ENZYMATIC SYNTHESIS OF N-GLUCOSYLUROニック
ACID CONJUGATES*

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Enzymes in the microsomes of mammalian liver have been shown to
transfer the glucuronosyl moiety from uridine diphosphate glucuronic
acid to phenolic (2-4) and carboxylic acid acceptors (5). The present
paper describes the enzymatic synthesis of another type of conjugate in
which the glucuronic acid is transferred to an amino group.

Materials and Methods

Chemicals—o-Aminophenyl glucosiduronic acid was kindly supplied by
Dr. R. T. Williams, St. Mary's Hospital Medical School, London. o-
Aminobenzoyl and p-aminobenzoyl glucosiduronic acids were prepared
enzymatically with UDP-glucuronic acid and aglycone (5). Phenol-
phthalein glucosiduronic acid and bacterial β-glucuronidase were obtained
from the Sigma Chemical Company, St. Louis, Missouri. UDP-glucuronic
acid was prepared enzymatically from UDPG, with UDPG dehydrogenase
and DPN (4).

An aniline-glucuronic acid complex was generously supplied by Dr. R. T.
Williams (6). This compound contained 2 moles of aniline per mole of
glucuronic acid. 1 mole of aniline could be removed by washing a solution
of the compound at pH 8.0 with ether. The resulting aniline-glucuronic
acid compound did not reduce triphenyltetrazolium in 0.5 N NaOH, but,
following acidification with 0.1 N HCl, both the reducing group of glu-
curonic acid and the amino group of aniline were liberated. These ob-
servations indicated that the compound was N-phenylglucuronosylamine,
in which the amino group was linked to carbon 1 of glucuronic acid.

Methods—Aniline and p-toluidine were extracted at pH 7.0 or above
into 5 volumes of ethylene dichloride containing 1.5 per cent isoamyl
alcohol. The compounds were returned to 1 ml. of 0.1 N HCl and de-
termined in 0.5 ml. of the acid extract by diazotization and by coupling

* A preliminary report of this work has appeared previously (1).

1 The following abbreviations will be used: DPN for oxidized diphosphopyridine
nucleotide, UDP-glucuronic acid for uridine diphosphate glucuronic acid, UDPG
for uridine diphosphate glucose, Tris for tris(hydroxymethyl)aminomethane.

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with N-1-naphthylethylenediamine dihydrochloride (7). p-Phenetidine was assayed by a procedure described previously (8). In the determination of N-glucuronosyl compounds produced during the enzymatic reaction, the unchanged free amines were removed by shaking the incubation mixture twice with 5 volumes of ether. An equal volume of 0.5 N HCl was then added to the extracted aqueous solution. After waiting 5 minutes, the solution was made alkaline with sodium hydroxide, and the liberated amine was determined as described above.

o-Aminophenyl glucosiduronic acid was measured by the procedure of Levy and Storey (9). o-Aminobenzoyl glucosiduronic acid and p-aminobenzoyl glucosiduronic acid were determined by the procedure described by Dutton (5). Phenolphthalein was measured at 540 mg after adjusting the pH to 10.0. Glucuronic acid was estimated by the carbazole reaction (10).

Preparation of Tissues—Microsomes from guinea pig liver were prepared by a procedure described previously (4). Nuclei, mitochondria, and soluble fractions were prepared by differential centrifugation of liver homogenates in isotonic sucrose (11).

Enzyme Assay—A typical incubation consisted of microsomes obtained from 300 mg. of guinea pig liver, 25 µmoles of MgCl₂, 0.1 µmole of UDP-glucuronic acid, 0.5 µmole of substrate, 100 µmoles of Tris buffer, pH 8.0, and water to make a final volume of 1.5 ml. The mixture was incubated at 37° in a 15 ml. glass-stoppered centrifuge tube. After 30 minutes, the mixture was cooled, and the N-glucuronosyl derivatives were determined as described under "Methods."

Results

Enzymatic Synthesis of N-Glucuronosyl Derivatives of Aniline and Other Amines—The formation of a glucuronic acid conjugate of aniline was indicated by the disappearance of the amine after incubation with guinea pig microsomes and UDP-glucuronic acid (cf. (1), Table I). In the absence of UDP-glucuronic acid or when this cofactor was replaced with UDPG or glucuronolactone, little or no disappearance of aniline was observed. To determine whether the aniline that disappeared was present in “bound” form, the residual aniline was removed from the incubation mixture by extraction with ether, and the aqueous residue was acidified with hydrochloric acid. When this solution was kept at room temperature for 5 minutes, its extraction with ethylene dichloride at an alkaline pH was demonstrated by an almost complete recovery of the free amino compound. The liberated amine was identified as aniline by the technique of comparative distribution ratios (7). These observations suggested that the aniline which disappeared upon incubation with UDP-
glucuronic acid and guinea pig liver microsomes was converted to an acid-labile glucuronide, presumably N-phenylglucuronosylamine.

Further evidence for the identity of the enzymatically formed product was obtained by comparing its solubility and rate of hydrolysis with those of a synthetic sample. The biosynthetic and authentic samples in 0.1 N sodium hydroxide were shaken with 10 volumes of n-butanol (previously saturated with water), and the aqueous phase was assayed for conjugated aniline. 25 per cent of each compound was extracted into the organic solvent.

The rates of hydrolysis of the enzymatically formed and synthetic aniline conjugates were compared at various pH values and were found to be the same (Fig. 1). N-Phenylglucuronosylamine was most stable at an alkaline pH. Upon incubation with β-glucuronidase at pH 7.0, the rates of hydrolysis of both compounds were no greater than that observed with the boiled enzyme.

Incubating amines such as p-phenetidine and p-toluidine with guinea pig microsomes and UDP-glucuronic acid resulted in the formation of N-glucosyluronic acids (Table I).

Significant conjugating activity was present only in the microsomal fraction of guinea pig liver.

Properties of Enzyme System—In Tris buffer the rate of aniline conjugation was most rapid between pH 7.5 and 8.0. At pH 6.0 the reaction proceeded at 6 per cent of the maximal rate, while at pH 9.0 the reaction was 80 per cent of maximal.

![Diagram showing rates of hydrolysis of enzymatic and synthetic N-phenylglucuronosylamine.](http://www.jbc.org/)
As might be expected, the rates were the same whether incubation was aerobic or anaerobic.

The time-course of \(N\)-phenylglucuronosylamine formation is shown in Table I.

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions (0.1 (\mu)mole each)</th>
<th>(N)-Glucosyluronic acid formed ((\mu)mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline</td>
<td>None</td>
<td>0.008</td>
</tr>
<tr>
<td>(p)-Phenetidine</td>
<td>UDP-glucuronic acid</td>
<td>0.060</td>
</tr>
<tr>
<td>(p)-Toluidine</td>
<td>None</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>UDP-glucuronic acid</td>
<td>0.037</td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>UDP-glucuronic acid</td>
<td>0.075</td>
</tr>
</tbody>
</table>

**Fig. 2.** Rate of \(N\)-phenylglucuronosylamine synthesis. Each sample contained microsomes from 300 mg of guinea pig liver, aniline, and cofactors as described under "Methods."

Fig. 2. A similar rate of biosynthesis of hemiacetal and ester type glucuronide linkages has been shown (2, 5).

Table II shows that the presence of phenolic or carboxylic acid acceptors reduced the amount of \(N\)-phenylglucuronosylamine formed even in the presence of excessive amounts of UDP-glucuronic acid and that this depression could be overcome by adding larger amounts of the amino substrate.

**Hydrolysis of Various Types of Glucuronide Linkages**—The rates of hydrolysis of hemiacetal, ester, and \(N\)-glucosyluronic acids under dif-
ferent conditions were compared (Table III). N-Glucuronosyl derivatives were easily hydrolyzed in dilute acid at room temperature, but resisted attack by β-glucuronidase. The reverse was found to be true with hemi-

**Table II**

*Inhibition of N-Glucosyluronic Acid Synthesis*

Each sample containing microsomes obtained from 300 mg. of guinea pig liver, 100 μmoles of Tris buffer, pH 8.0, 25 μmoles of MgCl₂, 0.4 μmole of UDP-glucuronic acid, aniline hydrochloride, and inhibitors was incubated for 30 minutes at 37° and assayed for N-phenylglucuronosylamine formed.

<table>
<thead>
<tr>
<th>Aniline (μmole)</th>
<th>Inhibitor (μmole)</th>
<th>Inhibition (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>Phenol, 0.05</td>
<td>44</td>
</tr>
<tr>
<td>0.1</td>
<td>&quot;</td>
<td>67</td>
</tr>
<tr>
<td>0.1</td>
<td>&quot;</td>
<td>82</td>
</tr>
<tr>
<td>0.4</td>
<td>&quot;</td>
<td>33</td>
</tr>
<tr>
<td>0.1</td>
<td>Benzoic acid, 0.1</td>
<td>13</td>
</tr>
<tr>
<td>0.1</td>
<td>&quot;</td>
<td>40</td>
</tr>
<tr>
<td>0.4</td>
<td>&quot;</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table III**

*Hydrolysis of Various Types of Glucuronic Acid Linkages*

The final concentration of glucuronides was 1 × 10⁻⁴ m. The results are expressed as per cent of substrate hydrolyzed.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>N-Glucuronosyls of</th>
<th>α-Glucuronosides of</th>
<th>Ester glucuronides of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aniline</td>
<td>β-Toluidine</td>
<td>α-Aminophenol</td>
</tr>
<tr>
<td>β-Glucuronidase, 700 units, pH 7.4, 15 min., 37°</td>
<td>0*</td>
<td>0*</td>
<td>90</td>
</tr>
<tr>
<td>0.2 N HCl, 5 min., 25°...</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.2 &quot; NaOH, 20 hrs., 37°</td>
<td>30</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

* The rate of hydrolysis of N-glucosyluronic acids with β-glucuronidase was no greater than that observed with boiled enzyme.

acetal and ester type linkages. In alkaline solution, ester glucuronides were readily hydrolyzed, whereas hemiacetal glucuronides were stable. On the basis of these properties, the type of glucuronide linkage may be established.

**Formation of N-Phenylglucuronosylamine in Vivo**—Three guinea pigs were given 50 mg. per kilo of aniline hydrochloride intraperitoneally.
30 minutes before the administration of the amine, each animal received 600 mg. of sodium acetate to insure the excretion of an alkaline urine. Urine was collected for 24 hours in a beaker containing 2 ml. of 1 N sodium hydroxide. The free aniline was removed by extracting an aliquot of the urine with ethylene dichloride, and bound aniline was liberated after treatment with hydrochloric acid was determined. About 22 per cent of the administered aniline was excreted as an acid-labile conjugate. The conjugated aniline found in the urine had the same partition coefficient in the butanol-0.1 N NaOH system, as well as rates of hydrolysis at various pH values, as an authentic sample.

**DISCUSSION**

The data presented here demonstrate the enzymatic transfer of the glucuronosyl moiety of UDP-glucuronic acid to an amine acceptor. These observations suggest that this biosynthetic reaction may represent an important pathway for the metabolism of amino compounds and would explain the findings of others that the administration of amines results in the excretion of N-glucosyluronic acids (12, 13).

It appears likely, on the basis of the evidence presented by Dutton (5) and the current investigation, that a single enzyme catalyzes the transfer of glucuronic acid to phenolic, alcoholic, carboxylic, and amino acceptors, although a definitive answer must await purification of the enzyme. The failure of UDPG to act as a donor, however, indicates at least a certain degree of specificity with regard to the "active" sugar. Because of its non-specificity, the glucuronosyl-transferring enzyme appears eminently suitable for the "detoxification" of a wide variety of foreign compounds as well as of those which are normally present. It is interesting in this regard that, of the numerous possible alcoholic acceptors tested, all but N-acetylglucosamine inhibited the formation of N-phenylglucuronosylamine, which may suggest that N-acetylhyalobiuronic acid formation is not catalyzed by this enzyme.

The present study, taken with the work of other investigators, indicates that the formation of glucosiduronic acid is a nucleophilic substitution reaction in which the electron-donating aglycone displaces the UDP moiety (3) from the anomic carbon atom of UDP-glucuronic acid by a backside attack. This formulation rationalizes the inversion of the α-glycosidic bond of the nucleotide to the β configuration of the glucosiduronic acid. On the basis of this mechanism, and in view of the lack of specificity of the transferring enzyme, one might predict that other nucleophilic substances, e.g. SH compounds, could also function as acceptors.

* Recently Clapp (14) has isolated an S-glucuronosyl derivative from urine after the administration of a sulfonamide.
SUMMARY

An enzyme in the microsomes of guinea pig liver that can transfer glucuronic acid from uridine diphosphate glucuronic acid to the nitrogen group of amines is described. The possibility is considered that a single enzyme is involved in the synthesis of all types of glucosiduronic acid linkages.

Methods for the differentiation of hemiacetal, ester, and N-glucosyluronic acids are presented.

BIBLIOGRAPHY

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