950. α-1 : 4-*Glucosans. Part VIII.** *Multiple-branching in Glycogen and Amylopectin.*

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The degree of multiple-branching in a glycogen or amylopectin can be evaluated from the chain lengths of the corresponding muscle-phosphorylase and β-amylase limit dextrins.

Fifteen samples of glycogen, from various biological sources, show small but significant differences in degree of multiple-branching. Amylopectins show a similar range of values. Accordingly, the marked physicochemical differences between glycogen and amylopectin cannot be related to differences in degree of multiple-branching.

A recent development in the chemistry of glycogen and amylopectin has been the recognition that multiple-branching is a characteristic structural feature. Multiple-branching was first postulated by Meyer,[1] and has been confirmed experimentally by Peat [2] and Cori [3] and their respective co-workers. We now describe a method for the quantitative estimation of the degree of multiple-branching in a branched α-1 : 4-glucosan. A preliminary account of part of this work has been published.[4]

The degree of multiple-branching may be conveniently expressed as the ratio ($\overline{A/B}$) of A-chains to B-chains. An A-chain (side-chain) is linked to the molecule only by the reducing group, whilst B-chains (main-chains) which are similarly linked, also have other chains attached to them.[2] In a glycogen-type molecule containing x chains, $\overline{A/B} = 1 : (x - 1)$ for a singly-branched " laminated " structure of the type originally suggested by Haworth, Hirst, and Isherwood,[5] whereas a multiply-branched " tree " structure as postulated by Meyer [1] contains approximately equal numbers of A- and B-chains.

For the proposed method of estimation of $\overline{A/B}$, a knowledge of the average chain length ($\overline{CL}$), muscle-phosphorolysis limit and β-amylolysis limit is required. From these, the difference (Δ) in $\overline{CL}$ values of the phosphorylase limit dextrin (ϕ-dextrin) and the β-amylase limit dextrin (β-dextrin) can be calculated. Normally, muscle phosphorylase removes 30—40% of glycogen as α-D-glucosyl phosphate, and β-amylase 40—50% as maltose, this degradation being limited to the exterior chains of the polysaccharide. The observed value Δ can be related to $\overline{A/B}$ as follows: in a β-dextrin, A-chain " stubs " contain 2 or 3 glucose residues,[2] whilst B-chain " stubs," which are probably of a similar

* Part VII, *J.*, 1957, 4430.

[1] Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875; Meyer and Fuld, *ibid.*, 1941, **24**, 375.
[2] Peat, Whelan, and Thomas, *J.*, 1952, 4546; *J.*, 1956, 3025.
[3] Larner, Illingworth, G. T. Cori, and C. F. Cori, *J. Biol. Chem.*, 1952, **199**, 641.
[4] Liddle and Manners, *Biochem. J.*, 1955, **61**, xii.
[5] Haworth, Hirst, and Isherwood, *J.*, 1937, 577.

length, are considered to contain n glucose residues. In a ϕ-dextrin, the A-chain " stubs " contain a single glucose residue, whereas the B-chain " stubs " contain 4 glucose residues more than those of the corresponding β-dextrin, *i.e.* $(4 + n)$.[6] In a branched α-1 : 4-glucosan with $\overline{A/B} = 1 : 1$, the average length of the exterior chains in the ϕ-dextrin is $[1 + (4 + n)]/2$, and in the β-dextrin is $(n + 2{\cdot}5)/2$, *i.e.* $\Delta = 1{\cdot}25$ glucose residues. [It will be noted that this calculation is independent of the length of the B-chain " stub " of a β-dextrin.] Similarly, when $\overline{A/B} = 1 : 2$, the exterior chain lengths of the ϕ- and β-dextrins are $[1 + 2(4 + n)]/3$ and $(2{\cdot}5 + 2n)/3$ so that $\Delta = 2{\cdot}17$. The values of Δ in the range $\overline{A/B} = 2 : 1 \longrightarrow 1 : 8$ have been calculated, and a graph of Δ against $\overline{A/B}$ prepared. The degree of multiple-branching can therefore be evaluated from experimental determinations of Δ.

In the present study, 15 samples of glycogen and 2 of amylopectin have been examined. Values of $\overline{CL}$ were determined by oxidation with potassium periodate,[7] and the β-amylolysis experiments are described in Part VI of this Series.[8] The polysaccharides were then incubated with rabbit-muscle phosphorylase [9] in presence of 0·1M-phosphate (pH 6·8) and 0·001M-adenylic acid (activator), and the percentage conversion into α-D-glucosyl phosphate determined. Control experiments showed that the enzyme was free from α-amylase and amylo-1 : 6-glucosidase (the " debranching " enzyme of rabbit muscle [6]), so that enzyme action must be confined to the outer chains of the polysaccharide. Although phosphorolysis was continued for 24 hr., enzyme action, with 90 ± 15 units * of phosphorylase per mg. of substrate, was complete within 1·5 hr.; moreover, the phosphorolysis limits were not altered by a four-fold increase in initial enzyme concentration, or by the addition of fresh enzyme after 4 hr. Since the enzyme was dissolved in a 0·03M-cysteine–1% glycerophosphate buffer, the amount of cysteine (traces of which are required for maximum solubilisation and activity of the enzyme [9]) could not be a limiting factor. Under these conditions, 14—36% of various glycogens and 40—41% of the amylopectin samples were converted into α-D-glucosyl phosphate. It will be noted that *Helix pomatia* II glycogen and rabbit liver I glycogen, both of which have low β-amylolysis limits,[8] have phosphorolysis limits of only 22 and 14%, respectively.

Comparable calculations based on phosphorolysis and β-amylolysis results published by G. T. Cori and her co-workers [3] show that rabbit liver glycogen, wheat amylopectin, and corn amylopectin have Δ values of 1·8, 2·6, and 2·7, respectively, equivalent to $\overline{A/B} = 1 : 1{\cdot}5$, $1 : 2{\cdot}9$, and $1 : 3{\cdot}2$.

It is concluded that different glycogens show small but significant differences in multiple-branching. The variation in $\overline{A/B}$ values is greater than that caused by a small experimental error in the analytical procedures. For example, if the phosphorolysis and β-amylolysis limits of rabbit liver I glycogen were 15 and 24%, respectively, then Δ would be 1·1, equivalent to $\overline{A/B} = 1 : 0{\cdot}9$; alternatively, if the limits were 13 and 26%, respectively, then the calculated $\overline{A/B}$ value is $1 : 1{\cdot}4$. Both of these $\overline{A/B}$ values are significantly different from those of the other rabbit liver glycogens.

Our previous studies [7,8] have already shown that glycogens differ in degree and position of branching, and it now seems clear that variations in degree of multiple-branching also exist. These properties do not appear to be related to the biological source of the glycogen. Furthermore, amylopectins show small variations in $\overline{A/B}$, over a similar range of values.

The above results are in agreement with other studies on multiple-branching. Calculations based on the yield of (*a*) maltose and maltotriose liberated by the action of

* For definition, see ref. 9.

[6] Cori and Larner, *J. Biol. Chem.*, 1951, **188**, 17.
[7] Bell and Manners, *J.*, 1952, 3641; Manners and Archibald, *J.*, 1957, 2205.
[8] Liddle and Manners, *J.*, 1957 3432.
[9] Green and G. T. Cori *J. Biol. Chem.*, 1943, **151**, 21; Illingworth and G. T. Cori, *Biochem. Preparations*, 1953, **3**, 1.

R-enzyme on amylopectin β-dextrin [2] and (*b*) glucose liberated from a glycogen or amylopectin *ϕ*-dextrin by amylo-1 : 6-glucosidase [10] indicate that all these polysaccharides contain a high proportion of A-chains, $\overline{A/B}$ ranging from *ca.* 1 : 1 to 1 : 3. The marked physicochemical differences [10] between glycogen and amylopectin (*e.g.* molecular shape, interaction with iodine and with concanavalin-A) are therefore not directly due to different degrees of multiple-branching, but arise from different conformations of A- and B-chains. This latter probably reflect differences in the site and mode of biosynthesis of the polysaccharides. In the animal cell, glycogen synthesis appears to be a three-dimensional polymerisation, whilst that of amylopectin must be sterically limited during the formation of mixed layers of amylose and amylopectin in the starch granule.

Multiple-branching in glycogen and amylopectin.

Polysaccharide	$\overline{CL}$	*ϕ*-limit (%)	$\overline{CL}$ of *ϕ*-dextrin	*β*-limit (%)	$\overline{CL}$ of *β*-dextrin	Δ	$\overline{A/B}$
Glycogens							
Rabbit liver I	13	14	11·2	25	9·8	1·4	1 : 1·1
,, III	13	31	9·0	51	6·4	2·6	1 : 2·9
,, V	14	32	9·5	51	6·9	2·6	1 : 2·9
,, XIII	15	30	10·5	46	8·1	2·4	1 : 2·4
Cat liver IV	13	36	8·3	53	6·1	2·2	1 : 2·0
,, VI	12	34	7·9	52	5·8	2·1	1 : 1·9
Fœtal sheep liver	13	29	9·2	49	6·6	2·6	1 : 2·9
Rabbit muscle I	13	25	9·8	45	7·2	2·6	1 : 2·9
Human muscle II	11	22	8·6	40	6·6	2·0	1 : 1·8
Mytilus edulis V	9	21	7·1	40	5·4	1·7	1 : 1·4
,, VI	13	28	9·4	46	7·0	2·4	1 : 2·4
Ascaris lumbricoides	12	31	8·3	49	6·1	2·2	1 : 2·0
Helix pomatia II	7	22	5·5	37	4·4	1·1	1 : 0·9
Tetrahymena pyriformis I	13	31	9·0	44	7·3	1·7	1 : 1·4
Brewer's yeast	13	30	9·1	44	7·3	1·8	1 : 1·5
Amylopectins							
Waxy maize starch	20	41	11·8	50	10·0	1·8	1 : 1·6
Waxy sorghum starch	22	40	13·2	52	10·6	2·6	1 : 2·9

Experimental

Analytical Methods.—Inorganic phosphate was estimated by Allen's colorimetric method,[11] except that a 10% solution of ammonium molybdate was used.

For the estimation of α-D-glucosyl phosphate in presence of 0·1M-inorganic phosphate a modification of Hanes's method [12] has been used. Aliquot parts of the enzyme digests (2 ml.) were diluted with distilled water (2 ml.), and magnesia solution [0·86% (w/v) magnesium chloride hexahydrate, 1·35% (w/v) ammonium chloride, 3·5% (v/v) aqueous ammonia; 10 ml.] was added, the mixture being warmed to *ca.* 50°. After about 45 min., the mixture was diluted to 25 ml. and the magnesium ammonium phosphate precipitate removed. An aliquot part of the filtrate (5 ml.) was hydrolysed with 11N-hydrochloric acid (0·65 ml.) for 7 min. at 98°, the solution cooled and diluted to 25 ml., and the inorganic phosphate content measured. A portion of the original filtrate, without acid hydrolysis, was also examined. Control experiments showed that glucose 6-phosphate was not hydrolysed under these conditions, and that adsorption of α-D-glucosyl phosphate on the magnesium ammonium phosphate precipitate did not occur. By contrast, if the inorganic phosphate is precipitated without dilution at room temperature, coprecipitation of the α-D-glucosyl phosphate occurs.

Phosphorylase.—Rabbit muscle phosphorylase was prepared by methods similar to those of Green and Cori, and Illingworth and Cori.[9] Several preparations were made; they varied in degree of crystallinity, and in the relative proportion of *a* and *b* forms. The phosphorylase was stored at 0° in 0·03M-cysteine hydrochloride–1% sodium β-glycerophosphate buffer.

We are indebted to Professor C. H. Waddington, F.R.S., and Dr. R. A. Beatty for the

[10] Manners, *Adv. Carbohydrate Chem.*, 1957, **12**, 261.
[11] Allen, *Biochem. J.*, 1940, **34**, 858.
[12] Hanes, *Proc. Roy. Soc.*, 1940, *B*, **128**, 426.

supply of rabbits, and to Mrs. R. M. Clayton who anæsthetised the rabbits; in some preparations, magnesium sulphate [13] was used in place of Nembutal.

Phosphorolysis of Polysaccharides.—Digests were prepared containing brewer's yeast glycogen [14] (40—50 mg.), 0·5M-phosphate buffer (pH 6·8; 4 ml.), 0·01M-adenylic acid (2 ml.), and phosphorylase solution (7500 units/ml.; 0·5 or 2·0 ml.) in a total volume of 20 ml. After incubation at 35°, portions (2 ml.) were removed for determination of α-D-glucosyl phosphate.

Time of incubation (hr.)	0·02	1·0	5·0	24·0
Phosphorolysis (%)				
(*a*) 80 units/mg. of glycogen	5	30	30	30
(*b*) 320 units/mg. of glycogen	19	31	31	29

In a subsequent experiment, glycogen (from *Helix pomatia*) on incubation with 110 units of phosphorylase/mg. gave 20% conversion into α-D-glucosyl phosphate within 1 hr. After 4 hr. the enzyme concentration was increased to 250 units/mg.; no further degradation occurred in the following 20 hr.

The phosphorolysis limits of the polysaccharides were determined, in duplicate, 75—105 units of phosphorylase/mg. of substrate being used. The α-D-glucosyl phosphate contents of samples of the digests examined after incubation for 1·5, 3, and 24 hr. were identical, or differed by only 1%.

Under the above conditions, normal mammalian glycogens had phosphorolysis limits of 29—36% and isolated *φ*-dextrins were resistant to further enzyme action. With less active enzyme preparations, the percentage conversions into α-D-glucosyl phosphate were 20—25%.[14]

The authors are grateful to Professor E. L. Hirst, F.R.S., for his interest, to the Rockefeller Foundation for a grant, and to the Department of Scientific and Industrial Research for a maintenance allowance (to A. M. L.).

Department of Chemistry, University of Edinburgh. [*Received, July 5th*, 1957.]

[13] Campbell, *Biochem. J.*, 1952, **52**, 444.
[14] Cf. Manners and Khin Maung, *J.*, 1955, 867.