PRODUCTION AND PROPERTIES OF BACTERIAL
β-GluCURONIDASE

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Hydrolysis of glucuronides is a prerequisite for the quantitative determination or the isolation of a number of physiologically important compounds which are conjugated with glucuronic acid in the animal body. The use of acid for this hydrolysis has frequently brought about chemical alteration of the product under study (1–4). The need for a hydrolytic agent which is specific and sufficiently mild to preclude the possibility of chemical alteration has led to the investigation of biological catalysts (enzymes). Obviously, an enzyme possessing the capacity to hydrolyze glucuronides at a low temperature and approximate neutrality should fulfill these requirements. Preparations containing such activity have been obtained from both mammalian and bacterial sources. Fishman et al. (5–7) and others (8, 9) have published reports on the preparation and purification of the enzyme from animal sources, and Buehler et al. (10) have presented details of the preparation and use of β-glucuronidase from Escherichia coli. The experimental work which is reported in this paper defines more clearly the conditions for the production of the latter and describes some of the characteristics of the partially purified product.

EXPERIMENTAL

Assay of Bacterial β-Glucuronidase—The determination of the glucuronidase activity is carried out by a modification of the procedure outlined by Talalay, Fishman, and Huggins (11). To 0.1 ml. of the enzyme solution are added 1.9 ml. of 0.05 phosphate buffer (pH 6.8) and 1 ml. of a sodium phenolphthalein glucuronidate solution (pH 6.8) containing the equivalent of 320 y of phenolphthalein. After incubation of the resulting solution for ½ hour at 37°, 1 ml. is removed and added to 5 ml. of 0.4 M glycine buffer (pH 10.4). The phenolphthalein liberated, which is proportional to the

* The material presented herein is taken, in part, from a thesis submitted to the Graduate School of St. Louis University by Miles L. Doyle in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

1 Since the amount of phenolphthalein liberated is influenced by the concentration of the substrate, a relatively constant concentration of substrate is maintained throughout the assay by using an appropriate dilution of the enzyme, which permits the liberation of only from 10 to 25 y of phenolphthalein.
time of incubation and enzyme concentration, is determined by means of a Klett-Summerson colorimeter with the use of a green filter (range 500 to 570 ma) and a standard curve. The activity is expressed as micrograms of phenolphthalein liberated in 1 hour (micrograms of phenolphthalein liberated in \( \frac{1}{3} \) hour \( \times 2 \times 10 \times 3 \)), or units per ml. of the original enzyme solution.

Production of \( \beta \)-Glucuronidase by E. coli

**Inoculum**—The strain of *E. coli* which was originally (10) isolated from a patient with cystitis has been maintained on nutrient agar slants by periodic transfers. Inocula were prepared by loop transfer of the organism from agar slants to nutrient broth (0.3 per cent beef extract, 0.5 per cent peptone). After incubation at 37° for 24 hours, 1 ml. of the resulting culture was added to each 250 ml. of the culture fluid used for enzyme production; an excess of this inoculum or the use of older cultures (more than 24 hours) has been found to result in decreased enzyme production. These cultures were then incubated for 24 hours at 25° without agitation before being placed on the shaking machine.

**Composition of Medium**—In a systematic study to evaluate each of the ingredients which was used in the culture medium (10), it was found that sodium chloride and beef extract were unnecessary. In fact, the presence of beef extract somewhat retarded the production of enzyme.

Furthermore, the use of ammonium menthol glucuronidate as the substrate for stimulating glucuronidase production gave results which were as good as those obtained with the free acid. In media containing 1.0 per cent Difco Bacto-peptone and 3.0 per cent sodium glycerophosphate, the amount of enzyme produced increased with increasing concentration of ammonium menthol glucuronidate up to 1.0 per cent. However, increasing the substrate concentration above 0.6 per cent gave only a slight increase in the production of enzyme. Maximal activity was attained by incubation at 25° with constant agitation on a rotary shaking machine (80 to 100 r.p.m.) for periods of from 5 to 7 days.

The results of the studies to ascertain the optimal concentrations of peptone and sodium glycerophosphate are shown in Fig. 1. All samples of the culture media contained 0.6 per cent ammonium menthol glucuronidate and were adjusted to between pH 6.5 and 6.8. Maximal activities in Fig. 1 were obtained by incubation for 6 days under the conditions described above. Media containing 5 and 7.5 per cent sodium glycerophosphate gave the highest activities with all concentrations of peptone studied. With these concentrations of sodium glycerophosphate, maximal production of enzyme was obtained with 3 per cent peptone.

For the original culture medium employed by Buehler et al. (10), the
optimal pH was found to be 7.3. The culture medium, as presently modified, affords maximal production of enzyme at an initial pH of from 6.5 to 6.8. It was noted that during incubation the pH increased about 0.2 unit and that this change occurred during the period in which production of enzyme was rapid.

The modified medium is, therefore, composed of 0.6 per cent ammonium menthol glucuronidate, 5.0 per cent sodium glycerophosphate, 3.0 per cent Difco Bacto-peptone, and HCl to adjust the pH between 6.5 and 6.8.

![Graph](http://www.jbc.org/)

Fig. 1. Relationship of peptone and sodium glycerophosphate to the production of bacterial β-glucuronidase by *E. coli*. Concentration of sodium glycerophosphate, Curve 1, 3 per cent; Curve 2, 4 per cent; Curve 3, 5 per cent; Curve 4, 7.5 per cent. The media contained 0.6 per cent ammonium methanol glucuronidate in addition to the peptone and sodium glycerophosphate indicated in the figure. Values are reported for the 6th day of incubation, at which time the highest activity was reached; longer incubation did not yield higher yields. This value was taken as the maximum and is expressed as 100 per cent (the actual activity was 4200 units per ml.). All other values are expressed as per cent of this.

Beall and Grant (12) have reported that *E. coli* produces glucuronidase more rapidly if menthol glucuronide is replaced by the borneol conjugate. With our modified medium, maximal activity was obtained with 0.3 per cent borneol glucuronide after from 3 to 5 days of incubation. The activity was approximately the same as that obtained with 0.6 per cent ammonium menthol glucuronidate in from 5 to 7 days.

Also in agreement with Beall and Grant, we found that the enzyme is distributed between the centrifuged culture fluid and the cells. With either menthol or borneol glucuronide, most of the enzyme is found in the cells during the early stages of incubation, but, as the cultures age, increasing amounts of the enzyme are liberated into the culture fluid.
Conditions Influencing Activity of Bacterial $\beta$-Glucuronidase—Attempts to separate the enzyme from the cold (5°) culture filtrate by dialysis (10), fractional precipitation with ethanol (13), or salting out (14) invariably resulted in a marked loss of activity. Only a small proportion of the activity was present in the protein fractions and, in most instances, none in the supernatant solutions or dialysate. However, if solutions of the former were combined with the latter, a marked increase of activity occurred which accounted for a considerable portion of the original activity. Furthermore, augmentation was observed to occur if any of the protein fractions was combined with any of the complementary fractions (13).

In an attempt to determine the nature of this non-protein material, a number of cofactors for other enzymes were examined for their ability to augment the activity of this bacterial $\beta$-glucuronidase. These included the nucleic acids, other purine-containing compounds, B vitamins, and mineral ions. None was found to be effective; in fact, most of these compounds had an inhibitory effect.

To ascertain whether the loss of activity incurred in the separation of the protein fraction was due to a greater lability of this fraction, a study was made of the effect of the buffer employed in the assay, since it had been observed that the activity of crude culture filtrates is influenced by moderately small concentrations of certain ions. During this study it was found that the addition of a 0.001 M solution of each of eighteen $\alpha$-amino acids, adjusted to pH 6.5, to a solution of the protein fraction of the culture fluid resulted in a 2-fold increase of activity, which in most instances accounted for from 90 to 95 per cent of the activity of the original preparation. A 0.001 M solution of the $\beta$-amino acids, $\beta$-alanine, and $\beta$-aminohydrocinnamic acid was totally ineffective; at higher concentrations, a small increase in activity was observed.

These findings suggested the possibility that the action of the $\alpha$-amino acids is due to their chelating properties and that bacterial $\beta$-glucuronidase itself might be acting as a chelating agent. Accordingly, the effect of the well known chelating agents, Versene and 8-hydroxyquinoline, on the reactivation of enzyme preparations was studied. From the data of Table I, it can be seen that both substances are very effective in this regard and, on a molar basis, are more effective than the $\alpha$-amino acids. However, at concentrations of 5 mg. or more per ml. both inhibit enzyme activity.

Since the loss of enzymic activity and its reversal seemed to be associated with chelation, a study of the effectiveness of various metal ions on inhibition of the activity of whole culture filtrates or partially purified preparations was made. For this purpose, sufficient solution was added to the as-

* This increase in activity was observed for the protein fraction obtained by precipitation with ethanol or ammonium sulfate or by dialysis.
say mixture to give a concentration of $1 \times 10^{-3}$ M of the salt at pH 6.5. The activities were compared with those of control solutions under the same conditions, but without added cations. With Cu++, Hg++, and Ag⁺ the inhibition was complete, while with Ni++, Sn++, and Zn++ only partial inhibition occurred. Inhibitory effects were also observed with other cations only at higher concentrations, but in these instances it was not possible to make accurate measurements because of the formation of precipitates. It is of

**Table I**

*Effect of Chelating Agents on Activity of Bacterial β-Glucuronidase*

<table>
<thead>
<tr>
<th>Concentration of chelating agent in assay mixture (mg. per ml.)</th>
<th>Versene</th>
<th>8-Hydroxyquinoline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Activity, per cent of controls</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.5</td>
<td>373</td>
<td>826</td>
</tr>
<tr>
<td>1.0</td>
<td>386</td>
<td>657</td>
</tr>
<tr>
<td>5.0</td>
<td>181</td>
<td>57</td>
</tr>
<tr>
<td>10.0</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>20.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

A, solution of material precipitated by (NH₄)₂SO₄ from previously centrifuged culture fluid. The activity of this solution without the addition of chelating agents (control activity) was 450 units per ml. and was taken as 100. B, solution of material precipitated from centrifuged culture fluid by 65 per cent ethanol. The activity of this solution in absence of chelating agents was 115 units per ml., and this activity was taken as 100. Solutions of Versene and 8-hydroxyquinoline at pH 6.8 were prepared in such concentrations as to give from 0.5 to 20.0 mg. per ml. in the assay solution. 1 ml. portions of these solutions were added to 0.9 ml. of phosphate buffer (pH 6.8, ¼/45), and to this solution were added 0.1 ml. of the enzyme preparation and 1.0 ml. of phenolphthalein glucuronide solution. The mixture was incubated for 1 hour at 37°, at which time 1 ml. was removed and added to 5 ml. of 0.4 M glycine buffer (pH 10.4).

interest that none of the polyvalent ions studied, Al⁺⁺⁺, Fe⁺⁺⁺, and Sn⁺⁺⁺⁺⁺, produced an inhibitory effect.

The data of Table II show the minimal concentrations of Hg++, Cu++, and Ag⁺ that caused inhibition and the effect of Versene and cysteine in restoring activity. Cu++ and Ag⁺, in a concentration of from $1 \times 10^{-5}$ to $1 \times 10^{-6}$ M, produced approximately 50 per cent inhibition; a lower concentration ($1 \times 10^{-7}$ M) of Hg++ was effective in this regard. Versene in the concentration used was not protective if the concentration was greater than $1 \times 10^{-7}$ M for Hg++, $1 \times 10^{-4}$ M for Cu++, and $1 \times 10^{-6}$ M for Ag⁺. The greater effectiveness of cysteine in reversing the inhibition produced by Ag⁺ might be due to the reducing activity of the sulfhydryl group, as well as to
its chelating activity. Cysteine also readily reversed the action of Cu++ and Hg++. More direct evidence that bacterial β-glucuronidase contains the sulfhydryl group was obtained by using p-chloromercuribenzoate, which Hellerman et al. (15) have shown is a highly specific sulfhydryl-binding agent, and the reversal of the action of this compound by cysteine, as shown by Harris and Hellerman (16), for the enzyme xanthine oxidase. Data from Table II

**Effect of Versene on Inhibition by Hg++ and Cu++ and Effect of Versene and Cysteine on Inhibition by Ag++**

<table>
<thead>
<tr>
<th>Concentration of metal ion</th>
<th>Hg++</th>
<th>Cu++</th>
<th>Ag+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Versene 1.3 × 10⁻⁴ M</td>
<td>No Versene</td>
<td>Versene 1.3 × 10⁻⁴ M</td>
<td>No Versene</td>
</tr>
<tr>
<td>Activity, per cent of control†</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>1 × 10⁻⁷</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1 × 10⁻⁴</td>
<td>1063</td>
<td>13</td>
<td>100</td>
</tr>
<tr>
<td>1 × 10⁻⁴</td>
<td>853</td>
<td>0</td>
<td>966</td>
</tr>
<tr>
<td>1 × 10⁻⁵</td>
<td>666</td>
<td>0</td>
<td>666</td>
</tr>
<tr>
<td>1 × 10⁻⁶</td>
<td>0</td>
<td>0</td>
<td>520</td>
</tr>
<tr>
<td>1 × 10⁻⁷</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* All concentrations listed are for the assay mixtures.
† Activity of the enzyme solution, without the addition of metal salts or either Versene or cysteine, taken as 100.
‡ A different enzyme preparation was used for the study of the effect of Ag⁺. These studies were carried out on solutions of the product obtained from culture fluid, which had previously been centrifuged, by half saturation with (NH₄)₂SO₄. The pH of the assay mixtures was maintained at 6.8 by the adjustment, of all solutions used, to that pH before mixing and the use of 1/45 phosphate buffer at that pH.

such studies are reported in Table III. The inhibition produced by p-chloromercuribenzoate, which was 90 per cent complete at a concentration of 1.7 × 10⁻⁴ M, was satisfactorily reversed by cysteine. These results seem to be explained best on the basis of a competition between the —SH groups of the enzyme and cysteine for the mercuribenzoate. Evidence for this view is supplied by the data in Fig. 2 in which the reciprocal of the concentration of the protective agent is plotted against the reciprocal of the activity of a given concentration of the inhibiting agent and enzyme. Data plotted according to Lineweaver and Burk (17) have been used to illustrate competitive inhibition in the simplest case of competition between substrate and inhibitor for an active site on the enzyme molecule. In such a presen-
tation of data with varying substrate levels, the intercept on the ordinate is the same for the curve for the inhibited enzyme and for the non-inhibited enzyme. However, in the case presented here, the analysis of the data, in

### Table III

**Inhibition of β-Glucuronidase by p-Chloromercuribenzoate and Reversal of Inhibitory Effect by Cysteine**

<table>
<thead>
<tr>
<th>p-Chloromercuribenzoate</th>
<th>Without cysteine</th>
<th>Cysteine $4.2 \times 10^{-4}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units per ml.</td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>1920</td>
<td>2592</td>
</tr>
<tr>
<td>$9 \times 10^{-7}$</td>
<td>1380</td>
<td>2400</td>
</tr>
<tr>
<td>$9 \times 10^{-6}$</td>
<td>624</td>
<td>2280</td>
</tr>
<tr>
<td>$1.7 \times 10^{-6}$</td>
<td>528</td>
<td>2256</td>
</tr>
<tr>
<td>$9 \times 10^{-6}$</td>
<td>276</td>
<td>2220</td>
</tr>
<tr>
<td>$1.7 \times 10^{-4}$</td>
<td>180</td>
<td>2100</td>
</tr>
</tbody>
</table>

The enzyme preparation was a solution of the material obtained from clarified culture fluid by precipitation with ammonium sulfate at half saturation. An alkaline solution of the sodium salt of p-chloromercuribenzoate was prepared and the pH adjusted to 6.8. This compound was added in the concentrations listed to buffered (phosphate buffer $M/45$, pH 6.8) enzyme solutions and allowed to stand for 15 minutes before the addition of cysteine.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Effect of Versene and cysteine on the activity of solutions of β-glucuronidase containing p-chloromercuribenzoate. The reciprocals of the molar concentrations of Versene and cysteine were plotted against the reciprocals of the activities, which were expressed in percentage of the maximal activity in the presence of only the protective agent. The lowest line, in each case, shows the effect of the protective agent on enzymic activity in the absence of p-chloromercuribenzoate (p-CMB).

The enzyme preparation was a solution of the product obtained by precipitation with ammonium sulfate.
which $1/V$ is plotted versus $1/(\text{cysteine})$, indicates a competition between enzyme and cysteine for $p$-chloromercuribenzoate. The reagents which react with mercuribenzoate would be expected to reverse the inhibition. The mechanism for reversal of inhibition by cysteine and Versene appears to be different. This difference may perhaps be explained by the consideration that cysteine possesses a sulfhydryl group, whereas Versene does not.

Since precipitation of the enzyme by various means usually yields products of relatively low activity and since full activity is restored by the action of chelating agents or compounds containing sulfhydryl groups (cysteine, sodium thioglycolate, or sodium hydrogen sulfide), the incorporation of such a protective substance in the assay has been adopted. Versene has been used routinely for this purpose because of its greater stability, while cysteine is used in those instances in which Versene is ineffective.

Purification of Bacterial $\beta$-Glucuronidase—Although the main purpose of this paper is to define more clearly the cultural conditions for the production of the enzyme and some of the properties of the enzyme, the inclusion of some notes on the purification may be helpful to those interested in using the enzyme.

Culture fluids prepared as described above and harvested at or near the time of maximal enzyme production were chilled to 5° in the cold room to aid the crystallization of free menthol. All procedures used in the purification of the enzyme were carried out at this temperature. After centrifugation (10,000 r.p.m. in a Servall S-1 angle head centrifuge) for 15 minutes to remove cellular material, the supernatant fluid was filtered to remove menthol crystals. The solution thus obtained usually contained from 65 to 80 per cent of the activity of the whole culture. The remainder of the activity has been recovered from the cellular material by a procedure described in a subsequent paragraph.

Fractionation with Ammonium Sulfate—The solution obtained by centrifugation and filtration was treated with an equal volume of saturated ammonium sulfate and permitted to stand for several days or until the precipitate settled. Most of the clear supernatant solution was removed by aspiration and the precipitate collected by centrifugation. This brownish material invariably contained approximately all of the activity of the culture filtrate and had a potency of from 400 to 700 units per mg. of protein. Protein was determined by the micromethod of Lowry et al. (18) with Armour's crystalline bovine albumin as a reference standard.

This precipitate was dissolved in a volume of $\pi/15$ phosphate buffer at pH 8 equivalent to about one-twenty-fifth of the volume of the culture fluid, and the solution was clarified by centrifugation. The clear solution was then fractionally precipitated with ammonium sulfate at concentrations of from 30 to 55 per cent of saturation. Precipitation at pH 6, 7, and 8 re-
sulted in essentially the same distribution of activity and potency; at pH 5, however, there was a marked loss of activity, which was not restored by cysteine. Most of the activity was present in the fractions which precipitated from solution with from 32 to 45 per cent of saturation with ammonium sulfate; the activity of these fractions ranged from 1200 to 4000 units per mg. of protein. Refractionation of the active fractions with ammonium sulfate increased the potency only slightly.

Adsorption on Calcium Phosphate Gel—Calcium phosphate gel containing 40 mg. of calcium phosphate per ml. of gel was prepared by the method of Kunitz (19) and the pH adjusted to 6.5 with acetic acid. The most active precipitates obtained by salting out with ammonium sulfate were dissolved in from 10 to 20 ml. of 0.003 M cysteine (adjusted to pH 7) to give a concentration of from 20 to 40 mg. of protein per ml. of solution. The addition of from 0.1 to 1.0 gm. of the gel adsorbed the enzyme completely. Elution, which was accomplished by the addition of the 0.05 M phosphate buffer containing cysteine (0.006 M) at pH 8, usually resulted in the recovery of more than 80 per cent of the enzyme. In order to repeat the adsorption, it was first necessary to precipitate the enzyme with ammonium sulfate to separate it from the phosphate which interfered with the adsorption. Each treatment with calcium phosphate has substantially increased the potency, resulting in the production of preparations with an activity of 13,500 units per mg. of protein after five successive treatments.

Liberation of Enzyme from Cells—The cells, obtained from each liter of the culture fluid, were suspended in 150 ml. of cold distilled water and allowed to stand for from 2 to 4 hours in the cold room. Each 100 ml. of suspension was then treated with 50 gm. of ammonium sulfate and after vigorous shaking and standing overnight was centrifuged. The supernatant solution was discarded, and the insoluble material was leached three times with small volumes of M/15 phosphate buffer at pH 8, with centrifugation after each leaching. The pooled supernatant solutions were treated with sufficient ammonium sulfate to give one-half saturation. The precipitated material thus obtained was light yellow, and several preparations had activities of between 1000 and 2000 units per mg. of protein.

SUMMARY

Increased production of bacterial β-glucuronidase has been obtained by culturing Escherichia coli on a medium containing 5 per cent sodium glycerophosphate, 3 per cent Difco Bacto-peptone, and 0.6 per cent ammonium menthol glucuronidate at pH 6.5 to 6.8.

Loss of activity during purification was prevented by the addition of α-amino acids, chelating agents, or sulfhydryl-containing compounds.

The enzyme is completely inhibited by Cu++, Hg++, and Ag+ at concen-
trations of $1 \times 10^{-4}$ M, $1 \times 10^{-7}$ M, and $1 \times 10^{-6}$ M, respectively. This effect is reversed by chelating agents and sulfhydryl-containing compounds. The complete inhibition by $1 \times 10^{-4}$ M $p$-chloromercuribenzoate is readily reversed by cysteine and to a lesser extent by Versene.

With the use of cysteine as a protective agent, it has been possible to achieve a 180-fold purification of this enzyme by fractional precipitation with ammonium sulfate and by repeated adsorption on calcium phosphate gel.

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BIBLIOGRAPHY

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