The preparation of an extract from animal tissues containing an enzyme specific for the hydrolysis of \( \beta \)-glucuronides was first described by Masamune \( (1) \). Oshima \( (2, 3) \) later studied the distribution of this enzyme in the tissues of the dog and the ox and improved the method of preparation of active extracts.

At the suggestion of Professor G. F. Marrian an investigation was undertaken to determine whether this enzyme might be concerned with the synthesis of estriol glucuronide in the female organism. Furthermore, it was suggested that the hydrolysis of conjugated sex hormones in human urine for assay purposes by a potent preparation of the enzyme might prove to be more efficient than hydrolysis by mineral acids.

As a preliminary to the main problems in this investigation, a study of the methods of preparation and the properties of \( \beta \)-glucuronidase has been made. The present paper deals mainly with a description of the methods of preparation of the enzyme. Further, experiments designed to indicate the possible role of the enzyme in the detoxification of glucuronogenic substances by the mammalian organism are now in progress and will be reported at a later date.

**Method of Assaying Activity of \( \beta \)-Glucuronidase Preparations**

**Determination of Glucuronic Acid**—The method developed by Miller and Van Slyke \( (4) \) for the microdetermination of blood sugar was modified for the determination of glucuronic acid. In

* The term \( \beta \)-glucuronidase is here used in preference to the term \( \beta \)-glucuronosidase as suggested by Masamune.
the blood sugar method, potassium ferricyanide is reduced by the sugar and the amount of ferrocyanide so produced is then determined by a direct titration with standard ceric sulfate solution, setopaline C being used as the oxidation-reduction indicator.

The routine assay of the activity of β-glucuronidase preparations was greatly simplified by substituting the ceric sulfate titration method for the Hagedorn-Jensen technique, as employed by the Japanese workers.

Glucuron, rather than glucuronic acid, was selected for the standardization, since it can be more readily prepared in a pure state. Assuming that the reducing powers of glucuron and glucuronic acid are proportional to their respective molecular weights, it is possible, after titrating known amounts of glucuron with ceric sulfate, to construct a standard graph correlating the corresponding amounts of glucuronic acid with the titration values. The ceric sulfate solution was therefore standardized against glucuron in the following manner.

50 mg. of pure glucuron were dissolved in 50 cc. of distilled water. Aliquots of this solution were then pipetted into 50 cc. boiling tubes, an excess (2.5 cc. per mg. of glucuron) of alkaline potassium ferricyanide solution (5 gm. per liter) was added, and the volumes were equalized with distilled water. The tubes were covered with blown glass bulbs and placed in a vigorously boiling water bath for 15 minutes. They were then cooled in running water and the contents of each tube were titrated with 0.0216 N ceric sulfate solution after the addition of 1 cc. of 18 N H₂SO₄ and 8 to 10 drops of setopaline C solution.

The amounts of glucuronic acid corresponding to the amounts of glucuron used were plotted, as in Fig. 1, against the ceric sulfate titration values.

By using a greater dilution of ceric sulfate, the method was applicable to the determination of much smaller amounts of glucuronic acid. For example, with 0.002 N ceric sulfate solution,

1 In view of the observation of Goebel and Babers (5) that there is a slight difference in the relative reducing powers of glucuronic acid and glucuron when determined by the Shaffer-Hartmann procedure, this assumption may be only approximately true.

2 A standard 10 cc. burette, graduated in 0.05 of a cc., was employed.

3 In the preparation and standardization of the ceric sulfate solution, the directions of Miller and Van Slyke were followed.
0.1 to 1.2 mg. of glucuronic acid could be estimated over a titration range of 14 cc.

Preparation and Treatment of Enzyme Digests—Sodium menthol glucuronidate was the substrate used in the experiments described in this paper. Pure menthol glucuronide was prepared by Bang's method, as described by Quick (6). In preparing standard solutions of the sodium salt, the calculated volume of N sodium hydroxide solution was added to a weighed amount of menthol glucuronide in a 100 cc. volumetric flask together with 75 cc. of distilled water. The pH of this solution was then adjusted to 4.8 to 5.4 (outside indicator) with a few drops of N acetic acid. The solution was finally diluted to 100 cc.

The experimental tubes (50 cc.) contained enzyme extract, 0.10 N acetate buffer of pH 5.2 to 5.4, and substrate solution usually in the ratio of 1:2:1, while the control tube contained buffer, substrate, and boiled enzyme in the same proportions. The tubes were stoppered and incubated for the desired length of time at 37.5°. At the end of this period, each digest was treated in the following manner.
After the digest was transferred to a 15 cc. centrifuge tube, an equal volume of 10 per cent trichloroacetic acid solution was added. Following centrifugation, the clear supernatant solution was returned to the original boiling tube and neutralized to phenolphthalein with 2 N NaOH. Potassium ferricyanide solution (10 cc.) was added and the reducing power determined by the modified ceric sulfate method. The amount of glucuronic acid liberated was read from the standardization curve (Fig. 1) by substituting the titration difference between control and experimental digests.

**Preparation and Purification of β-Glucuronidase Extracts**

Little success was realized in obtaining a potent purified enzyme extract by the procedure of Oshima (3). After a critical examination of the properties and solubilities of the enzyme, the present method was devised and found to be satisfactory. This process involves (1) initial extraction of the minced tissue with water, (2) acetone precipitation of the crude extract and subsequent extraction of the precipitate so produced with water, (3) acidification of this aqueous extract with acetic acid to pH 4.8 to 5.0, (4) evaporation to small volume in a current of air, (5) the application of an ammonium sulfate fractional extraction and precipitation technique to the concentrate. As high as 140-fold purifications have been achieved in this manner.

The resulting purified solution of the enzyme possessed as great a potency as seemed necessary for the purposes outlined in the introduction and accordingly no further purification studies were undertaken. The details of the method of preparation and purification are as follows:

Fresh beef spleen, free of fascia, was ground in a mincer. 585 gm. of the tissue were then stirred for an hour with 1170 cc. of distilled water. This crude extract (A) was shaken with diatomaceous earth (20 gm. per liter) and centrifuged for 30 minutes. The supernatant solution (B) was precipitated with 2 volumes of acetone. The reddish brown precipitate which formed was centrifuged immediately, the supernatant solution was poured off, and the residue transferred with the aid of a rubber-tipped glass rod to a 2 liter beaker. Inasmuch as acetone seemed to inactivate the enzyme, a 5 minute period of rapid centrifugation, followed by
the removal of some of the acetone from the precipitate with a stream of air, was adopted as a precautionary step at this stage of the purification. The precipitate was vigorously stirred for 1 hour with 1100 cc. of water.

The water-insoluble material was centrifuged off, leaving a very turbid supernatant solution containing the enzyme (C). The solution was adjusted to pH 4.8 to 5.0 with N acetic acid and warmed at 37.5° for 20 minutes. It was then centrifuged and the supernatant solution adjusted to pH 7 with N NaOH (D). The extract (1100 cc.) was poured into shallow porcelain dishes and evaporated to 160 cc. in a current of air at room temperature (E).

An equal volume of saturated ammonium sulfate solution was added to the concentrate; the precipitate was separated and dissolved in 100 cc. of water (F). The precipitate formed by adding to solution (F) 100 cc. of saturated aqueous ammonium sulfate was then stirred with 350 cc. of 35 per cent saturated ammonium sulfate solution. The insoluble fraction of this mixture was separated by centrifugation and preserved. The addition of 75 cc. of saturated ammonium sulfate solution to the centrifugate caused complete precipitation of the enzyme, which was separated and dissolved in 50 cc. of water. The enzyme was precipitated from this aqueous solution (G) by half saturation with ammonium sulfate and the precipitate was stirred with 300 cc. of 40 per cent saturated ammonium sulfate solution. The insoluble residue was preserved and the extract was then brought to 50 per cent saturation by the addition of 55 cc. of saturated ammonium sulfate solution. The precipitate, after centrifuging, was dissolved in 25 cc. of water (H).

An aqueous suspension (130 cc.) of the combined 35 and 40 per cent insoluble material was half saturated with ammonium sulfate and centrifuged. The precipitate was extracted with 300 cc. of 37 per cent saturated ammonium sulfate solution and to the supernatant solution, after centrifuging, 85 cc. of saturated ammonium sulfate solution were added. The precipitate was centrifuged off and dissolved in 25 cc. of water (H). Solutions H₁ and H₂ were combined, this final clear solution being denoted as (I) in Table I.

The activity and protein nitrogen content of the extract at each stage of the purification were determined in order to obtain quanti-
tative purification data (Table I). Digests contained 1 cc. of enzyme extract (boiled in the control digests), 2 cc. of acetate buffer at pH 5.4, and 1 cc. of 0.056 N sodium menthol glucuronidate. After incubation for 8 hours at 37.5° the liberated glu.
curonic acid was determined by the ceric sulfate titration method.

**Table I**

*Preparation and Purification of β-Glucuronidase from Ox Spleen*

Hydrolysis was carried out at pH 5.4, 37.5°, for 8 hours; substrate, 0.0156 N sodium menthol glucuronidate.

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage of preparation</th>
<th>Activity†</th>
<th>Volume of extract</th>
<th>Total activity†</th>
<th>Activity per mg. protein N†</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Aqueous suspension of minced tissue</td>
<td>4.90</td>
<td>1685</td>
<td>8200</td>
<td>0.63</td>
</tr>
<tr>
<td>B</td>
<td>Supernatant solution after centrifuging (A)</td>
<td>4.60</td>
<td>1140</td>
<td>5200</td>
<td>0.66</td>
</tr>
<tr>
<td>C</td>
<td>Aqueous extract of acetone ppt. of (B)</td>
<td>3.45</td>
<td>1190</td>
<td>4100</td>
<td>2.41</td>
</tr>
<tr>
<td>D</td>
<td>Acidified extract</td>
<td>3.25</td>
<td>1100</td>
<td>3500</td>
<td>8.10</td>
</tr>
<tr>
<td>E</td>
<td>Evaporated concentrate</td>
<td>15.5</td>
<td>160</td>
<td>2500</td>
<td>5.96</td>
</tr>
<tr>
<td>F</td>
<td>Solution of ppt. obtained from (E) by half saturation with (NH₄)₂SO₄</td>
<td>20.0</td>
<td>112</td>
<td>2200</td>
<td>16.80</td>
</tr>
<tr>
<td>G</td>
<td>Product of fractional extraction of ppt. salted-out from solution (F) with 35% saturated (NH₄)₂SO₄ solution</td>
<td>29.6</td>
<td>50</td>
<td>1500</td>
<td>45.50</td>
</tr>
<tr>
<td>H₁</td>
<td>(G) repeated on solution (G) with 40% saturated (NH₄)₂SO₄</td>
<td>34.0</td>
<td>25</td>
<td>850</td>
<td>63.00</td>
</tr>
<tr>
<td>I</td>
<td>Solution (H₁) combined with product of fractional extraction (37%) of insoluble residues from (G) and (H₁)</td>
<td>26.0</td>
<td>50</td>
<td>1300</td>
<td>87.00</td>
</tr>
</tbody>
</table>

* Mg. of glucuronic acid liberated per 1 cc. of extract.
† Number of cc. of extract times the number of mg. of glucuronic acid liberated per 1 cc. of extract.
‡ Mg. of glucuronic acid liberated per mg. of nitrogen precipitable by 10 per cent trichloroacetic acid.

Before the activity of highly concentrated glucuronidase solutions was assayed, they were first diluted to strengths such as 1:5, 1:10, and 1:20, depending on the amount of enzyme present. This course was necessary to avoid (1) a lowering of the pH of the digest by too great a liberation of glucuronic acid and (2) marked varia-
tions in the velocity of hydrolysis, the latter being eliminated when conditions were such that the final substrate concentrations in the digests were approximately the same. The activity of an extract was found to be directly proportional to the concentration of the enzyme.

In determining the protein nitrogen of the extracts, 1 cc. of the extract was diluted to 2 cc. with distilled water in a 15 cc. centrifuge cup and 2 cc. of 10 per cent trichloroacetic acid were added. After centrifuging, the supernatant solution was discarded, and the precipitate stirred with 5 cc. of water and centrifuged. Absence of sulfate ion indicated the removal of ammonium sulfate from the trichloroacetate precipitate likely to be adsorbed from extracts at steps (F) to (I). After the supernatant solution was discarded, 2 cc. of concentrated sulfuric acid were pipetted into the vessel and the mixture, after standing for 5 minutes, was transferred to a micro-Kjeldahl flask; washings were added and the total nitrogen was determined by a standard micromethod.

An experiment identical with that just described yielded a final preparation whose activity per mg. of nitrogen was appreciably greater than that of preparation (I) (Table I).

**SUMMARY**

1. A ceric sulfate titration method has been applied to the estimation of glucuronic acid liberated by the hydrolytic action of β-glucuronidase.

2. A rapid method for the concentration and purification of β-glucuronidase from ox spleen is described.

The author wishes to make grateful acknowledgment to Dr. G. F. Marrian, under whose direction this investigation has been carried out, and also to Dr. A. M. Wynne, for much helpful advice.

**BIBLIOGRAPHY**

STUDIES ON β-GLUCURONIDASE: I. A METHOD OF PREPARATION AND PURIFICATION
W. H. Fishman


Access the most updated version of this article at http://www.jbc.org/content/127/2/367.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/127/2/367.citation.full.html#ref-list-1