A SPECIFIC COLOR REACTION FOR GLUCURONIC ACID*

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The present report deals with a specific color reaction of glucuronic acid which can be used for the tentative identification in polyuronides. This reaction seems to be highly characteristic for glucuronic acid. It represents a specific form of a new general reaction of carbohydrates with SH compounds in H₂SO₄ which differentiates various classes of carbohydrate (methylpentoses, pentoses, hexoses, hexuronic acids) and individual hexose and hexuronic acids.¹

EXPERIMENTAL

Reaction of Glucuronic Acid with Thioglycolic Acid in Presence of Mannose; Simple Qualitative Test

Procedure—0.2 cc. of a 0.2 per cent solution of mannose is added to 0.8 cc. of a solution containing 0.02 to 0.04 per cent glucuronic acid. To this mixture are added with cooling in ice water and shaking 4.5 cc. of a water-sulfuric acid mixture prepared by mixing 6 volumes of concentrated H₂SO₄ with 1 volume of H₂O. After 2 minutes in ice water the reaction mixture is placed in water of 20–25° and later for 3 minutes in boiling water. It is then cooled to room temperature and 0.1 cc. of a 2.5 per cent solution of thioglycolic acid (commercial preparation, solution prepared every few days and kept on ice) is added. The mixture is shaken thoroughly and left at room temperature for 20 to 24 hours. A deep pink color develops. The blank prepared with a mixture of 0.2 cc. of the mannose solution and 0.8 cc. of H₂O shows a greenish yellow color.

Two Components of Color Reaction and Their Specificity—The color reaction of glucuronic acid is the sum of two different reactions: (1) a reaction of glucuronic acid with thioglycolic acid alone and (2) a reaction in which all three reactants, mannose, thioglycolic acid, and glucuronic acid, participate. The first will be called the thioglycolic acid reaction, the second the mannose-thioglycolic acid reaction, designated by the abbreviations ThR and MThR respectively.

ThR can be observed separately by adding 0.2 cc. of H₂O instead of mannose solution to 0.8 cc. of the solution of glucuronic acid and treating

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the sample in the same way as described above. The corresponding blank contains 1 cc. of water. Under these conditions glucuronic acid shows a brown-red color in lower concentration and a pure brown at 0.04 per cent. These colors are not characteristic for glucuronic but for hexuronic acids in general. Pentoses show an intensive pink, methylpentoses a green-yellow, and glucose and galactose a green and green-blue color respectively. The pink color observed in the presence of an excess of mannose, however, due to MThR, is completely specific for glucuronic acid and polyglucuronides. Galacturonic, pectic, and alginic acids and pneumococcus polysaccharide type I all show a non-characteristic brown color, and true sugars do not react with mannose at all. On the other hand not only mannose but all other hexoses react with glucuronic and thioglycolic acids with formation of a pink-colored product. These reactions, however, are not as specific as that with mannose.

Spectrophotometric Test for Glucuronic Acid

Procedure for Measurement of Absorption Due to MThR—In biological materials, we find in general glucuronic acid simultaneously with considerable amounts of true sugars. If the simple procedure described above is applied, the pink color of the ThR of pentoses may be mistaken for the MThR of glucuronic acid. Furthermore, when glucuronic acid is present in solution with a large excess of other hexuronic acids, the pink color of MThR may be covered by the intense brown color produced by the other compounds. In these cases, however, glucuronic acid still can be detected by spectrophotometric measurement of the intensity of MThR and with a slightly more elaborate procedure which consists of carrying out the reaction on two samples of the unknown solution (1) in the presence of mannose (MThR sample) and (2) in the absence of mannose (ThR sample). The absorption in the MThR sample gives the sum of the absorption due to MThR plus ThR (measured against a corresponding blank containing mannose); that in the ThR sample gives the absorption due to ThR (measured against a blank containing H₂O only). The difference between the two gives the absorption due to MThR alone. This value will not, in general, be influenced by true sugars.

Absorption Spectra for MThR of Various Hexuronic Acids and Polysaccharides

In Fig. 1 are recorded the absorption spectra of MThR of

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2 I am greatly indebted to Dr. Karl Meyer for preparations of hyaluronic and chondroitinsulfuric acids, to Dr. Michael Heidelberger for preparations of pneumococcus polysaccharides, and to Dr. Leonard Cretcher for a preparation of alginic acid.

3 The content in hexuronic acids of the preparations of galacturonic, hyaluronic, and chondroitinsulfuric acids was determined by the modification of Freudenberg and his colleagues of the Lefèvre-Tollens method, that of pneumococcus polysaccharides by titration.
glucuronic, galacturonic, hyaluronic, chondroitinsulfuric, pectic, and alginic acids and of pneumococcus polysaccharide type I. The concentration of the solutions was 0.01 per cent hexuronic acids and equivalent for the polyuronides. All spectra were measured in a Beckman spectrophotometer.

**FIG. 1.** Absorption spectra of MThR of some hexuronic acids and polyuronides. Curve I galacturonic acid, Curve II glucuronic acid, Curve III pneumococcus polysaccharide type I, Curve IV pectic acid, Curve V alginic acid, Curve VI hyaluronic acid, Curve VII chondroitinsulfuric acid. Wave-lengths in millimicrons (mμ).
As can be seen, the pink color given by the glucuronic acid is due to a
steep rise of the absorption starting at 5000A and reaching a maximum at
5400 A. The curves for hyaluronic and chondroitinsulfuric acids are almost
identical in shape with that of glucuronic acid, though the absolute values
for absorption are somewhat smaller for chondroitinsulfuric acid. This
may be due to the fact that not all of the hexuronic acid in this polyuronide
is glucuronic acid.

Table I

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Substance</th>
<th>$E_{4800}$</th>
<th>$E_{5100}$</th>
<th>$E_{5400}$</th>
<th>$E_{4800} - E_{5100}$</th>
<th>$E_{5100} - E_{5400}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Glucuronic acid</td>
<td>0.050</td>
<td>0.098</td>
<td>0.210</td>
<td>+0.068</td>
<td>+0.150</td>
</tr>
<tr>
<td></td>
<td>Alginic acid</td>
<td>0.090</td>
<td>0.073</td>
<td>0.110</td>
<td>-0.017</td>
<td>+0.020</td>
</tr>
<tr>
<td></td>
<td>Glucuronic + alginic acid</td>
<td>0.065</td>
<td>0.105</td>
<td>0.285</td>
<td>+0.040</td>
<td>+0.220</td>
</tr>
<tr>
<td>II</td>
<td>Glucuronic acid</td>
<td>0.060</td>
<td>0.180</td>
<td>0.310</td>
<td>+0.120</td>
<td>+0.250</td>
</tr>
<tr>
<td></td>
<td>Galacturonic acid</td>
<td>0.055</td>
<td>0.055</td>
<td>0.080</td>
<td>0</td>
<td>+0.025</td>
</tr>
<tr>
<td></td>
<td>Glucuronic plus galacturonic acid</td>
<td>0.055</td>
<td>0.170</td>
<td>0.365</td>
<td>+0.115</td>
<td>+0.310</td>
</tr>
<tr>
<td>III</td>
<td>Glucuronic acid</td>
<td>0.015</td>
<td>0.120</td>
<td>0.270</td>
<td>+0.105</td>
<td>+0.255</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid</td>
<td>0.025</td>
<td>0.120</td>
<td>0.245</td>
<td>+0.095</td>
<td>+0.220</td>
</tr>
<tr>
<td></td>
<td>Chondroitinsulfuric acid</td>
<td>0.085</td>
<td>0.115</td>
<td>0.225</td>
<td>+0.080</td>
<td>+0.190</td>
</tr>
<tr>
<td></td>
<td>Alginic acid</td>
<td>0.280</td>
<td>0.260</td>
<td>0.300</td>
<td>-0.020</td>
<td>+0.020</td>
</tr>
<tr>
<td>IV</td>
<td>Glucuronic acid</td>
<td>0.085</td>
<td>0.240</td>
<td>-0.030</td>
<td>0</td>
<td>+0.155</td>
</tr>
<tr>
<td></td>
<td>Pectic acid</td>
<td>0.065</td>
<td>0.035</td>
<td>0.065</td>
<td>0</td>
<td>+0.040</td>
</tr>
<tr>
<td></td>
<td>Pneumococcus polysaccharide type I</td>
<td>0.000</td>
<td>0.075</td>
<td>0.130</td>
<td>-0.015</td>
<td>+0.040</td>
</tr>
<tr>
<td></td>
<td>Alginic acid</td>
<td>0.105</td>
<td>0.060</td>
<td>0.115</td>
<td>-0.045</td>
<td>+0.010</td>
</tr>
<tr>
<td>V</td>
<td>Glucuronic acid</td>
<td>0.150</td>
<td>0.400</td>
<td>0.060</td>
<td>+0.250</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Galacturonic acid</td>
<td>0.076</td>
<td>0.076</td>
<td>0.138</td>
<td>+0.001</td>
<td></td>
</tr>
</tbody>
</table>

The absorption curves for the other hexuronides and galacturonic acid
are completely different. They show a flat minimum between 5000 and
5100 A.

Absorption Increment between Two Wave-Lengths As Test for Glucuronic
Acid—The characteristic differences in the shape of the absorption curve
between glucuronic acid and polyglucuronides, on the one hand, and other
hexuronic acids and corresponding polyuronides, on the other, permit the
detection of glucuronic acid, free or conjugated, in presence of an excess of
other hexuronic acids. For this purpose we have only to measure the in-
tensity of MThR of the solution at 5100 and 4800 Å and subtract the second value from the first. This difference will be designated \( \Delta E_{5100 - 4800} \). As can be seen from Table I this difference is positive for glucuronic acid and polyglucuronides and negative for the other hexuronic acids. Any positive value of \( \Delta E_{5100 - 4800} \) in an unknown solution indicates the presence of glucuronic acid. Positive values will be obtained even when glucuronic acid is present with a considerable excess of other hexuronic acids, because the negative values of \( \Delta E_{5100 - 4800} \) of other hexuronic acids are much smaller than the positive values for equivalent amounts of glucuronic acid.

If the purpose of the test is to exclude the presence of glucuronic acid in solution, it seems more convenient to determine the difference \( \Delta E_{6100 - 4800} \). Table I shows this difference to be \(+0.012\) for galacturonic acid (average of two determinations) and for alginic acid (average of four determinations) and zero for pectic acid (one determination). If \( \Delta E_{6100 - 4800} \) is not larger than 0.012, it can be assumed that no significant amounts of glucuronic acid are present in the solution.

**Influence of Hexuronic Acids and True Sugars on MThR of Glucuronic Acids**—When the concentration of every one of the hexuronic acids in solution does not exceed 0.01 per cent, \( \Delta E_{5100 - 4800} \) and \( \Delta E_{9300 - 4800} \) show no significant deviations from the additive behavior. When, however, the concentration of galacturonic or mannuronic acid exceeds that limit, the intensity of MThR of glucuronic acid is decreased and the test becomes less sensitive. It is therefore practical to work with solutions containing no more than about 0.01 per cent of total uronic acids.

True sugars also influence the intensity of MThR when their concentration exceeds certain limits; at 0.005 per cent galactose and arabinose are without influence, but glucose decreases MThR by about 20 per cent. The influence of glucose disappears at 0.002 per cent. A simple procedure for determining whether the concentration of hexoses in solution does not exceed this limit is the following. \( E_{5100} - E_{4800} \) is negative for the ThR of all hexuronic acids. When hexoses are present, the value of this difference is shifted towards the positive side. If after the addition of 0.01 cc. of glucuronic acid to the unknown solution, \( E_{6100} - E_{4800} \) of the ThR is not above zero, the concentration of hexoses in the solution is not high enough to influence MThR of glucuronic acid.

**DISCUSSION**

The validity of preliminary identifications based on color reaction always will appear subject to certain limitations. One of them is due in our case to the fact that the nature and mechanism of the reaction are unknown. It is, therefore, impossible to predict whether certain hexuronic or 5-keto-hexonic acids or related compounds, so far not investigated, may not give
the reaction. So far only three hexuronic acids were found in nature, but others may be found in the future. Under these circumstances conclusions can safely be drawn only when our color reaction is negative. This indicates absence of glucuronic acid.

**SUMMARY**

1. A new characteristic color reaction of glucuronic acid is described.
2. This reaction permits the detection of glucuronic acid in free form or in polyuronides in presence of an excess of other hexuronic acids.
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