **65**, 490.

[7] Larner, Ray, and Crandall, *J. Amer. Chem. Soc.*, 1956, **78**, 5890.

[8] Bryce, Cowie, and Greenwood, *J. Polymer Sci.*, 1957, **25**, 251.

[9] Bryce, Greenwood, and Jones, *J.*, 1958, in the press.

x_0 = distance (in cm.) of the meniscus from the axis of rotation; and c_0 = total concentration of the solution. However, the above conditions are obeyed by few polymers, and $g(S)$ has normally to be corrected for three boundary effects, (1) the spreading with time due to diffusion, (2) the anomalous apparent concentration of any individual molecular species due to heterogeneity—the Johnston–Ogston effect,[10] and (3) the narrowing due to the concentration-dependence of S. One can correct for diffusion by extrapolating an " apparent distribution " $g^*(S)$ *versus* $1/xt$ to infinite time.[2] One can correct for the other two effects either by extrapolating curves of $g^*(S)$ to infinite dilution,[4,5,11] or by correcting the curve of $g^*(S)$ at a single concentration for the dependence of S on c. The latter method [3] was adopted here. In view of the complex series of manipulations involved, the method is given in outline below.

Experimental

Glycogen Sample.—Glycogen was isolated from rabbit livers by extraction with hot 30% aqueous sodium hydroxide, and was purified and characterised as described elsewhere.[9] Sedimentation measurements showed that the sample was polydisperse, containing, in addition to the main component, both small and very large material (cf. ref. 9). A subfraction was obtained as follows: a 1% solution of glycogen in 0·1M-sodium chloride was centrifuged at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge) to remove very large material as gel. Cold ethanol was then slowly added to the supernatant liquid at 0°, to give a faint yet stable precipitate (approx. 30% by volume of alcohol was required). The precipitated glycogen was then removed by centrifugation at 1500 r.p.m. for 20 min. at 0°; under these conditions, the component of low molecular weight remained in solution.

Enzyme Preparation and Digest Conditions (with W. Banks).—β-Amylase was isolated from soya-beans as described by Peat, Pirt, and Whelan.[12] It contained only an insignificant trace of maltase, and no Z-enzyme as shown by experiments on potato amylose fractions.[13] α-Amylase was also absent as shown by the molecular size of the β-limit dextrin of both amylose [14] and the glycogen (see below). The activity of the enzyme in Hobson, Whelan, and Peat's units [15] was *ca.* 20,000 units/ml.

Glycogen (1 mg./ml.) was incubated with β-amylase in the presence of 0·2M-acetate buffer of pH 4·6 at 35°. Although conversion was complete within about 2 hr., the digest was left for 24 hr. before the enzyme was deactivated by heating it on a boiling-water bath for a few moments, and the glycogen-product (Found: 41% conversion into maltose) precipitated from solution with ethanol. After centrifugation, the residual β-limit dextrin was washed with alcohol and dried with ether. A polymer-product at an intermediate stage of β-amylolysis was isolated similarly (Found: 13·2% conversion into maltose).

Concentrations of glycogen were determined by hydrolysis to glucose. The latter and the amount of maltose liberated on β-amylolysis were determined by alkaline ferricyanide.[16]

Physical Measurements.—Sedimentation measurements were made as described earlier.[9,17] The time representing the effective start of the sedimentation was obtained from the point where the curve of $\log_{10} X$ against t cut the time-axis at the value of $\log_{10} X$ for the meniscus.[17] The glycogen samples were dissolved in 0·1M-sodium chloride. Sedimentation runs for distribution analysis were carried out at 20,000 r.p.m., and a series of five photographs were taken at 6 min. intervals after the boundary had completely left the meniscus (the latter requiring about 10 min.). The concentration of glycogen was approx. 8 g./l. for these measurements.

Optical System of the Ultracentrifuge and the Measurement of the Photographic Plates.—The Spinco ultracentrifuge is equipped with a Philpot–Svensson [18] optical system, which gives the refractive index gradient curve directly. However, measurements of the height and area of the

[10] Johnston and Ogston, *Trans. Faraday Soc.*, 1946, **42**, 789.
[11] Gralén and Lagermalm, *J. Phys. Chem.*, 1952, **56**, 514.
[12] Peat, Pirt, and Whelan, *J.*, 1952, 705, 714.
[13] Cowie and Greenwood, *J.*, 1957, 4640.
[14] Cowie, Fleming, Greenwood, and Manners, *J.*, 1958, 697.
[15] Hobson, Whelan, and Peat, *J.*, 1950, 3566.
[16] Lampitt, Fuller, and Coton, *J. Sci. Food Agric.*, 1955, **6**, 656.
[17] Greenwood and Das Gupta, *J.*, 1958, 707.
[18] Philpot, *Nature*, 1938, **141**, 283; Svensson, *Kolloid Z.*, 1939, **87**, 181.

peak can be complicated by Fresnel fringes.[19] For this work, we have found an inclined bar to be more satisfactory than a wire.

When an appropriate base line has been fitted, the height (dn/dx) of at least 20 equi-spaced lamellæ at distances x_1, x_2, etc., throughout the refractive-index gradient curve has to be measured. These heights, when corrected for the angle of the inclined bar and the magnification factor of the optical system, give dn/dx values which are related to the total concentration (c) by the expression:

$$c = [\int (x/x_0)^2 \,.\, dn/dx \,.\, dx]/\Delta n$$

where x, x_0 have the values given above and Δn = specific refractive-index increment of the solute. (Trapezoidal integration is sufficiently accurate for this type of work.)

Our initial measurements were made directly from the photographic plates by using a two-dimensional travelling microscope (reading to 0·001 cm.) after the "vertical" traverse had been carefully aligned parallel to the meniscus. Heights of the two edges of the Schlieren pattern were measured. It was found most convenient to record these values and subsequent calculations directly on to a Remington–Rand printing calculator. Later measurements were made easier by printing an enlargement (9 ×) and tracing this on graph paper, corrections then being made for the additional magnification factor.

In both instances, base lines were fitted from the average of the two areas (A) under the peak and a knowledge of the concentration (c) of the solution, since

$$c = (\mathrm{A} \tan \theta / m_1 m_2 H_1 H_2 \Delta n)(x/x_0)^2$$

where θ = angle of the inclined bar in the optical system; m_1, m_2 = magnification of the cylindrical and camera lenses; H_1 = distance between the nodal point of the condensing lens and the inclined bar; H_2 = thickness of fluid column; Δn, x, x_0 are as defined above. The calculated and the actual concentrations agreed within experimental error.

Method of Determining the Molecular-weight Distribution.—(a) *The apparent distribution of sedimentation coefficients* $g^*(S)$. This function can be derived from the relations[20] $c = c_0(x_0/x)^2$ and $S = (1/\omega^2 t) \ln (x/x_0)$. It follows that $dc = dc_0(x_0/x)^2$ and $dS = (1/\omega^2 xt)dx$, and the combination of these equations gives $dc_0/dS = (dc/dx)(x/x_0)^2 \,.\, \omega^2 xt$. The curve of dc_0/dS *versus* S is not a conventional distribution since the area under it is not unity but c_0. Normalisation of the function therefore gives the apparent distribution of sedimentation coefficients $g^*(S)$ as:

$$g^*(S) = (dc_0/dS)c_0^{-1} = dc/dx \,.\, (x/x_0)^2 \,.\, \omega^2 xtc_0^{-1}$$

This function was calculated for each sedimentation diagram for about 20 incremental values of x (*i.e.*, x_i etc.) by taking the corresponding values of $(dn/dx)_{x_i}$ for $(dc/dx)_{x_i}$ and $\Delta x \,.\, \sum_{x=0}^{x=\infty} (dn/dx)$ for c_0; the proportionality factors disappear in the quotient $(dc/dx)/c_0$. Conversion of the values of x_i into the corresponding values of sedimentation constant S_i[by $S_i = (1/\omega^2 t) \ln (x_i/x_0)$ after correction for viscosity and temperature] then enabled the graph of $g^*(S)$ *versus* S to be plotted for the different times of sedimentation.

(b) *Elimination of the diffusion effect.* From the graphs of $g^*(S)$ *versus* S, values of $g^*(S)$ for discrete values of $10^{13}S_i$ (*i.e.*, 10, 20, 30, etc.) were taken and plotted as $g^*(S_i)$ *versus* $1/x_i t$. A graphical extrapolation was then made to $1/x_i t = 0$ to yield values of the apparent distribution corrected for diffusion effects [$g'(S)$]. In agreement with Larner, Ray, and Crandall's results,[7] the data were best represented by straight lines, and all extrapolations were made on this basis. Our results were similar to those shown in Fig. 1 of ref. 7. The graph of $g'(S)$ *versus* S was thus obtained.

(c) *Transformation of* $g'(S)$ *into* dc/dx. Before corrections can be applied for the Johnston–Ogston effect,[10] the function $g'(S)$ *versus* S has to be transformed into dc/dx *versus* x. The distribution equation can be re-written in this instance as:

$$dc/dx = g'(S) \,.\, x_0^2 c_0/x^3 \omega^2 t$$

where t is now chosen as the average time, *i.e.*, the time in the middle of the run.[7] Values of $(dc/dx)_{x_i}$ were therefore calculated from corresponding values of $g'(S)$. When values of S_i

[19] Cf. Kegeles and Gutler, *J. Amer. Chem. Soc.*, 1951, **73**, 3770.
[20] Cf. Svedburg and Pedersen, "The Ultracentrifuge," Oxford Univ. Press, 1940.

were converted into x_i by the expression $x_i = x_0 \exp(S_i\omega^2 t)$, the graph of dc/dx *versus* x was obtained.

(d) *Correction for heterogeneity.* In order to correct for heterogeneity, the distribution curve dc/dx *versus* x is divided into a number of equi-spaced lamellæ, and these are regarded as different components. Every molecular species i present in a given plane x_j changes in concentration at that plane, if its sedimentation coefficient S_i varies with the total concentration c_1. Baldwin [3] has shown that the change in concentration of the species (Δc_i) is related to the change in its sedimentation coefficient (ΔS_i) in terms of a parameter ($r/\omega^2 x$), where

$$r/\omega^2 x = \ln(x_j/x_0)/\omega^2 t = S_i + c_i(\Delta S_i/\Delta c_i)$$

whence

$$\Delta c_i = c_i \Delta S_i/\{[\ln(x_j/x_0)/\omega^2 t] - S_i\}$$

To carry out these calculations, about 20 values of dc/dx at a fixed increment, Δx, were tabulated against x, and the parameter $\ln(x/x_0)/\omega^2 t$ was calculated. The change in concentration of each of the components in each successive plane was then calculated in a stepwise manner by Baldwin's method.[3]

For the first lamella (x_1), only component 1 is present and therefore its concentration $c_1 = \Delta x \,.\, (dc/dx)_{x_1}$.

For the second lamella (x_2), the total concentration is $\Delta x \sum_{x=0}^{x=x_2} (dc/dx)$, which is an increase of $\Delta x(dc/dx)_x$. If $S = S_0(1 - kc)$ (see p. 3562), the sedimentation coefficient of component 1 in this lamella $(S_1)_{x_2}$ therefore decreases by an amount $(\Delta S_1)_{x_2}$ given by $-kS_{01}\Delta x(dc/dx)_{x_2}$ where $S_{01} = S_1/(1 - kc_1)$. From Baldwin's work,[3] the corresponding change in concentration $(\Delta c_1)_{x_2}$ is thus equal to:

$$(\Delta c_1)_{x_2} = c_1 \,.\, (\Delta S_1)_{x_2}/\{[\ln(x_2/x_0)\omega^2 t] - (S_1)_{x_2}\}$$

The true concentration of component 2 (c_{02}) is thus greater than $\Delta x(dc/dx)_{x_2}$ by $-(\Delta c_1)_{x_2}$.

This calculation is repeated for all the components (i) in each lamella until the corrected concentrations (c_{oi}) of each are known. Then since $c_{oi} = \Delta x(dc/dx)_{x_i}$, it follows that $(dc/dx) = c_{oi}/\Delta x$. Hence values of the corrected distribution function $g(S)$ were calculated from $g(S) = (c_{oi}/\Delta x) \,.\, (x/x_0)^2 \,.\, (\omega^2 x t/c_0)$, and the graph of $g(S)$ *versus* S obtained.

(c) *Correction for the concentration dependence of S; the extrapolation of g(S) to infinite dilution.* The distribution of sedimentation coefficients at infinite dilution $g(S_0)$ is derived from $g(S) \times (dS/dS_0)$, since $g(S_0) = c_0^{-1} \,.\, (dc_0/dS_0) = c_0^{-1} \,.\, (dc/dS) \,.\, (dS/dS_0)$. Here dS/dS_0 was obtained from tabular differentiation of S_{oi} and S_i values, S_{oi} being calculated from $S_i = S_{oi}(1 - kc_i)$ where $c_i = \Delta x \sum_{x=0}^{x=x_1} (dc/dx)$, as above. Values of $g(S_0)$ when plotted against the corresponding values of S_0 gave the true sedimentation coefficient distribution curve.

(f) *Calculation of the molecular-weight distribution curve.* Since the distribution of molecular weight $g(M)$ is given by:

$$g(M) = c_0^{-1}(dc_0/dM) = c_0^{-1}(dc_0/dS_0)(dS_0/dM)$$

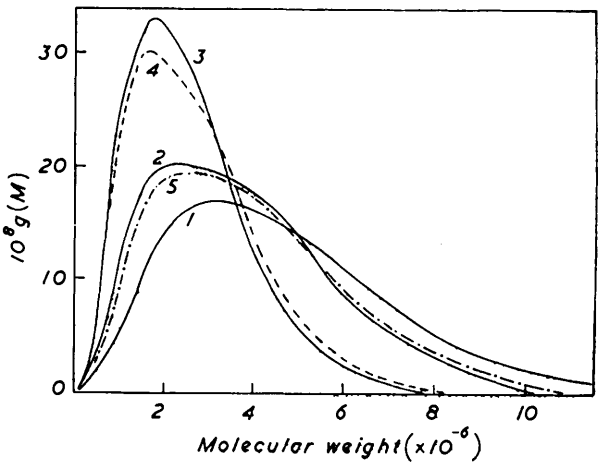
values can be calculated from $g(S_0) \times dS_0/dM$. The results described elsewhere [9] enabled the value of dS_0/dM to be obtained from differentiation of the relation (obtained by the method of least squares) between S_0 and M. This value was then utilised to calculate the curve for $g(M)$ against M.

Results and Discussion

The resultant molecular-weight distribution curves for the original glycogen (curve 1), the intermediate dextrin at 13% conversion into maltose (curve 2), and the β-limit dextrin (curve 3) are shown in the Figure. (It should be noted that these curves are weight- and not number-distributions.) Although the original glycogen was subfractionated, it still possessed a very wide distribution—from about 1×10^6 to about 13×10^6—illustrating the difficulties inherent in obtaining sharp fractions of glycogen.[9]

Molecular-weight distribution curves obtained by the above methods are not absolute unless ideal polymers which are molecularly homogeneous are available: the form of the $g(S)$–S curve depends entirely on the relation between S and c, and this can be influenced

by heterogeneity in molecularity. Also the transformation of the $g(S)$–S curve into the $g(M)$–M curve depends on $S = f(M)$, and this relation presents several difficulties experimentally when non-ideal samples are used: heterogeneous molecularity may influence the results, and there are often inaccuracies in measurements of the diffusion coefficients. [The method suggested by Williams and Saunders [4] for combining sedimentation-equilibrium and -velocity measurements appears to have many advantages in that diffusion measurements are avoided by using a double plot of integral distribution of M (from equilibrium measurements) and S (from velocity measurements).] For our calculations, we used the relation previously obtained [21] of $S_{20} = (S_{20})_0(1 - kc)$. Other results [9] have indicated that $dS/dM = (4{\cdot}79 \times 10^{-25})S^{-0{\cdot}59}$. The latter equation is necessarily not extremely accurate, but is as good as can be expected.[9] It would appear that an *absolute* molecular-weight distribution is not yet available by this method. However, the distributions obtained here are satisfactory for comparisons on a molecular basis of



Molecular-weight distribution curves.

1, *Original glycogen.*
2, *Intermediate dextrin.*
3, *β-Limit dextrin.*
4, *Theoretical curve for β-limit dextrin (see text).*
5, *Theoretical curve for intermediate dextrin (see text).*

various types of degradation processes. (Since Larner and his co-workers [7] used different equations, no direct comparison is possible between the shapes of his distribution curves and those reported here.)

Mode of Action of the β-Amylase.—Although an accurate estimate of the enzyme concentration in these digests was not possible,[22] conditions were such that the substrate : enzyme ratio was high. The action pattern of the enzyme was investigated by comparing theoretical distributions calculated from the original for various mechanisms with those experimentally determined for the intermediate and limit dextrin. This was achieved by dividing the distribution curve into about 20 lamellæ of discrete molecular weight, and regarding each of these as a homogeneous polymer. If the attack of the β-amylase is random with regard to molecular size, then the decrease in molecular weight during β-amylolysis will be proportional to the number of non-reducing terminal units, *i.e.*, to the molecular weight, and the molecular-weight distribution for the limit dextrin (M_{LD}) will be simply related to that of the original (M_O) by $M_{LD} = (100 - c)M_O/100$, where c = the percentage conversion into maltose (*i.e.*, 41%).

Curve 4 is the result of such a calculation. Comparison with the experimental curve (3) shows agreement within experimental error. It appears that it is essentially correct that all glycogen molecules are degraded to the same relative extent after β-amylolysis; there is no appreciable preferential and more extensive degradation of material of either low or high molecular weight.

[21] Bryce, Greenwood, Jones, and Manners, *J.*, 1958, 711.
[22] Banks and Greenwood, unpublished experiments.

For the intermediate dextrin, a theoretical curve was first calculated on the assumption that 32% of the molecules over the whole molecular-weight range were converted to the limit of 41% of maltose (*i.e.*, the percentage necessary to account for the observed limit of 13%), while the remainder were unchanged. However, although the maximum in the resultant distribution was correct, the high-molecular-weight leading edge was very much higher than the experimental curve, and the amount of material in the molecular-weight range of 3—6 $\times$ 10^6 was too low, the differences in each case being outside experimental error. This mechanism was therefore not compatible with the experimental results. However, when the theoretical curve (curve 5) was calculated by assuming a 13% conversion of all molecular species, good agreement was obtained with the experimental curve. This suggests that during β-amylolysis, all glycogen molecules are degraded to the same extent independently of molecular size, and the enzyme does not in fact degrade one molecule completely before attacking another. Rather it appears that action must be random with regard to individual external chains. This is in agreement with unpublished kinetic experiments carried out by Mr. W. Banks. First-order kinetics are virtually non-existent, being obeyed for only the first 10% of the total reaction, and thereafter there is a gradual decrease in rate. This suggests that as β-amylolysis progresses, it becomes increasingly more difficult to remove successive maltose units from any chain; such a mechanism implies that essentially all molecules will be degraded to the same extent throughout the reaction.

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