Structure of a Low Molecular Weight Form of Glycogen Isolated from the Liver in a Case of Glycogen Storage Disease*

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SUMMARY

An incompletely branched, low molecular weight form of glycogen has been isolated from liver tissue of a patient with glycogen storage disease. Digestion with pullulanase and $\beta$-amylase, oxidation by periodate, Smith degradation, spectral studies of the iodine complexes, osmotic pressure measurements, and end group analysis gave results which indicate a molecular weight range of 8,000 to 15,000 with an average chain length of 17 to 18 glucose units per branch point. The substance was found in heart and kidney tissues in addition to liver. In an attempt to evaluate the role of branching enzyme in the disease, it was found that the branching enzyme was not stable in normal frozen liver and disappeared with a half-life of about 5 weeks. Clinical, chemical, and pathological factors in this case were similar to those previously reported in the case of glycogen storage disease type IV.

An unusual form of glycogen has been found in a liver, taken at autopsy, of a patient who had exhibited storage of polysaccharide in several tissues. Osmotic pressure measurements and reducing end group analysis of the polysaccharide indicated an average molecular weight of 8,000 to 15,000. Normal liver glycogen has been reported to have a molecular weight range of 2 to $30 \times 10^6$ (2). The analogous plant polysaccharide, amylpectin, has been reported to have a molecular weight of between 10 and $100 \times 10^6$. Chemical determination of molecular weights of glycogen have not generally been made because the very high molecular weight yields very few reducing terminals and also due to the destruction of the reducing terminal sugar in the usual methods of glycogen extraction by digestion of the tissue with strong base (3). Another important structural characteristic of glycogen is the high degree of branching in the molecule. A typical glycogen sample contains between 10 and 18 glucose units for each $\alpha$-(1,6) branch point while the number for amylpectin is 20 to 30. The material studied in this work has 17 to 18 glucose units in each chain.

Only two glycogen storage diseases have been previously described in which glycogen with an abnormal structure was isolated from the tissues. Limit dextrinosis (type III), a disease in which the debranching enzyme activities (amylo-1,6-glucosidase and oligo-1,4 $\rightarrow$ 1,4-glucan transferase) are missing, results in an accumulation of a glycogen having abnormally short outer chains with a higher than usual number of $\alpha$-(1,6)-linked glucosyl units (4). Amylpectinosis (type IV) has been characterized by Brown and Brown (5) as a condition in which the branching enzyme is missing. This enzyme, $\alpha$-1,4-glucan: $\alpha$-1,4-glucan $\beta$-glycosyltransferase (EC 2.4.1.18), forms branches by transfer of segments of the $\alpha$-(1,4)-linked glucan to position 6 of glucose units within the chain. When branching enzyme is absent, the resultant storage product is a form of glycogen which exhibits many of the properties of amylpectin. These include a shift toward higher wave lengths in the spectrum of the iodine complex and an increased extent of degradation by $\beta$-amylase and phosphorylase; both of these effects are caused by the polymer having fewer $\alpha$-(1,6) branch points than normal glycogen. Recently Mercier and Whelan (6) investigated the storage polysaccharide from a case of type IV glycogenosis and concluded that at least some of the branches contained as few as 5 glucose units.

In a preliminary report (1), we showed that the polyglucan isolated from the diseased liver had properties consistent with the unusually low molecular weights indicated above and a degree of branching intermediate between glycogen and amylpectin. In the present paper, we have confirmed the molecular weight by an independent method and established the average chain length by direct measurement. In addition enzyme studies are presented which were carried out in an attempt to establish the factors which might be responsible for deposition of this material in the liver and other tissues. The clinical and pathological factors which were found in the present case to be similar to those first described by Anderson (7) for amylpectinosis are in a separate publication (8). Possible relationships between the material isolated in the present case and the material stored in amylpectinosis (type IV) are discussed.

* These studies were supported in part by an American Cancer Society Institutional Research Grant, a University of Minnesota Graduate School Grant-in-Aid and United States Public Health Service Grant AM-10127. A portion of this work has been published in preliminary form (1).
EXPERIMENTAL PROCEDURE

MATERIALS—Pancreatic RNase and pancreatic DNase were purchased from Worthington Biochemical Corp., Freehold, N. J., sweet potato β-amylase from Sigma Chemical Co., St. Louis, Mo. Promase, rabbit liver glucoamylase, and amylpectin were obtained from Calbiochem, Los Angeles. Pullulanase was a gift of Dr. Thomas Nelson, Baylor University. Tritium-labeled sodium borohydride (New England Nuclear Corp., Boston) (300 mCi per mmole) was dissolved in 1 N sodium hydroxide for storage and diluted just prior to use. DEAE-cellulose was obtained from Distillation Products, Rochester, N. Y.

Analytical Methods—Sialic acid was measured after hydrolysis in 0.1 N H₂SO₄ for 30 min at 100° by the method of Aminoff (9). Hexose was determined by the anthrone method (10). For specific determination of the glucose content, the polysaccharides were hydrolyzed in 2 N HCl for 2 hours at 100°. After neutralization of the acid with 2 N NaOH, the glucose was determined with Glucostat (Worthington Biochemical Corp., Freehold, N. J.). Hexosamine was estimated by the method of Elson and Morgan (11) as modified by Roseman and Daffner (12). After saponification with 1 N KOH at 100° for 30 min, acetylation with CHCl₃, and extraction with chloroform, fatty acids were estimated by the method of Duncombe (13). Estimation of 6-deoxyhexose content was carried out by the method of Dische and Shettles (14). Uronic acids were determined by the carbazole method (15). Total phosphate analysis was performed by the method of Amco and Dublin (16). Protein was estimated by the method of Lowry et al. (17). Amino acid content was determined after hydrolysis under nitrogen gas in 6 N HCl at 115° for 21 hours by a Spinco amino acid analyzer. Reducing sugar was assayed by the alkaline ferricyanide procedure (18) and by the method of Conrad et al. (19). In this latter method, the reducing end groups are converted to gluconol by reduction with NaPH₄. The amount of tritium in the gluconol is proportional to the reducing group content. Radioactivity was determined by liquid scintillation counting.

Paper chromatography was used for qualitative analysis of the polysaccharide after hydrolysis. Descending chromatography was used with Whatman No. 1 paper and the following solvent systems: Solvent System A, butane-1-ol-acetic acid-water (5:1:2); Solvent System B, propane-1-ol-water (8:2); Solvent System C, butane-1-ol-ethanol-H₂O (10:2:1). Paper electrophoresis was carried out on Whatman 3MM paper with a Savant Flat-plate apparatus. Buffers were: Buffer I, 1 M formic acid; Buffer II, 0.05 M sodium germanate, pH 10 (20); and Buffer III, 0.05 M triethylenammonium acetate, pH 4.5. Sugars were detected by the periodate-benzidine dip of Gordon et al. (21) or the alkaline silver stain of Trevelyan (22).

Periodate oxidation of the polysaccharides was carried out in the dark. Samples of the polysaccharides (30 mg) were dissolved in 22 ml of water by heating to 100°. The results of the following line were treated with 3 ml of 1 N sodium periodate. Samples (3 ml) were withdrawn at various times and 0.2 ml of ethylene glycol was added to destroy the residual periodate. After 30 min in the dark, the formic acid was titrated with 0.01 N NaOH to a bromcresol purple end point (pH 5.2 to 6.8).

Iodine spectra were determined on solutions containing 0.1 mg of polysaccharide (sodiumized by heating) in 1 ml of 0.2% KI + 0.02% I₂ against a blank containing no polysaccharide.

Assays for branching enzyme (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase) were performed by the method of Krisman (23). Enzyme was prepared from liver samples by the method of Brown and Brown (5). The decrease in the absorbance of the LiK₁ complex with amylopectin at 520 nm is taken as the measure of branching activity after correction for α-amylase activity.

Molecular weight determinations by osmotic flow measurement were carried out by the method of Johnson (1). A Technicon autoanalyzer continuous flow dialysis unit was used with the standard Technicon cellophane membrane. Volume changes in the two compartments were measured while pumping the solvent (water) and the solution through the membrane-separated chambers at identical rates with a standard Technicon metering pump. With the intake and outlet lines connected to calibrated reservoirs, to allow continuous flow, it was possible to measure changes in volume of 5 μl in a total sample volume of 8 ml.

Isolation of Glycogen—The usual methods of glycogen isolation by digestion of the tissues with strong base could not be used due to the destruction of the reducing end group of the polymers by the alkali (30% KOH). Two alternate procedures were developed to permit isolation of minimally degraded glycogen. The two methods summarized below have been described in detail in an earlier publication (1). Portions of the frozen liver (10 g) were homogenized in cold 0.9% NaCl. The homogenate was immediately added to 9 volumes of acetone and the precipitate washed with acetone and ether, then dried in a vacuo at 50° (yield was 2.1 g). Method A depends on removal of protein and nucleic acids by enzymatic means followed by fractionation into the cold water-soluble (Preparation I) and the hot water-soluble (Preparation II) fraction. Starting with 1 g of acetone powder, the average yield of Preparation I was 23 mg (range, 21 to 24) (0.48% wet tissue) and for Preparation II the average was 55 mg (range 50 to 66) (1.2% wet tissue). Method B utilized the direct extraction of the acetone powder by hot water without the use of enzymes. The procedure yielded an average of 92 mg of Preparation III (1.9% wet tissue) per g of powder with a range of 63 to 122.

A special preparation of rabbit liver glycogen was made with the procedure for preparation of polymer I, Method A above. After killing by exsanguination, the liver was removed from a rabbit, and immediately frozen in Dry Ice. From 10 g of frozen liver were obtained 2 g of acetone powder which yielded 142 mg of glycogen after three reprecipitations with 50% ethanol (glucose content 95%).

Smith Degradation—Samples of polysaccharide (10 mg) were dissolved in 5 ml of water at 100°. After cooling to room temperature, 5 ml of 0.1 M NaCl were added. After 18 hours at room temperature, 200 μl of ethylene glycol was added and the samples were dialyzed. The oxidized polymer was treated with 1 ml of 1 M NaOH (specific activity 5 mCi per mmole) for 10 hours at room temperature followed by 3 ml of 1 N HCl. After evaporation, the dry residue was dissolved in 2 ml of 2 N HCl and heated at 100° for 1 hour. The boric acid was removed by distillation as methyl borate and the entire sample was electrophoretically treated in Buffer III for 1 hour at 2500 volts. The radioactive material which remained at the origin was eluted and chromatographed on 3-mm paper in Solvent System C for 24 hours. The strips were scanned for radioactivity and the

1 Personal communication from Dr. J. Johnson, Department of Physiology, University of Minnesota.
2 An error in the preliminary report indicated 19 volumes of acetone (1).
areas in the peaks corresponding to the glycerol and erythritol standards were determined by triangulation.

RESULTS

Structural Studies

Qualitative and Quantitative Analysis of Polysaccharides I, II, and III

Composition—Quantitative analysis of the three preparations indicated that they were homopolymers containing only glucose in appreciable amounts (95 to 100%). Since only negligible amounts (<1%) of phosphate, fatty acids, uronic acid, 6-deoxyhexoses, sialic acid, or hexosamines were found in preparations I and II, contamination by nucleic acids, mucopolysaccharides, glycolipids, or glycoproteins was minimal. The presence of nucleic acids in Preparation III was indicated by an absorption at 260 nm equivalent to 2% nucleic acid based on an assumed average molar extinction coefficient of $10^4$ M$^{-1}$ cm$^{-1}$. The only other significant, detectable contaminating substance was a variable but small amount of protein (2 to 5%). Repeated fractionation by the procedures described for isolation of the glycogen reduced the values to less than 1%. Quantitative analysis indicated a complete spectrum of amino acids in this protein contaminant. Paper chromatography of acid hydrolysates (2 N HCl, 100°, 2 hours) of Preparations I and II showed only one reducing sugar spot corresponding to glucose in Solvent Systems A and B. In Preparation III a weak spot corresponding to ribose was also found (presumably from nucleic acid).

Table I

<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>No previous treatment*</th>
<th>After pullulanase digestion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>II</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>III</td>
<td>58</td>
<td>84</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>Glycogen</td>
<td>45</td>
<td>45</td>
</tr>
</tbody>
</table>

* Reaction mixtures contained: polysaccharide, 0.23 mg; sodium acetate buffer, pH 4.6, 0.10 M; and $\beta$-amylase, 500 $\mu$g. Total volume of 1 ml. Portions, 50 $\mu$l, were analyzed for reducing sugar at intervals up to 90 min. The extent of degradation was maximal at 15 min.

* Reaction mixtures contained: polysaccharide, 2 mg; sodium acetate, pH 5, 0.03 M; and pullulanase, 250 $\mu$g. Total volume = 1 ml. After 25 hours at 37°, there was no further release of reducing groups. The samples were heated to 100° for 2 min, cooled to 37°, and 50 $\mu$g of $\beta$-amylase added. Portions, 10 $\mu$l, were analyzed for reducing sugar at intervals up to 18 hours. No increase in reducing value occurred after 2 hours.

Iodine Spectra—In Fig. 1 are shown the spectra of the iodine complexes of the three polysaccharides compared with those of amylopectin and rabbit liver glycogen. The data are presented in the form of logarithm of absorbance as a function of wave length to allow comparison of the shape and position of the peak without regard to the extinction coefficients. The longer wave length of maximal absorption for amylopectin is due to the greater average chain length per branch of 20 to 30 compared to 10 to 15 glucose units for glycogen. Preparations I, II, and III have absorption maxima of 525, 510, and 518 nm, respectively, compared to 480 and 530 nm for glycogen and amylopectin. These spectra are suggestive of a greater average number of glucose units per branch for the three fractions than is found in normal glycogen. The rather low absorbance of the iodine complex with Preparation III may be due to the same factors which cause its tendency toward aggregation and low solubility. This insolubility phenomenon is reminiscent of the retrogradation of starch solutions which results in a decrease in ability to form blue complexes with iodine (24).

$\beta$-Amylase Digestion—Polyglucans such as glycogen and amylopectin which contain $\alpha$-(1,4) linkages are digested by $\beta$-amylase from the nonreducing ends by successive removal of maltose units. Since the enzyme is unable to pass the $\alpha$-(1,6) branch points, the extent of degradation is an indication of the amount of glucose in the external chains. In Table I, the maximal extents of degradation of the three fractions by $\beta$-amylase are displayed as compared to the hydrolysis of glycogen and amylopectin. The greater degree of hydrolysis of the three preparations than is found for rabbit liver glycogen is consistent with an average outer chain glucose content somewhat greater than is normal for glycogen. The finding that the samples are more susceptible to $\beta$-amylosylation than was amylopectin was somewhat surprising. The lower wave length of maximal absorption for the I$\beta$ complexes of Preparations I, II, and III compared to that of amylopectin indicated a slightly shorter average chain length.
for the three preparations compared to amylopectin. A possible reconciliation between these two sets of data could be made if an unusually large amount of the glucose was present in the outer chains, i.e., those in which none of the glucose units are bound in the six positions. A second set of data in Table I shows the extent of β-amylolysis after treatment with the α(1,6)-glucanosylase, pullulanase. This enzyme can completely debranch amylopectin but has little effect on the more highly branched glycogen molecule (6).

Smith Degradation—In treatment of glycogen or amylopectin by this procedure, erythritol would result from all glucose units except those at the nonreducing ends of the chains, where glycerol would be found. The ratio of erythritol to glycerol is a direct measurement of the number of glucose units per branch. The results for two independent runs were: rabbit liver glycogen, 13.4; 12.4; amylopectin, 23.1, 25.6; polysaccharide III, 17.6, 18.7.

Periodate Oxidation—The time course of formic acid production by periodic acid oxidation of polysaccharides I and II is shown in Fig. 2. Oxidation of amylopectin and rabbit liver glycogen are shown for comparison. The final yields of formic acid (mole of HCOOH per mole glucose) were polysaccharide I, 0.086; polysaccharide II, 0.079; rabbit liver glycogen, 0.071; amylopectin, 0.037. In polymers of α-(1,4)-linked glucose, 1 mole of formic acid is produced by periodate oxidation of each nonreducing terminus while as many as 2 moles may be produced at the reducing end. In highly branched, high molecular weight glucans such as glycogen or amylopectin, the ratio of formic acid to glucose units may be insignificant, the average chain length (moles of glucose per branch) would be the reciprocal of the quantity of reducing ends. The results for two independent runs were: rabbit liver glycogen, 13.4, 13.4; amylopectin, 23.1, 25.6; polysaccharide III, 17.6, 18.7.

Measurement of molecular weight by sedimentation in the ultracentrifuge or passage through calibrated gel filtration columns were not successful due to the tendency for aggregation and precipitation of the polysaccharides.

Molecular Weight Determination—Attempts at estimation of molecular weight by sedimentation in the ultracentrifuge or passage through calibrated gel filtration columns were not successful due to the tendency for aggregation and precipitation of the polysaccharides.

Measurement of molecular weight by determination of osmotic flow is based on the permeability equations of Kedem and Katruchsky (20).

Flux through a membrane \( J_x \) is

\[
J_x = -L_x uRT \Delta C_x
\]

where \( L_x \) is a filtration coefficient; \( u \) is the reflection coefficient (when \( u = 1 \) the membrane is impermeable to solute, when \( u = 0 \), solvent and solute are equally permeable); \( R \) is the gas constant \((8.31 \times 10^{-5} \text{ dyne cm/deg mole})\); \( T \) is the absolute temperature; \( \Delta C_x \) is the difference in molar osmotic concentration across the membrane.

When the pressure is equal on both sides, \( \Delta p = 0 \) and

\[
J_x = -L_x uRT \Delta C_x
\]

The rate of flow \( (Q) \) from Side 1 to Side 2 is

\[
Q = -A \cdot J_x = -L_x A \sigma \rho RT \Delta C_x
\]

where \( A \) is the area of the membrane. By rearranging Equation 4

\[
C_x = Q/(A \cdot L_x \sigma \rho RT)
\]

\( L_x \) is evaluated from Equation 1 by measurement of \( J_x \) when \( \Delta C_x = 0 \) at a known \( \Delta \rho \). and was found to be \( 2.0 \times 10^{-11} \text{ cm}^3/\text{dyne sec} \).

\( A \) was found to be 35.4 cm². The reflection coefficient, \( \sigma \), was estimated to be very close to 1 on the basis of negligible amounts of glucose containing material being found on the solvent side (Side 1) of the membrane at the end of the run (membrane permeable to solvent). Accurate determination of \( \sigma \) for inulin (molecular weight 4600) yielded a value of 0.8 to 0.9 for the same system. It seems reasonable that the assumption of \( \sigma = 1.0 \) could involve an overestimate of molecular weight by more than 20%. In any case, an underestimate would not be possible.

For a solution containing \( 2.6 \times 10^{-3} \text{ g per cm}^3 \) at \( 37^\circ \text{C} \), \( Q \) was determined to be \( 5.2 \times 10^{-4} \text{ cm}^3/\text{sec} \). This value yielded a molar concentration (from Equation 4) of

\[
C_x = 2.9 \times 10^{-4} \text{ mole/cm}^3
\]

which gives a molecular weight of 9100.
Table II

Molecular weight of polysaccharides

<table>
<thead>
<tr>
<th>Method of analysis</th>
<th>Polysaccharide molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Alkaline ferricyanide</td>
<td>8,600</td>
</tr>
<tr>
<td>Reduction by NaBH₄</td>
<td>7,900</td>
</tr>
<tr>
<td>Osmotic pressure</td>
<td>9,100</td>
</tr>
</tbody>
</table>

* Rabbit liver glycogen, isolated by the method used for polymer I.
* This value for polymer III by the borotritide reduction is based on electrophoretic purification of [3H]glucitol after acid hydrolysis.
* Due to the insolubility of polysaccharides II and III, it was not possible to obtain osmotic pressure measurements.

Table III

Paper chromatography and electrophoresis of tritium-labeled hydrolysis product

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rglucitol</th>
<th>Electrophoresis</th>
<th>Chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>1.67</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>Galactitol</td>
<td>1.13</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>Xylitol</td>
<td>1.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosaminitol</td>
<td></td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>3H-product</td>
<td>1.00</td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

* System II.
* Solvent A.

Table IV

Summary of structural data

<table>
<thead>
<tr>
<th>Structural feature</th>
<th>I</th>
<th>II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles glucose/reducing group</td>
<td>51</td>
<td>88</td>
</tr>
<tr>
<td>HCOOH to glucose</td>
<td>0.098</td>
<td>0.079</td>
</tr>
<tr>
<td>HCOOH to reducing group</td>
<td>5.0</td>
<td>6.9</td>
</tr>
<tr>
<td>Number of chains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HCOOH/reducing group) - 1.2</td>
<td>3.8</td>
<td>5.7</td>
</tr>
<tr>
<td>Average chain length</td>
<td>13.4</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Enzyme Studies

The storage of an unusual polysaccharide such as this must be the result of deletion or aberration of enzyme activity. The following studies were undertaken to see if such a defect could be substantiated.

Branching Enzyme—Assays for branching enzyme activity in the frozen diseased liver sample showed negligible amounts to be present after 18 months storage at −21°C. Because the activity was not measured at the time of death, it was of interest to see if branching activity in the tissue specimen would survive 18 months under those conditions of storage. Portions of liver were removed at autopsy from individuals aged 0 to 5 years who had died of accidental causes or at birth. The tissue samples were frozen immediately in Dry Ice and stored at −21°C. Samples were taken at weekly intervals and the branching enzyme was assayed. Values are expressed as changes in absorbance at 520 nm per min per mg of protein. The resultant activities were analyzed by least squares and found to fit the equation y = aXᵇ where y is the amount of activity at time t, b is the first order rate constant, and a is the extrapolated value of activity at zero time. Fig. 3 shows the rate of loss of branching activity in the tissue during storage. The average value for all five samples is plotted...
The failure of the enzyme preparation to cause a decreased absorbance in the iodine complex of rabbit liver glycogen at 460 nm. Samples were removed at the beginning and after 24 min of incubation of branching enzyme with polymer III and their I₂ complex spectra were taken. The wave length of maximum absorbance shifted from the region of 515 nm to the 490 to 495 area during that 24-min period indicating that branching did occur.

### Table VI

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Amount (mg/g tissue)</th>
<th>I₂ wave length maximum</th>
<th>Percentage glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart I</td>
<td>1.4</td>
<td>510</td>
<td>%</td>
</tr>
<tr>
<td>Heart II</td>
<td>26</td>
<td>515</td>
<td>78</td>
</tr>
<tr>
<td>Kidney I</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Kidney II</td>
<td>1.5</td>
<td>505</td>
<td>50</td>
</tr>
<tr>
<td>Liver I</td>
<td>7.5</td>
<td>525</td>
<td>95-100</td>
</tr>
<tr>
<td>Liver II</td>
<td>14.6</td>
<td>510</td>
<td>99</td>
</tr>
</tbody>
</table>

### Discussion

The present work described the characterization of an abnormal glycogen which differs in several respects from the usual form.

The results of structural studies on the isolated material were all consistent with a low molecular weight, branched polysaccharide. The various preparations were found to have molecular weights in the range of 8,000 to 15,000 with an average chain length of 17 to 18 glucose units per branch point. The spectra of the iodine complexes indicated a chain length of between that usually found for glycogen (10 to 15) and the 20 to 30 normally associated with amylopectin. The unusually large amount of formic acid produced during periodate oxidation was attributed to the formic acid arising by oxidation of carbon atoms 1 and 2 of the glucose at the reducing end of the carbohydrate chain. Digestion of the polysaccharide by β-amylase produced significantly more maltose than would be expected for a high molecular weight polymer having an average outer chain length between glycogen and amylopectin. However, as the molecular weight of such a polysaccharide is drastically reduced, much more of the glucose is contained in outer, unbranched chains. This concept is based on the findings of Gunja-Smith et al. (28) that nearly all of the glucose chains in glycogen have lengths close to the average so that there are few, if any, very long, multiply branched chains. This concept is based on the findings of Gunja-Smith et al. (28) that nearly all of the glucose chains in glycogen have lengths close to the average so that there are few, if any, very long, multiply branched chains as in the usual description of the model of Meyer and Bernfeld (29). Using models based on the proposal of Gunja-Smith et al. for polymers having the properties demonstrated for Preparations I and II (four and six chains of lengths between 13.4 and 15.4 glucose units), an average of 61 and 63% of the glucose was...
found to be external to all 1-6 branches. Thus, it is then possible for the degree of degradation by an exo-enzyme such as β-amylase to increase markedly without an increase in the average number of glucose units per chain.

Determination of molecular weights of polysaccharides by analysis of reducing end groups has not always been successful in the case of high molecular weight polymers. By using the reducing agent sodium borohydride labeled with tritium and then purifying and quantifying the labeled glucitol, it is possible to avoid the overestimations due to polysaccharide degradation and the presence of contaminating substances. Physical measurement of molecular weight of preparation by an osmotic flow method gave a value which was in agreement with the two chemical methods.

The usual method for the isolation of glycogen from tissues is that of Somogyi (30). Digestion with 30% KOH destroys the proteins, nucleic acids, and saponifies the triglycerides leaving the glycogen more or less intact. According to Orrell et al. (31), the degradation occurring by this method may reach an extent sufficient to cause a 50-fold loss in molecular weight. Recently, Boudin et al. have studied the sedimentation efficiencies of glycogens from human storage disease types I, II, III, and VI (32). With a cold water extraction method, they isolated native glycogens from a variety of sources having sedimentation coefficients in the range of 179 to 997. Such values would correspond to molecular weights of several millions of daltons. In the case under present discussion, the glycogen was not extractable by cold water. Orrell et al. have also stated that glycogen extracted with hot water is somewhat degraded with respect to particle weight (31). Greenwood and Manners using hot water extraction of rabbit liver glycogen found molecular weights in the range of 2 to 6 x 10⁶ daltons (33). Therefore, a reduction to the level of a molecular weight of 10,000 from over 10⁶ could not be achieved by a simple heating at 100°C in neutral solution. The similar results found with both methods indicates that there was no significant degradation of the polysaccharides by the enzyme treatments in Method A. Additionally, rabbit liver glycogen isolated by Method A retained a high molecular weight. The range in yields from the combined preparations (Preparations I and II) of Method A was 71 to 90 mg per g of acetone powder, compared to a range of 63 to 122 mg per g of acetone powder for Method B (Preparation III). The variations in yields may have been due to the difficulty of keeping the slightly soluble material in solution during purification.

Since analyses were done on autopsy material, post-mortem changes must be considered. The following arguments can be made against such a possibility. The liver was removed at autopsy within 2 hours of death and placed in a -70°C freezer. After freezing, the tissues were transferred to a -21°C freezer where they have remained. In electron micrographs of sections taken at biopsy, the polysaccharide material had the same morphological appearance as in sections removed from the frozen organ (8). In earlier, preliminary studies done on liver biopsy samples, it was found that little glucose-containing material could be extracted from homogenates with cold water (5 mg per g of tissue), while hot 0.1 M H₂SO₄ did release substantial amounts of glucose-containing materials (15 mg per g of tissue). Unfortunatel, by the time the storage product was identified as glycogen, biopsy material was no longer available.

The clinical and pathological findings in this case are similar to those found for the six previously reported cases of glycogen storage disease type IV (5, 7, 34-37). In all of these cases the storage product has been described as being similar to amylopectin based on some or all of the following criteria: (a) an iodine spectrum having an absorbance maximum in the 510- to 535-nm region, (b) relative insolubility in cold water, and (c) an unusually high extent of digestion by phosphorylase (50 to 58%) or β-amylase (57 to 66%). In one of those cases periodate oxidation was performed and the production of formic acid measured (35). The results indicated an average chain length of 20. The glycogen was isolated in that case by digestion of the tissue with KOH which converts the reducing end group to a saccharinic acid (3). Such an end group would not yield 2 moles of formic acid upon treatment with periodate as was the case with material under present investigation. Thus, their finding that periodate oxidation yielded an amount of formic acid consistent with an average chain length of about 20 should not be interpreted to exclude the possibility of a low molecular weight polymer. The only information concerning the size of the glycogen molecules isolated from the tissues of a case of IV glycogenesis is a brief statement by Cori that unpublished ultracentrifugation studies showed a molecular weight smaller than that of normal liver glycogen (38).

Mercier and Whelan (6) analyzed the glycogen isolated from the liver in one of the previously described cases of type IV disease (36). They took advantage of the enzyme pullulanase which cleaves the α-(1,6) linkages in amylopectin to the extent of 95% but has no action on the same linkages in normal human liver glycogen. Treatment of the resultant products with β-amylase causes complete degradation of amylopectin and 47% degradation for glycogen. They found type IV glycogen to be degraded to the extent of 80% by this method. The 84% degradation found for Preparation III in this study is strikingly similar.

An enzymatic deficiency has been shown in type IV glycogenesis by Brown and Brown (5). They were unable to detect branching enzyme in either the liver or leukocytes. More recently, Legum and Nitowsky have shown that both parents of a child having died of type IV glycogenesis had approximately 50% of the level of branching enzyme in their leukocytes as compared to normal controls (39). Because of the many similarities between the present case and the authentic type IV glycogenesis, the frozen, diseased liver was tested for branching enzyme activity. Although none could be detected, subsequent studies on the rate of disappearance of branching activity from normal liver under identical conditions of storage showed that it could not be detected after 18 months even if it had been present originally. The polysaccharide under present study contains fewer branches than normal glycogen in addition to having a much smaller molecular weight. There does not appear to be any basis for postulating a causal relationship between these two factors. With purified branching enzyme, it was possible to show increased branching of the isolated polysaccharide as shown by changes in absorption spectrum with iodine. Thus, the polymer would appear to be a suitable substrate for the enzyme.

Because little is known about either the regulation of polymer size in glycogen biosynthesis and degradation, or the de novo
initiation of glycogen synthesis, one may only speculate on the causes of accumulation of such a product. There are at least three possible causes for the formation of such material: (a) a partial degradation of a polysaccharide having an unusually low number of branch points, (b) an inability of the synthesizing system to extend and branch low molecular weight chains, or (c) excessive initiation of glycogen molecule synthesis. The first of these would require the presence of a depolymerizing activity which would act only on high molecular weight substances having an unusually low number of branch points. There is no such enzyme known to be present in liver tissue. A plausible argument for the second possibility can be found in the work of Goldemberg who has shown that glycogen synthetase (α-glucan-UDP:α-glucose glucosyltransferase) (EC 2.4.1.11) works better with larger branched molecules than with oligosaccharides or only slightly branched acceptors such as amylose or amylopectin (40). Such a selective affinity together with the relative insolubility of the smaller slightly branched polymers once formed might be sufficient to account for their accumulation. The third possibility cannot be discussed since little is known concerning the initiation of glycogen synthesis.

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