ON RABBIT LIVER GLYCOGEN AND ITS PREPARATION.

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The importance of studying glycogen that has been prepared without the use of strong caustic alkali has been pointed out in previous papers.\(^1,2\) In one of them (Petree and Alsberg), a method of preparation based on the removal of protein with picric acid was described. In the present paper, an improved method with trichloroacetic acid is presented, and rabbit liver glycogen prepared by it is described with especial reference to its phosphorus content.

Trichloroacetic Acid Method for Preparation of Glycogen.

The rabbit livers were rapidly removed from the animals, cut to pieces, and thoroughly ground in a mortar with 3 parts of 3 per cent trichloroacetic acid solution. The ground mixture was then strained through cheesecloth and pressed by hand. The pressed tissues were replaced in the mortar and thoroughly mixed with fresh 3 per cent trichloroacetic acid solution. This second extraction was found essential, for about 30 per cent, roughly, of the glycogen remains in the ground, pressed tissues. A third extraction might also be worth while.\(^3\) The various extracts were collected and centrifuged, since the rate of filtration was very slow. To 1 volume of the opalescent filtrate, 2 volumes of 95 per cent ethyl alcohol were added. The glycogen precipitated

\(^3\) In this regard, we might mention an experiment with the tissues after the second extraction. About one-third of the tissue residue was kept in trichloroacetic acid for about 1 week. 0.7 gm. of glycogen was recovered.
Rabbit Liver Glycogen Preparation

rapidly, and upon standing for a short time it settled at the bottom of the vessel, so that most of the liquid could be decanted. The glycogen was then centrifuged and the remaining liquid decanted. It was then redissolved in a small amount of 3 per cent trichloroacetic acid and centrifuged to remove any proteins still present. The trichloroacetic acid-glycogen solution was then shaken with ether several times in a separatory funnel to remove any fatty acids present. The extracted solution was then precipitated with 2 volumes of alcohol and centrifuged. The centrifuged glycogen was redissolved in distilled water, precipitated with alcohol, then centrifuged, and the process repeated twice. If the glycogen solution is so dilute that it does not precipitate rapidly upon the addition of alcohol, a trace of solid ammonium acetate added to the mixture will hasten precipitation. Ammonium acetate is superior to either sodium or potassium salts, since it does not appear in the ash and is soluble in dilute alcohol. From the dilute alcohols decanted in the course of preparation, an appreciable amount of glycogen can be recovered by allowing them to stand for several days.

The glycogen is finally taken up in the least possible amount of water and filtered through a Büchner filter, precipitated with alcohol, taken up in 95 per cent alcohol, and finally in absolute alcohol before drying. Ether was not used, for it dries the surface too rapidly and leaves the interior wet. The glycogen was dried in vacuo over calcium chloride. In this way, Preparation A was produced. From 240 gm. of liver used for one of the preparations, a total of 18 gm. of glycogen in the dry form was obtained.

Drying to constant weight in the above manner required a minimum of 2 weeks at room temperature with frequent stirring. It is not safe to dry at elevated temperatures, as the following experience demonstrates.

While drying about 40 gm. of glycogen, prepared during the summer of 1929, in an oven at 60°, the temperature rose accidentally to 105° and caused alteration in the nature of the preparation. Since it acquired different physical and, perhaps, chemical properties, we speak of it as "denatured" glycogen. Its color changed from pure white to slightly brown. The viscosity of its solutions was greatly increased, though no measurements were made. It
became less soluble, and in strong solutions most of it settled at the bottom of the beaker in the form of a sticky, gelatinous mass. The weak solutions of glycogen above the gelatinous mass and this gelatinous material, itself, had the same characteristics. It was filtered through charcoal to remove impurities, and then subjected to boiling with 60 per cent potassium hydroxide solution for more than 7 hours under a reflux condenser in order to destroy other substances than glycogen that might have been formed. The process of purification by precipitation with alcohol was then repeated. Upon drying the final product a shining, transparent surface of extreme hardness was formed on the outside that retarded drying to a considerable extent, and we, therefore, believe that about 10 per cent by weight of water remained present. This "denatured" glycogen is designated as Preparation B.

The removal of proteins by means of the Folin-Wu sodium tungstate and sulfuric acid reagent was also investigated. The substitution of these reagents for trichloroacetic acid is not advantageous, since tungstic acid is precipitated by alcohol along with glycogen. However, investigations are in progress to determine the value of these reagents for a quantitative, rapid, micro estimation of glycogen in tissues.

Analysis of Glycogen.

Ash.—The ash content of glycogen preparations depends to a large extent upon (1) the method of preparation, (2) the source, and (3) the presence of such ions as calcium, sodium, potassium, iron, etc.

McDowell,1 using Pfüger's method, reported the ash content of a glycogen preparation from the edible mussel, Mytilus edulis, as 0.254 per cent before electrodialysis with ultrafiltration, and 0.0487 to 0.0828 per cent after dialysis. Petree and Alsberg,2 using the picric acid method, obtained from abalone, Haliotis rufescens, Swainson, preparations with 0.069 per cent before dialysis, and 0.057 per cent after dialysis. In a more recent investigation of glycogen, Barbour4 prepared his samples from livers of rabbits by Pfüger's method, and reported that his preparation was 99 to 100 per cent pure. He did not state the ash

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1 Barbour, A. D., J. Biol. Chem., 85, 29 (1929).
content exactly. Though the ash content of glycogen, carefully prepared, is in general very small, yet it cannot be regarded as negligible. Earlier investigations on this subject are reviewed by Petree and Alsberg.²

Preparation A was dried to constant weight in a finely powdered form, and the ash content of each of two samples of 500 mg. determined. Results were as follows:

<table>
<thead>
<tr>
<th>Weight of ash (gm.)</th>
<th>Percent ash (%)</th>
<th>Percent P₂O₅ (%)</th>
<th>Ca</th>
<th>Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0009</td>
<td>0.18</td>
<td>16.5</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>0.0010</td>
<td>0.20</td>
<td>15.5</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

It is quite possible to obtain ash-free glycogen, in spite of the fact that phosphorus may be present, for unless there are such ions as calcium, iron, sodium, potassium, etc., phosphorus will volatilize upon ashing. In the presence of such ions, however, phosphate is formed, and consequently they determine the percentage of ash formed.

Reducing Sugars.—Though Preparation A had all the appearance of a pure material, its purity was verified by hydrolyzing a portion of it in order to determine the reducing sugar equivalent as glucose, and from the value obtained the amount of glycogen was computed by multiplying by the factor 0.927.

Two samples of Preparation A and two others of Preparation B were accurately weighed and dissolved in 100 cc. of distilled water in a 250 cc. volumetric flask. The weight of each sample was 1 gm. 2.2 per cent of hydrochloric acid was added to each solution, and the flasks were placed in boiling water for 3 hours. Upon cooling, the samples were neutralized to litmus with sodium hydroxide and made up to volume. The results for reducing sugars, determined by the sugar method of Folin and Wu, and recorded as glucose, were as follows:

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Weight (gm.)</th>
<th>Sugars as glucose (gm.)</th>
<th>Glycogen computed (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.000</td>
<td>1.040</td>
<td>0.964</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>1.075</td>
<td>0.996</td>
</tr>
<tr>
<td>B</td>
<td>1.000</td>
<td>0.980</td>
<td>0.906</td>
</tr>
<tr>
<td></td>
<td>1.000</td>
<td>1.010</td>
<td>0.936</td>
</tr>
</tbody>
</table>
As can be seen from the data, the glycogen value approaches very closely the theoretical value for Preparation A, but is too low for Preparation B, which is to be expected since this preparation is "denatured" and not anhydrous.

Phosphorus.—While there is as yet no final and absolute proof that phosphorus as P$_4$O$_8$ is an integral component of the glycogen molecule, the investigations of McDowell$^1$ and Petree and Alsberg$^2$ support that view, since they were unable to free glycogen from phosphorus by means of prolonged electrodialysis with ultrafiltration. The amounts reported are small. The literature has been reviewed by Petree and Alsberg.$^2$ In a recent investigation by McBride$^5$ on glycogen, phosphorus was determined and found to agree with the findings of former investigators.

When a sample of Preparation A was dissolved in water, it showed no positive test for phosphorus. Preparation B, "denatured" glycogen, also gave negative results when freshly dissolved, but upon standing 24 hours the same solution gave a positive test.

After hydrolysis, however, tests for phosphoric acid were positive. Hydrolysis was accomplished with hydrochloric acid, as described above in connection with the estimation of reducing sugar. Two samples of Preparation A and two of Preparation B were hydrolyzed and phosphoric acid estimated by the method of Fiske and Subbarow.$^6$ The reagents used were thoroughly tested and found free from phosphorus. The method is quite satisfactory, as the close checks obtained in analyzing a sample of commercial takadiastase indicated.

100 mg. of takadiastase were dissolved in a 100 cc. volumetric flask, and samples were removed and analyzed for phosphorus. 50 cc. of this solution were electrodialyzed and the ultrafiltrable fraction as well as the non-ultrafiltrable fraction were analyzed.

<table>
<thead>
<tr>
<th>Takadiastase</th>
<th>Total P as P$_4$O$_8$</th>
<th>Ultrafiltrable P$_4$O$_8$</th>
<th>Non-ultrafiltrable P$_4$O$_8$</th>
<th>Total ultrafiltrable and non-ultrafiltrable P$_4$O$_8$.</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>100</td>
<td>2.04</td>
<td>1.85</td>
<td>0.215</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>2.05</td>
<td>1.82</td>
<td>0.210</td>
<td>2.03</td>
</tr>
<tr>
<td>100</td>
<td>2.04</td>
<td>1.83</td>
<td>0.210</td>
<td>2.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.82</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Two samples of glycogen, Preparation A, and two others of "denatured" glycogen, Preparation B, were hydrolyzed with hydrochloric acid, as described above. The results of the phosphorus determinations were as follows:

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Weight of glycogen hydrolyzed</th>
<th>% P as P₂O₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1000 mg</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>1000 mg</td>
<td>0.033</td>
</tr>
<tr>
<td>B</td>
<td>1000 mg</td>
<td>0.028</td>
</tr>
<tr>
<td></td>
<td>1000 mg</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Though we employed a different procedure, a different source of glycogen, and a different method of phosphorus determination from McDowell, Petree and Alsberg, and McBride, yet our results are in general agreement with their findings.

Attention is to be called to the fact that in Preparation B the glycogen had been subjected during preparation to drastic treatments such as filtering through charcoal and boiling for 7 hours in a solution of 60 per cent potassium hydroxide; yet the phosphorus content was only slightly less than that of Preparation A, although Preparation B was not anhydrous and distinctly altered.

Aside from its relative insolubility, noted above, the characteristics of Preparation B, so far as investigated, were as follows:

**Iodine Test.**—A solution gave the same port wine color with iodine as ordinary glycogen, but it differed from the pure preparation in showing a change of color in fading. Instead of the original color's becoming gradually fainter, the color turned pink as it vanished.

**Opalescence.**—It was considerably less opalescent, so that 4 to 5 times the concentration was required to match the opalescence of pure glycogen.

**Reducing Sugars.**—It did not reduce Benedict's alkaline copper solution and did not precipitate silver from an ammoniacal silver nitrate solution.

**Adhesion.**—It acquired very strong adhesive properties, even to smooth surfaces, such as glass.
SUMMARY.

A method is described for the preparation of glycogen with the use of 3 per cent trichloroacetic acid.

Glycogen prepared by this method was found to contain between 0.20 and 0.18 per cent ash. The samples analyzed were not dialyzed. The ash was found to contain phosphorus as $\text{P}_2\text{O}_5$ and traces of calcium and iron.

Upon hydrolysis, the computed value of this glycogen was from 0.964 to 0.996 per cent of the theoretical.

The phosphorus content, calculated as $\text{P}_2\text{O}_5$, of this glycogen was 0.032 per cent.

Glycogen is very easily altered by heating to 105°. Some of the properties of such a glycogen are described.
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