STRUCTURE OF GLYCOGENS AND AMYLOPECTINS

I. ENZYMATIC DETERMINATION OF CHAIN LENGTH*

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Glycogen and amylopectin from a variety of natural sources have been subjected to a structural analysis by means of enzymatic end-group determination and determination of the average number of glucose residues of inner and outer chains. The linear portions of these branched polysaccharides are made up of 1,4-linked glucose residues. Inner chains are linear portions situated between two branch points (glucose units in 1,6 linkage), while outer chains have a branch point at one, and the non-reducing end-group at the other end. Enzymatic degradation by phosphorylase begins at the non-reducing end; in the presence of inorganic phosphate glucose units in 1,4 linkage are removed one at a time as glucose-1-phosphate until the enzyme approaches the 1,6 linkage at branch points, when its action is stopped. Phosphorylase can neither break nor by-pass this linkage. A second enzyme, amylo-1,6-glucosidase (1), splits off the glucose residues in 1,6 linkage by hydrolytic action, permitting phosphorylase to continue its action, until by a repetition of the process the entire molecule is digested. The detailed structural requirements for the activity of these two enzymes (phosphorylase and amylo-1,6-glucosidase) have been previously investigated and reported (1) (see also Paper II, this series).

The degree of branching (number of α-1,6 linkages in relation to number of α-1,4 linkages) can now be determined by three independent methods; a methylation technique, periodate oxidation, and an enzymatic method. In the two chemical methods, the non-reducing terminal glucose units of the outer branches yield 2,3,4,6-tetramethylglucose in the methylation procedure and formic acid in the periodate oxidation method. Hence, the per cent of such end-groups can be determined, and it can be shown that, independent of what structure is assumed, the number of end-groups is related to the number of branch points as $n$ is to $n-1$. In the enzymatic method, it is not the terminal non-reducing glucose residues but the glucose residues in α-1,6 linkage which are uniquely

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determined; they are split off as free glucose by amylo-1,6-glucosidase, while all other glucose units are split off as glucose-1-phosphate through the action of phosphorylase.

The values obtained by the three methods of end-group determination will be shown to be in general agreement. The average number of glucose residues of outer and inner branches has been found to be a characteristic property that distinguishes various polysaccharides of the glycogen-amylopectin class.

EXPERIMENTAL

The enzymatic end-group determination was carried out as described previously (1). Considerable experience with this method has shown that it gives reproducible results and that the accuracy compares favorably with that obtainable by the chemical methods. Only 10 to 15 mg. of polysaccharide are needed for a determination.

The length of the outer branches of the polysaccharides was determined by exhaustive digestion with rabbit muscle phosphorylase (recrystallized eight to nine times) in the presence of an excess of inorganic phosphate and measurement of the amount of glucose-1-phosphate formed. As a rule, enough phosphorylase (about 0.4 mg.) was added to 3 to 4 mg. of polysaccharide to reach the end-point in 1 to 2 hours. The mixture was then incubated for another hour to ascertain that no further reaction had taken place. After deproteinization with 2.5 per cent HgCl₂ in 0.5 N HCl, the mercury- and polysaccharide-free acid filtrates were heated for 5 minutes at 100° in order to hydrolyze glucose-1-phosphate. After neutralization, the reducing power was determined by the method of Nelson (2). A blank determination was carried out on the digestion mixture before incubation.

For the determination of the outer chain length, as well as for the stepwise degradation of polysaccharides, it is essential that the phosphorylase be entirely free of glucosidase. Each batch of phosphorylase was tested as follows: From 3 to 4 mg. of the phosphorylase limit dextrin of glycogen were incubated with inorganic phosphate and about 1 mg. of phosphorylase for 4 hours at 30°; when glucosidase is absent, no glucose-1-phosphate is formed. The test is a sensitive one, since removal by glucosidase of only a few glucose residues in 1,6 linkage opens the way for considerable phosphorylase activity.

Isolation of Glycogen—The method of isolation and purification of glycogen merits some discussion. It has been repeatedly shown that glycogen is resistant to digestion with strong alkali. On ultracentrifugation no significant difference could be detected in the distribution of particle

1 As mentioned previously (1), Hg is removed by H₂S and the polysaccharides by adsorption on the HgS precipitate. The filtrates contained about 0.3 N HCl.
weights between samples of glycogen obtained with or without treatment with hot strong alkali (3, 4). When the glycogen content of the liver is high, it is possible to isolate by digestion with alkali and precipitations with ethanol, as described below, highly purified glycogen in a yield of up to 95 per cent of the analytically determined content of the alkali digest. Such glycogen samples will therefore be representative of the distribution of molecular sizes originally present. This is of importance in ultracentrifugation studies, which will be reported in a separate paper.

It is not possible to give a description of a method of isolation of glycogen which is generally applicable, because a number of variables, such as fat content, type of tissue, and glycogen content, require slight modifications of the procedure in order to obtain a highly purified product.

The method of isolation used in this laboratory for many years follows the outline given by Somogyi (5). The tissue is digested in a covered flask in 30 per cent NaOH (1.5 to 2 volumes per weight of tissue) for 3 hours in a boiling water bath. On cooling, a solid layer or cake of soap forms on the surface (in the case of most livers) from which the gelatinous fluid below can be separated before it too congeals. If a clear separation from the soap is not achieved, the fluid is filtered through glass wool. In digests of human muscle the soaps did not congeal and were removed in a separatory funnel. Removal of the soaps greatly facilitates subsequent purification, and, although they occlude some glycogen, they are discarded. The next steps, precipitation of glycogen with ethanol at a concentration of 33 per cent and washing of the precipitate, are those described by Somogyi. A marked purification is obtained in the step in which impurities are removed by the addition of about 0.5 volume of ethanol after adjusting the glycogen solution with HCl to pH 4 to 5 (Congo paper showing a brownish blue color). A brownish or yellow precipitate forms over the course of some hours, which is removed by centrifugation. This precipitate includes some glycogen and is reworked by dissolving and precipitating again with about 0.5 volume of ethanol at slightly acid reaction. To the combined supernatant fluids more ethanol is added to make the concentration 45 to 50 per cent. If the precipitate is not pure white, the whole procedure, beginning with the acid step, is repeated. The final steps are those described by Somogyi for the preparation of salt- and P-free glycogen.

After being dried, the white powders are completely soluble in water and form a transparent 1 per cent solution with a bluish opalescence. Muscle glycogen is less opalescent than liver glycogen. A milky solution is obtained with incompletely purified samples. That turbidity of solution is a sign of impurity can be demonstrated by further fractionation, which leads to clarification without appreciable loss of glycogen.
The purity of the isolated products was determined as follows: The sample was dried to constant weight in vacuo at 100°. A weighed sample was (a) completely hydrolyzed in acid (4 hours in 1 N HCl at 100°) and determined as glucose by copper reduction, (b) completely digested enzymatically by means of amylase-1,6-glucosidase plus phosphorylase and determined as the sum of free and phosphorylated glucose by copper reduction. The determinations under (a) and (b) were generally within ±2 per cent of theory (based on the molecular weight of 162 for anhydroglucose units) for glycogen purified by the method outlined above.

**Glycogen Samples**—We are greatly indebted to the following colleagues. Dr. L. B. Flexner collected about 10 gm. each of livers of fetal, new-born, and young adult guinea pigs. These were shipped in 30 per cent NaOH and yielded on isolation in this laboratory 60, 208, and 330 mg. of glycogen, respectively. Dr. Max Schlamowitz supplied glycogen from the livers of two rabbits which had been given an intravenous infusion of glucose or fructose after a fasting period. Dog liver glycogen was supplied by Dr. W. Z. Hassid, the corn glycogens by Dr. T. J. Schoch. Finally, in 1946 we obtained a number of glycogen samples from the collection of Dr. D. J. Bell (see Tables I and II). These had been purified by precipitation with glacial acetic acid as used in the method of isolation of Bell and Young (6). Some of these samples could be digested enzymatically only to the extent of 60 to 70 per cent (see below).

**Determination of Degree of Branching**—In Table I are assembled values obtained by the three methods of end-group analysis. The reciprocal of the per cent end-group (× 100) is the average chain length, defined as the number of 1,4-linked glucose residues per 1,6-linked glucose residue. Thus, an average chain length of 15 means 14 glucose residues linked 1,4 and 1 residue linked 1,6.

The most valid comparisons are those in which the identical material was analyzed by two or more methods; such cases are identified in Table I by bold-faced type. If the samples analyzed by the three methods are not identical, differences in the end-group value may arise in the following manner.

The glycogen content of such organs as liver and muscle is subject to rapid turnover, and it seems that the composition of a particular glycogen sample depends on the previous history of the animal from which the tissue is taken. This was suggested by the finding of Bell (17) that the average chain length of rabbit liver glycogen may be either 12 or 18 glucose units under different conditions and was definitely indicated in recent experiments of Schlamowitz (18). Examples of the effect of the previous nutritional history on the degree of branching are given in Table II. Glycogen isolated from rabbit liver with low content (1 to 2 per cent) was more
highly branched than glycogen isolated from the livers of well-fed rabbits, average chain length 12 and 14.7, respectively. These differences are beyond the error of the enzymatic method. (Examples of the extent of agreement of duplicate determinations are given in Table I.) Similarly, the two samples of rabbit muscle glycogen in Table I (average chain length of 13 and 15, by the enzymatic method) deviate significantly from each other. Age may also play a rôle; there is a progressive increase in average chain length of glycogen isolated from liver of fetal, newborn, and young adult guinea pigs and from immature, mature, and over-mature sweet corn (Table II).

It might be said that with three different methods showing in many cases good agreement the problem of the degree of branching of the glycogen-

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Enzymatic end-group</th>
<th>Average chain length</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>enzymatic</td>
<td>enzymatic</td>
</tr>
<tr>
<td></td>
<td>per centi</td>
<td></td>
</tr>
<tr>
<td>Ascaris lumbricoides glycogen (Bell)</td>
<td>6.6</td>
<td>15</td>
</tr>
<tr>
<td>Dog liver glycogen (Hassid)</td>
<td>8.3</td>
<td>12*</td>
</tr>
<tr>
<td>Guinea pig liver glycogen</td>
<td>6.5</td>
<td>15†</td>
</tr>
<tr>
<td>Horse muscle glycogen (Bell)</td>
<td>6.9</td>
<td>15</td>
</tr>
<tr>
<td>Mytilus edulis glycogen (Bell)</td>
<td>6.7</td>
<td>11*</td>
</tr>
<tr>
<td>Potato amylopectin (Hassid)</td>
<td>4.6†</td>
<td>22</td>
</tr>
<tr>
<td>Rabbit liver glycogen</td>
<td>6.6</td>
<td>15†</td>
</tr>
<tr>
<td>Rabbit muscle glycogen (Bell)</td>
<td>7.8</td>
<td>13</td>
</tr>
</tbody>
</table>

* Personal communication from the donor of the preparation.
† Authors' sample.
‡ Values for other amylopectins (1) were sago 5.9, wheat 5.4, corn (two samples) 4.4 and 4.1.

The Potter and Hassid (12) periodate method gives values for average chain length which are higher than the enzymatically determined values (1), while results by the periodate method of Brown et al. (13) tend to deviate in the other direction.
CHAIN LENGTH OF GLYCOCEN

The range of the average chain length for glycogen with the enzymatic method is from 12 to 17 glucose units, while the range for different amylopectins (1) is from 17 to 24 glucose units. There is thus a continuous series of polysaccharides showing different degrees of branching.

Effect of Glacial Acetic Acid—The finding that purified glycogen and amylopectin can be digested completely by the action of phosphorylase plus glucosidase did not hold in three out of the six glycogen samples received from Dr. Bell (human leg muscle, Mytilus edulis, fetal sheep). These could be degraded only 61 to 70 per cent by exhaustive action of the two enzymes. The possibility was considered that the exposure of glycogen to glacial acetic acid in the method of Bell and Young (6) may lead to acetylation of a few glucose residues. Such acetylation could protect a considerable portion of the glycogen molecule from enzymatic digestion. In order to test this idea the above samples were treated with 15 per cent NaOH at 100° for 1 hour and the glycogen isolated by several precipitations with alcohol. After this treatment the samples became completely digestible enzymatically. That saponification with alkali was the essential step was shown by the fact that mere precipitation with alcohol did not improve enzymatic digestibility.

The analytical values obtained with the enzymatic method differed significantly before and after treatment with alkali, and only the latter are recorded in Tables I and II. To give examples of the magnitude of the change, the respective per cent end-groups for glycogen from human leg muscle, Mytilus edulis, and fetal sheep liver were 6.9, 6.3, and 5.0 before and 8.0, 7.7, and 8.7 after treatment with alkali. It seems clear that glycogen purified by treatment with glacial acetic acid is not satisfactory for enzymatic analysis and may also be unsuitable for physical measurements, such as ultracentrifugation.

The above results have a bearing on previous experiments in which glycogen was degraded incompletely by the two enzymes on purpose (1). In this case the per cent end-group did not change significantly when enzyme action was interrupted at 42 to 64 per cent degradation as compared to complete degradation. As a possible explanation it was suggested that the two enzymes degraded 1 glycogen molecule completely before attacking a new one. As will be shown in the next section, the outer chains are longer than the inner chains; hence a partial degradation, if it involved all glycogen molecules, should give end-group values lower than those obtained on complete degradation. This is the case in the enzymatic degradation of the partially acetylated glycogens; assuming random acetylation, there must have been present intermediates of all stages of degra-

1 Suggested by Dr. Schoch.
dation when enzyme action came to an end because of hindrance by an acetyl group.

**Outer and Inner Chains**—The length of the outer branches can be determined by exhaustive degradation with either phosphorylase or β-amylase. The limit dextrin which remains after phosphorylase action is larger than that which remains after β-amylase action, and, on isolating the former and treating it with β-amylase, there is split off 1 maltose unit per outer chain (19) or rather 2 units per every other chain (1). How close the glucose unit in the 1,6 linkage at the branch point can be approached by β-amylase action will depend on whether the number of glucose units in the chain is even or odd, the probable remainder being 2 or 3 glucose units, respectively. In calculating the length of the outer branches, i.e., (per cent degraded by enzyme)/(per cent end-group), 2 glucose units per chain were added when the degradation was with β-amylase and 4 glucose units per chain when the degradation was with phosphorylase. If the average chain length for the whole structure and the average number of residues for the outer chains is known, that of the inner chains is obtained by difference.

Surveying the data (Table II), one finds that the range of the average outer chain length for normal animal glycogen is from 7 to 13 glucose residues and for the few amylopectins so far studied from 13 to 16 glucose residues. Meyer (25), by means of degradation with α-amylase, estimated the outer branches of amylopectin to be from 15 to 18 units long. The average inner chain length is shorter in all cases, ranging from 3 to 5 glucose units for glycogens and from 5 to 6 glucose units for amylopectins. Individual chains, both inner and outer, probably vary considerably in length. For the inner chains this was shown by Swanson (26), who found that there must be some in amylopectin which have 8 to 10 glucose residues. This estimate was arrived at by comparing the absorption spectrum of the limit dextrin-iodine complex with the iodine color of chains of known length.

A few special cases may be pointed out. Tubercle bacillus "glycogen"

This assumption is based on a somewhat analogous case examined by Posternak (20). A phosphohexaose, containing a phosphate group esterified on carbon 6 of either the 4th or 5th glucose unit (counting from the non-reducing end from which the enzyme attacks), was not acted upon by β-amylase, while after removal of the phosphate group by kidney phosphatase the resulting hexaose was completely degraded to maltose. Thus, the phosphate group on carbon 6 (a C—O—P bond instead of the C—O—C bond at branch points) protects a maltose unit from being split off by β-amylase.

The number of residues in outer and inner branches is additive with respect to "average chain length" (as defined above), because there are twice as many branches (outer plus inner) as there are branch points. The models of polysaccharide structure in Paper II of this series illustrate this point.
(27) was not attacked at all by phosphorylase alone, behaving in this respect as if it were a phosphorylase limit dextrin. Phosphorylase plus glucosidase could degrade it only 40 per cent and its apparent end-group was 16 per cent. Treatment with hot alkali and subsequent isolation by ethanol precipitations removed the turbidity from solutions of this mate-

**Table II**

*Partial Structure Determination of Glycogens*

Except as indicated, the samples were prepared by the authors.

<table>
<thead>
<tr>
<th>Glycogen source</th>
<th>Enzymatic end-group</th>
<th>Average chain length</th>
<th>Degraded by phosphorylase</th>
<th>Average No. of glucose residues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal guinea pig liver</td>
<td>7.7</td>
<td>13.0</td>
<td>42.7</td>
<td>9.5</td>
</tr>
<tr>
<td>New-born guinea pig liver</td>
<td>6.9</td>
<td>14.5</td>
<td>40.8</td>
<td>9.9</td>
</tr>
<tr>
<td>Young adult guinea pig liver</td>
<td>6.5</td>
<td>15.4</td>
<td>39.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Immature corn (Schoch)</td>
<td>9.3</td>
<td>10.8</td>
<td>19.6</td>
<td>6.1</td>
</tr>
<tr>
<td>Mature corn (Schoch)</td>
<td>8.4</td>
<td>11.9</td>
<td>24.2</td>
<td>6.9</td>
</tr>
<tr>
<td>Overmature corn (Schoch)</td>
<td>7.6</td>
<td>13.2</td>
<td>24.9</td>
<td>7.3</td>
</tr>
<tr>
<td>Fetal sheep liver (Bell)</td>
<td>8.7</td>
<td>11.5</td>
<td>36.6</td>
<td>8.2</td>
</tr>
<tr>
<td>Rabbit liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low glycogen content (1-2%)</td>
<td>8.3</td>
<td>12.0</td>
<td>29.6</td>
<td>7.6</td>
</tr>
<tr>
<td>High &quot; (pooled)</td>
<td>6.8</td>
<td>14.7</td>
<td>36.2</td>
<td>9.3</td>
</tr>
<tr>
<td>Glucose-infused (Schlamowitz)</td>
<td>6.3</td>
<td>15.9</td>
<td>44.7</td>
<td>11.1</td>
</tr>
<tr>
<td>Fructose-infused &quot;</td>
<td>5.8</td>
<td>17.2</td>
<td>49.4</td>
<td>12.5</td>
</tr>
<tr>
<td>Rat liver, Busch strain</td>
<td>8.0</td>
<td>12.5</td>
<td>38.0</td>
<td>8.8</td>
</tr>
<tr>
<td>&quot; &quot; Sprague-Dawley</td>
<td>7.7</td>
<td>13.0</td>
<td>38.0</td>
<td>8.9</td>
</tr>
<tr>
<td>Cat liver</td>
<td>8.1</td>
<td>12.4</td>
<td>28.0</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Periodate 10 (21) and 11 (22); methylation 11 (23).
† Periodate 13 (personal communication).
‡ Periodate 22 (18).
§ Periodate 21.5 (18).
∥ Periodate 11 (24).

Material without affecting at all its behavior towards the two enzymes. More work is required to characterize this polysaccharide (see also Cori et al. (28)).

Guinea pig liver glycogen shows an increase in the length of the outer and inner chains from fetal to adult glycogen, and a similar change occurs in corn glycogen with increasing maturity of the kernels (Table II). The outer branches of rabbit liver glycogen are short when the glycogen content is low and increase in length as new glycogen is being deposited. The basis for these differences may well be a varying ratio of activity of phosphorylase.
which synthesizes straight chains in $\alpha$-1,4 linkage and of branching enzyme (29) which makes $\alpha$-1,6 linkages by a shift of chain segments. The kinetics of the branching enzyme when acting alone or in combination with phosphorylase are being investigated at the present time in the hope that this will be an aid in the analysis of structure.

SUMMARY

1. A partial determination of structure has been carried out on glycogens isolated from livers and muscles of different animal species as well as from the livers of the same species under different conditions. This consisted in the determination of the degree of branching and of the average number of glucose residues in the outer and inner branches.

2. It was shown that enzymatic and chemical end-group determinations carried out on the same sample were in fair to excellent agreement. Some samples purified with glacial acetic acid were resistant to complete enzymatic digestion, presumably because of partial acetylation; after treatment with hot alkali they became completely digestible.

3. The per cent end-group for different glycogens varied from 8.3 to 5.8 and that for amylopectins from 5.9 to 4.1, showing that there exists a fairly continuous series of branched polysaccharides. In all cases the outer branches were longer than the inner branches.

4. The per cent end-group varied considerably in the same species, depending on experimental conditions. It was 8.3 in glycogen isolated from rabbit livers with low content, 6.8 in two pooled samples from well fed rabbits, and 6.0 in glycogen freshly deposited after a fasting period. The degree of branching decreased in glycogens isolated from the livers of embryonal, new-born, and young adult guinea pigs. A similar decrease was also noted in phytoglycogen isolated from immature, mature, and overmature sweet corn.

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