Isolation of Uridine Diphosphate N-Acetylglucosamine-6-phospho-1-galactose from Hen Oviduct

Othmar Gabriel* and Gilbert Ashwell

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda 14, Maryland, and the Research Foundation of the Children's Hospital, Washington, D. C.

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The isolation and identification of uridine diphosphate N-acetylglucosamine 6-phosphate from extracts of hen oviduct was first reported by Strominger in 1955 (1). Since that time, essentially no information concerning the metabolic role of this compound has become available. Recently, however, brief reports from two independent laboratories (2, 3) have described the isolation of a modified compound in which the terminal phosphate of the hexosamine is found in phosphodiester linkage to the anomeric carbon of galactose. The possibility must be considered, therefore, that the original compound may represent a partial degradate product of the more highly substituted nucleotide conjugate.

The present paper provides a detailed description of the isolation procedure as well as further documentation in support of the proposed structure of the galactose-containing nucleotide (Fig. 1).

EXPERIMENTS AND RESULTS

Materials and Methods

In the preparation and analysis of various nucleotide components, extended use was made of a commercially available high voltage electrophorator, model D, obtained from Gilson Medical Electronics, Middletown, Wisconsin. Electrophoresis was routinely carried out on Whatman No. 1 paper with 0.05 M citrate buffer, pH 4.0, at 80 volts per cm for 30 minutes. The origin was set at 10 cm from the cathodic end of the paper.

Among the various solvent systems used for the paper chromatographic separation, the following proved useful: solvent 1, ethanol-1 M ammonium acetate (70:30); solvent 2, isobutyric acid-0.5 M NH₄OH (5:3); solvent 3, pyridine-butanol-water (90:90:45); solvent 4, pyridine-ethyl acetate-acetic acid-water (5:5:1:3). All paper chromatographic separations were carried out by the descending technique at room temperature.

Colorimetric assays for hexosamines and N-acetylhexosamines were performed according to the modifications by Boss (4) and Levvy and McAllan (5), respectively, of the Elson-Morgan and Morgan-Elson procedures. The cysteine-H₂SO₄ reaction of Dische, Shettles, and Osnos (6) was used for the determination of hexoses. This assay proved of special value with galactose, since, in this case, an additional maximum at 600 μ μ appears after 24 to 48 hours at room temperature and thereby provides a second parameter for the identification as well as the quantitative measurement of this compound. Total phosphate was determined by a highly sensitive method described by Ames and Dubin (7). As used in this paper, acid-labile phosphate is defined as that amount of inorganic phosphate release by heating the sample in 1 M HCl at 100° for 7 minutes. In the case of galactose 1-phosphate, the less sensitive phosphate method of Fiske and SubbaRow (8) was employed, since this compound is partially dephosphorylated under the more drastic conditions of the ascorbic acid procedure (9).

Preparation of Extract—The oviducts* from 60 to 70 freshly slaughtered laying hens were removed and immediately packed in ice for transportation to the laboratory. Approximately 5 kg, wet weight, of tissue were so obtained. The oviducts, still packed in ice, were minced by passage through a meat grinder kept at 0°. The resulting pulp-like material was suspended in 80% ethanol in a stainless steel container and rapidly brought to boiling (20 to 30 minutes) by passing steam into the suspension. The steam was discontinued, and the suspension was kept simmering for another 30 minutes on a large electric hot plate. At the end of this time, the insoluble residue, collected by filtration through several layers of cheesecloth, was re-extracted with an additional 4 liters of 80% ethanol as before. The residue from the second extraction was discarded, and the pooled filtrates were stored overnight in the cold room. The next morning, a large fat layer was removed from the surface of the extract, and the resulting turbid, yellowish solution was clarified by filtration through Celite. The resulting filtrate was concentrated under reduced pressure at an external bath temperature of 50° to a final volume of 1 liter. The concentrated material was extracted three times with an equal volume of ether, and the aqueous extract was passed through a fine sintered glass filter to yield 560 ml of a clear, dark brown colored liquid. As measured in a Beckman DU spectrophotometer at 260 μμ, this material contained 135,000 optical density units.

Isolation of Unknown Sugar Nucleotide

1. Column Chromatography—All of the following chromatographic procedures were carried out in a cold room at 0°-3°. The clarified, aqueous oviduct extract was carefully layered onto a column of Dowex 1-X2 (Cl⁻ form, 200 to 400 mesh) measuring 10 cm in diameter and 23 cm in height. The column was then washed with 16 liters of cold distilled water. Approximately 50,000 optical density units, measured at 260 μμ, were recovered in the wash. This material, representing the bases and nucleotides, was retained on the column and further analyzed.

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† We are greatly indebted to Dr. Henninger of the Dover Poultry Company of Baltimore, Maryland, for the generous gift of this material.
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FIG. 1. UDP-\(\text{N-acetylglucosamine-6-phospho-1-galactose}\)

Asides as well as non-nucleotide components, was rejected. A second wash of 20 liters was passed through the column with no further appearance of ultraviolet-absorbing products.

Consequently, a linear gradient elution was arranged whereby a mixing chamber containing 38 liters of 0.01 \(\text{N HCl}\) was connected by a siphon to a reservoir containing an additional 38 liters of 0.01 \(\text{N HCl}\) made 0.50 \(\text{M NaCl}\). The flow rate was adjusted to 25 ml per minute, and 1-liter samples were collected every 40 minutes. In this manner, a total of 76 liters was collected, and the optical density was determined at 260 \(\text{nm}\). The resulting elution pattern is plotted in Fig. 2. Including the original water wash, a total of 128,000 optical density units, or 95% of the starting material, was recovered.

To each of the pooled fractions shown in Fig. 2 a sufficient amount of acid-washed Norit was added to remove at least 95% of the 260 \(\text{nm}\) absorbing material. For the particular charcoal preparation used in these experiments, it was found that 2 mg were required for the complete adsorption of 1.0 optical density unit. The Norit suspension was stirred vigorously and allowed to settle overnight in the cold room. The clear supernatant solution was siphoned off and discarded; the residual Norit was collected on a sintered glass filter and washed thoroughly with cold distilled water.

Elution of the nucleotides from the washed Norit was carried out by suspending the charcoal in 3 to 4 volumes of a 50% ethanol solution containing 0.1% \(\text{NH}_{2}\text{OH}\). After 1 to 2 hours at room temperature, the suspension was again filtered, and the optical density of the filtrate was determined at 260 \(\text{nm}\). In most cases, 3 to 4 extractions were required to remove the bulk of the nucleotide material. The eluates were then concentrated under reduced pressure at room temperature. This procedure resulted in an average recovery of 80 to 90%.

The present paper is concerned with the identification of a nucleotide complex recovered in Peak E of Fig. 2. Consequently, only this fraction will be treated in detail here. The identification of compounds in the remaining fractions will be described elsewhere.

2. Paper Chromatography—Fraction E was concentrated to 5.0 ml of a pale yellow liquid containing 4800 optical density units of 260 \(\text{nm}\) absorbing material. The ultraviolet spectrum closely paralleled that of authentic UDP in both acid and alkaline \(\text{pH}\) ranges. In order to purify this fraction further, it was subjected to large scale paper chromatography in solvent 1.

Whatman No. 3MM paper was cut into strips 9 inches wide. To each of four such strips, 300 \(\mu\text{l}\) of Fraction E were applied in 10 applications of 30 \(\mu\text{l}\) each. Descending chromatography was carried out for 24 hours, at the end of which time the papers were removed, dried, and examined in ultraviolet light. The resolution was incomplete, and the papers were replaced in the chromatography tank for an additional 24 hours. At the end of this time, three distinct ultraviolet-absorbing bands could be seen. However, when guide strips were sprayed with aniline phthalate (10) only the large, diffuse middle band gave a positive reaction, as evidenced by the formation of an orange-brown color. Hence, both the slow and fast moving compounds were rejected.

Upon closer examination of the middle fraction, two incompletely separated areas were observed, which were arbitrarily divided into two unequal portions by a line separating the faster running two-thirds from the slower moving one-third. Both fractions were eluted from the paper and purified by treatment with Norit as described earlier. The faster running fraction, which contained 326 optical density units at 260 \(\text{nm}\), was shown to contain a single major component when subjected to high voltage electrophoresis (see "Materials and Methods"). The second fraction, containing a mixture of the above material plus an additional component identified as UDP-glucuronic acid, was set aside for subsequent resolution.

3. Ethanol Fractionation—Although the above fractionation permitted the recovery of a single carbohydrate-containing nucleotide complex, contamination of this material by several minor ultraviolet absorbing impurities was readily demonstrated by high voltage electrophoresis. In early experiments, this difficulty was overcome by subjecting the unknown nucleotide to large scale paper electrophoresis. However, the losses entailed...
in solution from the paper and subsequent purification by treatment with Norit were large, and alternate purification procedures were investigated.

The simplest and most reproducible technique for the further purification of this material proved to be precipitation with ethanol. An aliquot of the charcoal-treated eluate containing 85 optical density units was concentrated under reduced pressure at 37°C to a yellow syrup. This was dissolved in a small amount of water and chilled to 0°C, and cold ethanol was added to a final concentration of 90%, volume for volume. A cloudy white precipitate formed which was collected by centrifugation in the cold and dissolved in a small amount of water to yield a clear colorless solution. Spectrophotometric determination at 260 nm revealed that 74% of the original material had been precipitated. When this material was re-examined by high voltage electrophoresis, a single spot corresponding in location to the original absorbing material plus a small amount of the unknown nucleotide. Subsequent experiments indicated that a concentration of 95% ethanol could be safely employed.

### Table I

**Ultraviolet absorption data recorded for UDP-N-acetylglucosamine 6-phospho-1-galactose (Compound I) and UDP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 1</th>
<th>pH 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>260</td>
<td>220</td>
</tr>
<tr>
<td>A (UDP)*</td>
<td>261</td>
<td>225</td>
</tr>
<tr>
<td>Authentic UDP</td>
<td>290</td>
<td>229</td>
</tr>
</tbody>
</table>

* Isolated by high voltage electrophoresis after hydrolysis in 0.01 N HCl.

### Table II

**Summary of analytical data obtained on original nucleotide and isolated degradation products**

The figures recorded represent the calculated molar ratios for each of the constituents.

<table>
<thead>
<tr>
<th>Compound</th>
<th>N-Acetylglucosamine</th>
<th>Galactose</th>
<th>Acid-labile phosphate</th>
<th>Total phosphate</th>
<th>Anodic electrophoretic migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.0</td>
<td>1.1</td>
<td>1.06</td>
<td>3.06</td>
<td>26.5</td>
</tr>
<tr>
<td>II</td>
<td>0.93</td>
<td>1.0</td>
<td>1.03</td>
<td>1.91</td>
<td>23.0</td>
</tr>
<tr>
<td>III</td>
<td>0.91</td>
<td>1.0</td>
<td>1.00</td>
<td>1.09</td>
<td>11.5</td>
</tr>
<tr>
<td>Isolated N-acetylglucosamine 6-phosphate*</td>
<td>1.0</td>
<td>0.0</td>
<td>1.04</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Authentic N-acetylglucosamine 6-phosphate</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>16.0</td>
<td></td>
</tr>
<tr>
<td>Isolated galactose 1-phosphate†</td>
<td>0.0</td>
<td>1.0</td>
<td>1.02</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>Authentic galactose 1-phosphate</td>
<td>0.0</td>
<td>1.0</td>
<td>1.0</td>
<td>17.0</td>
<td></td>
</tr>
</tbody>
</table>

* Isolated by high voltage electrophoresis after hydrolysis of Compound I in 0.01 N HCl.
† Isolated after alkaline hydrolysis of Compound III. Details are described in the text.

4. **Additional Fractionation Procedures**—In an attempt to determine whether the ethanol precipitated material could be fractionated further, aliquots were subjected to column chromatography on diethylaminoethyl (DEAE) cellulose, Dowex 1-X2 (formate), and Dowex 1-X2 (chloride). In each case, a single compound was recovered which appeared to be identical in every respect with the starting material when subjected to electrophoresis, paper chromatography in solvents 1 and 2, and colorimetric analysis. On the basis of these findings, together with the degradation studies described later, it was concluded that this material represented a single sugar-nucleotide complex free from extraneous ultraviolet-absorbing or carbohydrate-containing impurities.

### Identification of Isolated Sugar Nucleotide

An examination of the ultraviolet absorption spectrum of the intact nucleotide, carried out on a Cary recording spectrophotometer, indicated the presence of a uridine base. The spectral characteristics of authentic UDP and the unknown compound are listed in Table I. Calculations based upon the molar extinction coefficient of UDP, 10 × 10³ at 262 nm and pH 2.0, revealed a molar ratio of ultraviolet absorption to total phosphorus to hexosamine to hexose of 1:3:1:1 (Table II). For these determinations, authentic N-acetylglucosamine and galactose were used as standards for the hexosamine (6) and hexose (6) assays, respectively.

The reducing sugar assay (11) on the intact nucleotide was negative. Similarly, upon treatment with periodic acid (12), no demonstrable amount of formaldehyde could be detected (13). However, mild acid hydrolysis with 0.01 N HCl at 100°C for 15 minutes resulted in decomposition of the compound, accompanied by the liberation of material with strongly reducing properties. When the acid-hydrolyzed products were again subjected to periodate oxidation, exactly 1.0 mole of formaldehyde was formed.

In an attempt to isolate the individual degradation products, 1 µmole of the intact nucleotide was brought to pH 2.0 with HCl and heated at 100°C for 15 minutes. The reaction mixture was then evaporated to dryness at 37°C in a vacuum, taken up in a small amount of water and streaked on Whatman No. 1 chromatographic paper. High voltage electrophoresis was carried out as described in “Materials and Methods.” After the paper was dry, a single band of ultraviolet-absorbing material was observed close to the anodal region (Compound A). A second negatively charged product and a third neutral component were visualized when guide strips, cut from each side of the paper, were treated with AgNO₃ (14) and/or aniline phthalate (10). The three components were eluted from the paper with water and identified as UDP, N-acetylglucosamine 6-phosphate, and galactose, respectively.

### Enzymatic Degradation Studies

In order to establish the sequence and linkage of the various constituents of the intact nucleotide (Compound I), a systematic

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* Since the experimental procedures employed in the identification of these compounds were essentially similar to those described in the accompanying papers by Strominger (15) and Suzuki (16), further details have been omitted in an attempt to minimize unnecessary duplication. The analytical data are summarized in Tables I and II.
enzymatic and chemical degradation of the original complex was undertaken.

1. Isolation of Phospho-1-N-acetylglucosamine-8-phospho-1-galactose (Compound I)—A partially purified bull semen preparation was utilized to cleave the pyrophosphate bond of Compound I to yield a new diphosphorylated disaccharide. This material was isolated and characterized as follows. A reaction mixture containing 4 μmoles of Compound I, 0.04 ml of 0.10 M MgSO4, and 0.70 mg of the bull semen preparation (17), in a total volume of 1.0 ml, was adjusted to pH 8.5 with 1 N NH4OH and allowed to incubate for 3 hours at 37°. The pH was maintained between 8 and 8.5 by the occasional addition of NH4OH. At the end of the incubation period, the reaction was adjusted to pH 2.0 with Dowex 50 (H+). The resin was washed three times with 1.0 ml of water, and the wash was pooled with the original supernatant solution. Determination of the inorganic phosphate content revealed that 4.0 μmoles had been formed. The reaction mixture was treated with Norit as described earlier. The charcoal eluate was concentrated under reduced pressure at 37° to 0.40 ml, streaked onto Whatman No. 1 chromatographic paper, and subjected to high voltage electrophoresis (see "Materials and Methods"). Residual uridine, resulting from the phosphatase activity of the semen enzyme on UMP, was detected as a broad ultraviolet-absorbing band at the origin. Guide strips cut from the sides of the paper revealed a single aniline phthalate-positive area with an anodal migration of 23 cm. This material was eluted from the paper and analyzed for hexosamine, hexose, and acid-labile and total phosphorus. The values, recorded in Table II, indicate a molar ratio corresponding to 1:1:1:2. Treatment of an aliquot of this material with 0.01 N HCl at 100° for 15 minutes, followed by high voltage electrophoresis, yielded two reducing sugars which were characterized as N-acetylglucosamine 6-phosphate and galactose.

2. Isolation of N-Acetylglucosamine-6-phospho-1-galactose (Compound III)—The above eluate, containing approximately 1 μmole of Compound II, was concentrated to 1.0 ml; the pH was adjusted to 8.5 with 1 N NH4OH, and the eluate was incubated at 37° with 0.01 ml (0.035 mg of protein) of a purified Escherichia coli phosphatase. After 1 hour, 50% (0.53 pmole) of the acid-labile phosphate had been cleaved from the molecule. Consequently, an additional 0.01 ml of enzyme was added, the tube was stoppered, and the incubation was allowed to continue overnight. The next morning, the reaction was stopped by adjustment of the pH to 2.0 with Dowex 50 (H+). The resin was removed by filtration and washed with water, and the washings were pooled with the original filtrate. Phosphate analysis revealed the formation of 1.00 μmoles of inorganic phosphate. The reaction product was concentrated to dryness under reduced pressure at 37°, dissolved in 0.05 ml of water, and resolved by high voltage electrophoresis. The reaction product, which was free from ultraviolet-absorbing material, was identified by the aniline phthalate spray. A single band of material was detected as N-acetylglucosamine A-phosphate and galactose.2

3. Isolation of α-N-Galactose 1-phosphate—Although the phosphodiester bond of Compound III was readily cleaved in acid to yield free galactose, it proved to be resistant to all of the commonly available phosphatase preparations examined. Alkaline hydrolysis of Compound III was shown to be converted to free galactose and N-acetylglucosamine-6-P upon mild acid hydrolysis.3

4. Demonstration of the stereospecificity of galactose 1-phosphate. Assay conditions were as described by Kurahashi and Anderson (20). Curves 1, 2, and 3 were obtained in the presence of 0.039, 0.064, and 0.16 μmole of authentic α-N-galactose 1-phosphate, respectively. Curve 4 represents the reaction obtained upon incubation of 0.10 μmole of the isolated alkaline degradation product of Compound III. See text for details.

![Fig. 3. Comparison of the reactivity of authentic and isolated galactose 1-phosphate in the uridyl transferase system. Assay conditions were as described by Kurahashi and Anderson (20). Curves 1, 2, and 3 were obtained in the presence of 0.039, 0.064, and 0.16 μmole of authentic α-N-galactose 1-phosphate, respectively. Curve 4 represents the reaction obtained upon incubation of 0.10 μmole of the isolated alkaline degradation product of Compound III. See text for details.](http://www.jbc.org/)

![Fig. 4. Demonstration of the stereospecificity of galactose 1-phosphate. Assay conditions were the same as those in Fig. 3. Authentic α- and β-N-galactose 1-phosphate (0.07 μmole) were added to separate cuvettes at zero time. After incubation for 12 minutes (1/2), an equal amount of α-N-galactose 1-phosphate was added to the cuvette containing the inactive β isomer.](http://www.jbc.org/)
hydrolysis, on the other hand, resulted in the rupture of this bond and the consequent formation of galactose 1-phosphate. Thus, 0.50 μmole of Compound III, made 1 n with respect to KOH, was heated at 100° for 1 hour. The slightly yellow-colored solution was then chilled in an ice bath and adjusted to pH 6.0 by the slow addition of 0.5% perchloric acid. The mixture was stored overnight at 0° to obtain a maximal precipitation of KClO₄. The salt crystals were removed by filtration, and the solution was concentrated to 0.10 ml. Upon high voltage electrophoresis, an aliquot of the alkaline degradation product exhibited a single spot migrating together with known galactose 1-phosphate. In this case, the FeCl₃-sulfosalicylic acid reagent used for the presence of a cyclic phosphate. The unknown phosphate was eluted from the paper in 0.1 N HCl for 7 minutes at 100° in 1 N HCl. After treatment with Dowex 50 (H⁺) to remove excess Fe⁺ and extraction with ether to remove the sulfosalicylic acid, the eluate was concentrated to 0.01 ml and subjected to paper chromatography in solvent 3. This material cochromatographed with authentic galactose.

A second aliquot of the alkaline degradation mixture was heated in 1 N HCl for 7 minutes at 100°. All of the phosphate was recovered as inorganic phosphate. Since under these conditions a cyclic phosphate ester is not dephosphorylated (19), the presence of such a compound could be excluded.

Finally, a third aliquot was assayed enzymatically by galactose 1-phosphate uridyl transferase in the presence of uridine diphosphoglucose, phosphoglucomutase, glucose 6-phosphate dehydrogenase, and TPN (20). A comparison of the rate and extent of TPNH formation in the presence of the alkaline degradation product with that produced by varying concentrations of authentic α-D-galactose 1-phosphate is shown in Fig. 3. That the enzymatic system is specific for the α isomer and inert in the presence of β-D-galactose 1-phosphate is illustrated in Fig. 4.

DISCUSSION

On the basis of the evidence presented in this paper, the structure of the heretofore unrecognized sugar nucleotide isolated from hen oviduct is proposed to be uridine diphosphate N-acetylglucosamine-6-phospho-1-galactose (Fig. 1).

The compound appears to represent a new class of sugar nucleotides in that it is a disaccharide in which the two carbohydrate components are found to be linked by a phosphodiester bond. In this sense, it appears to be markedly different from the recently described nucleotide-oligosaccharides of Jourdian, Shimizu, and Roseman (21), in which the individual sugars are presumably linked by glycosidic bonds. At the present time, little can be said concerning the possible role of this compound in biosynthetic processes. It is of interest to note, however, that Slodki (22) has described the isolation of a mannose polymer in which the individual units are joined in 1,6 phosphodiester linkages.

A major point upon which uncertainty still exists concerns the linkage of the anomeric carbon of galactose to the terminal phosphate of the amino sugar (2). Unfortunately, thus far, the only successful method for the isolation of galactose 1-phosphate has been by drastic degradation in hot alkali. It would seem clear, however, from the hydrolytic and enzymatic studies reported here, that this compound exists in the α form as isolated. Entirely upon the basis of this evidence, the galactose fragment is presumed to occur in the intact nucleotide as α-D-galactose 1-phosphate.

SUMMARY

The isolation of an unusual sugar nucleotide from an extract of hen oviduct has been described, and evidence is presented to document the identification of this material as uridine diphosphate N-acetylglucosamine-6-phospho-1-galactose. On the basis of the data presented, the anomeric carbon of galactose is presumed to possess an α configuration in the intact molecule.

REFERENCES

Isolation of Uridine Diphosphate $N$-Acetylglucosamine-6-phospho-1-galactose from Hen Óviduct
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