Structure and Distribution of a Disaccharide-carrying Nucleotide and Related Nucleotides in Hen Oviduct*

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SUMMARY

A comparison has been made among the free nucleotide patterns of three different regions (magnum, isthmus, and uterus) of hen oviduct. More than 30 nucleotides have been separated from the three regions and characterized.

One of the nucleotides isolated from the isthmus region has been shown, by a series of degradative procedures followed by comparison with authentic samples and oxidation with periodate, to be a new sugar nucleotide in which a disaccharide, 0-α-L-fucopyranosyl-(1 --f 4)-2-acetamido-2-deoxy-a-D-glucopyranose, and uridine 5'-diphosphate are joined through a glycosidic link.

A unique feature of the oviduct of the laying hen is the diversity of its function as a polysaccharide-producing mechanism. The organ forms large quantities of various mucopolysaccharides and glycoproteins, not only as its own structural elements but also as the constituents of the egg albumen, shell membranes, and shells (1-7). The oviduct is made up of four morphological regions, namely, the magnum, isthmus, uterus, and vagina, each having a fairly specific function in egg formation (8). Accordingly, it has been our expectation that analysis of sugar nucleotides in each region of the oviduct will lead to the definition of the sugar nucleotide pattern of each region, which must provide useful information regarding the mechanism of polysaccharide biosynthesis.

In 1962, a new nucleotide was isolated from the isthmus region by Suzuki (9), and characterized as uridine diphosphate-2-acetamido-2-deoxy-alpha-D-glucopyranose, and uridine 5'-diphosphate are separated from the same region and partially characterized by Takahashi and Suzuki (10). The former compound was independently obtained from the homogenate of whole oviduct by Gabriel and Ashwell (11) and by Strominger (12), and evidence of its complete structure was given by the three laboratories. However, only limited information could be obtained about the structure of the latter compound, owing to the small amount of material available. Subsequent work done in this laboratory has recently assigned the structure, O-α-L-fucopyranosyl-(1 --f 4)-2-acetamido-2-deoxy-a-D-glucopyranose, to the disaccharide moiety (see Fig. 3). The primary purpose of this paper is to describe the isolation of this nucleotide and the evidence to support this structure.

In view of morphological and functional differences within the oviduct system, it seemed likely that the magnum, isthmus, and uterus may differ in the proportions of this nucleotide in their pools. For the preparation of the nucleotide, therefore, the three regions were separately extracted and each of the extracts was fractionated. This has resulted in providing valuable information concerning the distribution patterns of various other nucleotides as well. This paper describes, as a second subject, how a variety of nucleotides may be isolated in a high state of purity and summarizes the values which were found for their normal equilibrium levels.

EXPERIMENTAL PROCEDURE

Materials—NAD(P) glycohydrolase (EC 3.2.2.6) was prepared from Neurospora crassa (13), UDP-glucuronate decarboxylase (EC 4.1.1.35) from wheat germ (14), UDP-2-acetamido-2-deoxy-D-glucose pyrophosphorylase from Azotobacter vinelandii (15), and D-fucose isomerase from Escherichia coli Bas (16). An enzyme system capable of catalyzing the conversion of GDP-mannose to GDP-fucose was prepared from Aerobacter aerogenes (ATCC 12658) according to the method of Ginsburg (17). The strain was a generous gift of Dr. V. Ginsburg, National Institutes of Health, Bethesda, Maryland. Formyltetrahydrofolate synthetase (EC 6.3.4.3) was prepared from Clostridium cylindrosporum according to the method of Rabinowitz and Pricer (18). The strain was generously provided by Dr. J. C. Rabinowitz, University of California, Berkeley. Purified α-L-fucosidase of abalone (Haliotis gigantea Gmelin), with specific activity of 16.5 units per mg of protein, was a gift of Dr. K. Tanaka, Osaka Gakugei College, Osaka, Japan, and Dr. W. Pigan, New York Medical College, New York. For providing technical information, we are especially indebted to

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Dr. K. Tanaka. 5' Nucleotidase (EC 3.1.3.5) purified from the venom of 
Agkistrodon blomhoffii was supplied by Dr. S. Iwana, Kyoto University, Kyoto, Japan. Alkaline phosphatase, glucose-6-P dehydrogenase, and snake venom phosphodiesterase were purchased from Worthington, and UDP-glucose dehydrogenase from Sigma.

UDP-2-acetamido-2-deoxy-d-glucose-(4 → 1)-d-galactose-(2 → 1)-l-fucose was prepared from human milk according to the method of Kobata (19). ADP-glucose, GDP-glucose, UDP-glucose, and dTDP-glucose were prepared by a slight modification of the method of Rosenman, Distler, Moffatt, and Khorana (20). UDP-2-amino-2-deoxy-d-glucose was synthesized from UTP and 2-amino-2-deoxy-a-D-glucose-1-P by the enzyme preparation from Azotobacter vinelandii as previously described (15). UDP-glucuronic acid synthesized from a-D-glucuronic acid-1-P and uridine 5'-phosphoramide (21) was generously provided by Dr. M. Honjo, Takeda Chemical Industry, Osaka, Japan. 6-Deoxy-d-glucose was a generous gift of Dr. R. K. Crane, Rutgers-The State University, New Brunswick, New Jersey. dTDP-rhamnose, dTDP-4-amino-4,6-dideoxy-d-glucose, and dTDP-4-acetamido-4,6-dideoxy-d-glucose were provided by Dr. R. Okazaki of this laboratory. Other chemicals were purchased from either Sigma or the Wako Chemical Company, Osaka, Japan.

Hydrolysis of Nucleotides with Potato Nucleotide Pyrophosphatase—In the experiments reported below, a nucleotide pyrophosphatase preparation purified from potato was used as a test for cleaving a variety of nucleotides at the pyrophosphate linkage. This enzyme was first reported by Kornberg and Pricer (22) as one capable of cleaving β-NAD, β-NADP, ADP, ATP, and thiamine pyrophosphate. A thermal inactivation procedure has been devised in this laboratory, which provides a simple procedure for the preparation of nucleotide pyrophosphatase free from contaminating 5'-nucleotidase.

The combined ethanol fraction of Kornberg and Pricer (22) was diluted with water to give a protein concentration of 1 to 2 mg per ml, and was incubated with mechanical stirring for 20 min in a water bath at 60°. The mixture was allowed to stand for 30 min at 0°, after which solid ammonium sulfate was added to give 30% saturation. After standing for 2 hours, the precipitates were removed and the ammonium sulfate concentration of the supernatant was raised to 70% saturation. The resulting precipitates were collected, dissolved in water, and dialyzed against a large excess of distilled water at 2°. These procedures resulted in a 2-fold purification over the ethanol fraction (180-fold over the crude extract) with a yield of 82% on the basis of β-NAD-cleaving activity. With this preparation, it is possible to obtain quantitative degradation of various nucleotides (see below) at the pyrophosphate linkage; the reaction mixture contained 10 μl of 0.1 m Tris-HCL, pH 8.0, 5 μl of 0.1 m KH₂PO₄, 0.1 μmole of substrate, and sufficient amounts of water and the enzyme, in a total volume of 50 μl. The mixture was incubated at 37°.

Our test has indicated an interesting specificity of this enzyme. The nature of the base part of the substrate molecule has a considerable influence on the rate of hydrolysis; the relative rates with ADP-d-glucose, GDP-d-glucose, CDP-d-glucose, UDP-d-glucose and dTDP-d-glucose were 100, 86, 47, 33, and 1.5, respectively. Furthermore, the enzyme preparation shows an increase of activity with increase of positive charge in substrate ion; with adenosine nucleotides, the order is β-NAD > ADP-d-glucose > β NADP; with guanosine nucleotides, GDP-d-glucose = GDP-d-mannose = GDP-d-fucose; with cytidine nucleotides, CDP-ethanolamine > CDP-choline > CDP-d-glucose; with uridine nucleotides, UDP-2-amino-2-deoxy-d-glucose > UDP-2-acetamido-2-deoxy-d-glucose = UDP-2-acetamido-2-deoxy-d-galactose = UDP-d-glucose > UDP-d-glucuronic acid; and with deoxythymidine nucleotides, dTDP-4-amino-4,6-dideoxy-n-glucose > dTDP-4-acetamido-4,6-dideoxy-n-glucose = dTDP-rhamnose = dTDP-d-glucose. Also to be noted is the fact that the rate decreases sharply with increase of chain length of the sugar moiety. Thus, UDP-2-acetamido-2-deoxy-d-glucose-(4 → 1)-l-fucose (from isthmis, see below) and UDP-2-acetamido-2-deoxy-o-glucose-(4 → 1)-α-galactose-(2 → 1)-l-fucose (from human milk (19)) were hydrolyzed at only 41% and 23%, respectively, of the rate with UDP-2-acetamido-2-deoxy-d-glucose. A detailed description of the nature of this enzyme will be presented in a forthcoming paper.

Conditions for Hydrolysis with Phosphodiesterase, Alkaline Phosphatase, and a-L-Fucosidase—Hydrolysis of oligonucleotides with snake venom phosphodiesterase was carried out in the reaction mixture containing 0.1 μmole of oligonucleotide, 5 μl of 0.1 m Tris-HCL, pH 8.8, 5 μl of 0.1 m MgCl₂ and sufficient amounts of water and the enzyme, in a total volume of 25 μl at 37°.

Hydrolysis of nucleoside monophosphate or diphosphate or sugar phosphate with E. coli alkaline phosphatase was carried out in the reaction mixture containing 0.1 μmole of substrate, 5 μl of 0.04 m Tris-HCl, pH 8.0, 5 μl of 0.02 m MgCl₂ and sufficient amounts of water and the enzyme, in a total volume of 25 μl at 37°.

Hydrolysis of the fucose-containing nucleotide with abalone a-L-fucosidase was carried out in the reaction mixture containing 0.3 μmole of the nucleotide, 15 μl of 0.1 m phosphate-0.05 m citrate, pH 5.0, and sufficient amounts of water and the enzyme, in a total volume of 150 μl at 37° for 4 hours.

Analytical Procedures—The procedures for measurements of phosphate (23), ribose in purine nucleotide (24), acetylamino sugar (25), 6-deoxyhexose (26), and reducing capacity (27) were modified to a micro scale, so that the amount of sample was in the range of 0.01 to 0.1 μmole. Ribose in the pyrimidine nucleotides (see Table I) was determined by the orcinol reaction following destruction of the pyrimidine ring by hydrogenation and ultraviolet irradiation as follows. The solution (80 μl, containing 0.01 to 0.1 μmole of nucleotide) was mixed with 30 mg of NaBH₄ in a test tube, 0.8 X 7.5 cm, and illuminated for 2 hours from the top of the tube by a 15-watt germicidal lamp (Matzuda). The resulting solution was analyzed for ribose by the orcinol reaction (24), with 5'-UMP (for uridine nucleotides) or 5'-CMP (for cytidine nucleotides) as a standard. A proportionality between absorbance and the quantity of substance was achieved, although the millimolar extinction coefficients for cytidine nucleotide (18.1) and uridine nucleotide (28.4) were not identical.

The staining of reducing sugars and acid-labile phosphate esters on the chromatogram was previously described (9). Glycans were detected by spraying the periodate-benzidine reagent (28) onto the dried chromatogram.

Periodate oxidation was carried out with 0.2 μmole of sample in 1.5 ml of 1.25 mm sodium periodate-0.05 m acetate buffer, pH 4.8, at room temperature in the dark. Periodate consumption was determined by measurement of the decrease in absorbance
at 265 μm (29), with glycerol as a standard. Formaldehyde in the oxidation mixture was determined as previously described (30). Formic acid formation during the oxidation was measured by the method of Rammler and Rabinowitz (31), with the use of formyltetrahydrofolate synthetase.

Preparation of Extracts—The oviducts were excised from about 3000 white Leghorn laying hens to secure sufficient material for extensive chemical work. Each oviduct was dissected into the four regions, like regions (except vagina) were combined, and each region was extracted separately with an equal volume of boiling water for 5 min. The extract obtained from 10 kg of each region was then fractionated for free nucleotides.

Separation of Nucleotides from Non-nucleotide Materials—All operations were carried out in a cold room (3°). The extract was first passed through a column (5 × 52 cm) of Dowex 1 resin (chloride form, 50 to 100 mesh, 2% cross-linked) at the rate of 5 ml per min. The column was then washed with 4 liters of cold water. The nucleotide material was eluted from the column with a solution that was 0.003 N with respect to HCl and 1 M with respect to NaCl at the rate of 5 ml per min. Elution was followed by measuring the absorbance at 260 μm, and fractions with absorbance over 0.05 were pooled. The resulting solution was clear and light yellow in color. Nucleotides were recovered from this solution by the charcoal method described by Strominger (12). The ethanol extracts were combined and concentrated to about 0.33 volume on a rotating evaporator. The solution was then washed three times with 0.33-volume portions of l-butanol in a separatory funnel. A considerable amount of ultraviolet light-absorbing impurities (including nucleosides) was removed by this means. The apparent yield of nucleotide at this stage, calculated from ultraviolet light absorption measurements with the use of 10 as an average millimolar extinction coefficient, were 5.1 mmole from the magnum, 9.5 mmole from the isthmus, and 5.8 mmole from the uterus.

Ion Exchange Chromatography—The method used to separate nucleotides was based on anion exchange chromatography on a column (5 × 39 cm) of Dowex 1 resin (chloride form, 100 to 200 mesh, 2% cross-linked), with the use of stepwise elution with NaCl. The experimental details are indicated in the legend to Fig. 1. Since much sharper elution peaks were obtained in the column chromatography when EDTA was present in the system, 0.01% EDTA was routinely added to the eluting solutions. The fractions were pooled as indicated in Fig. 1, desalted, and concentrated by the charcoal method.

Paper Chromatography and Paper Electrophoresis—The fractions obtained by ion exchange chromatography had to be separated further by paper chromatography and paper electrophoresis on a preparative scale, since all of the fractions contained more than one component. The concentrated solution obtained from each of the peaks of ion exchange chromatography was applied as a zone on Toyo No. 50 filter paper, which was then chromatographed by descending technique in Solvent A, 1-butanol-ethanol-water (52:32:16), in which nucleotides have only slight mobility. After drying, the nucleotide zone near the origin, from which a considerable amount of ultraviolet light-absorbing impurities had been removed, was cut out, eluted with water, and dried. The sample was subjected to the second paper chromatography in Solvent B, 95% ethanol-1 M ammonium acetate, pH 7 (7.5:3) (32). After the paper was dried, all of the ultraviolet light-absorbing materials were eluted from the paper.

Fig. 1. Dowex 1-chloride separation of nucleotides from 10 kg each of magnum, isthmus, and uterus of hen oviduct. The butanol-treated extract (see text) was run through a column, 5 × 39 cm, of Dowex 1-chloride (100 to 200 mesh, 2% cross-linked), and the adsorbed compounds were eluted at 5 ml per min with the indicated eluents. Tubes (20 ml) were analyzed for absorbance at 260 μm. Separation and identification of some of the nucleotides found in each peak are not described in detail in the text, and they are indicated in this figure only as an index to the relative mobilities of the more interesting compounds listed in Table I.
Fig. 2. A schematic representation of paper chromatogram (Toyo No. 50 filter paper) and paper electrophoretogram (Toyo No. 51A filter paper) of nucleotides. The load was 0.1 μ mole. The positions of nucleotides are indicated by the following reference numbers: 1, AMP; 2, GMP; 3, CMP; 4, UMP; 5, dTMP; 6, IMP; 7, dCMP; 8, ADP; 9, CDP; 10, GDP; 11, UDP; 12, dTDP; 13, dCDP; 14, ATP; 15, GTP; 16, CTP; 17, UTP; 18, dTTP; 19, dATP; 20, dCTP; 21, NAD; 22, NADP; 23, nicotinic acid mononucleotide; 24, nicotinic acid adenine dinucleotide; 25, ADP-glucose; 26, UDP-glucose; 27, UDP-glucose-6-phosphate; 28, UDP-glucose; 29, 3′-phosphoadenosine 5′-phosphosulfate; 30, ADP-glucose; 31, GDP-mannose; 32, GDP-fucose; 33, CDP-glucose; 34, UDP-glucose, -galactose, -xylose, and -arabinose; 35, UDP-gluconic acid; 36, UDP-glucuronic acid; 37, UDP-2-acetamido-2-deoxy-α-glucose; 38, UDP-2-acetamido-2-deoxy-α-galactose-4-sulfate; 39, UDP-2-acetamido-2-deoxy-α-glucose-6-phosphogalactose; 40, UDP-2-acetamido-2-deoxy-α-glucose-4-sulfate; 41, UDP-2-acetamido-2-deoxy-α-glucose-4-sulfate; 42, dTDP-glucose; 43, dTDP-glucose-6-phosphate; 44, dTTP-glucose; 45, dTDP-glucose; 46, dTDP-glucose; 47, dTDP-glucose.

which, if desired, can be removed by paper chromatography in Solvent A.

During paper chromatography and paper electrophoresis, a number of standard nucleotides were run in parallel with the test samples. The relative mobilities of these standards are schematically represented in Fig. 2, which clearly indicates the high resolving power of the system used.

RESULTS

Comparative Nucleotide Patterns in Three Separate Regions of Hen Oviduct—The isolated nucleotides were characterized by the following criteria: (a) chromatographic and electrophoretic behavior, (b) ultraviolet light absorption spectra, (c) total phos-
phate content and phosphate liberated after hydrolysis with 1 N H$_2$SO$_4$ at 100° for 15 min, (d) type and content of pentose, (e) susceptibility to alkaline phosphatase before and after nucleotide pyrophosphatase digestion, and (f) reducing value before and after hydrolysis with 10% acetic acid at 100° for 7 min. In addition, specific tests for certain nucleotides were used (see Table I). The results of the analyses are tabulated in Tables I and II.

Ten sugar nucleotides have been isolated and identified. Digestion of the compounds with nucleotide pyrophosphatase followed by alkaline phosphatase yielded the respective sugars, which were then determined by the ferricyanide method (27) and identified by paper chromatography in Solvent D, ethyl acetate-acetic acid-water, 3:1:3 (upper layer), and Solvent E, 1-butanol-pyridine-water (6:4:3). The compounds were GDP-mannose, GDP-fucose, UDP-glucose, UDP-galactose, UDP-2-acetamido-2-deoxy-n-glucose, UDP-%acetamido-2-deoxy-n-galactose, UDP-glucuronic acid, UDP-2-acetamido-2-deoxy-n-glucose-6-phosphogalactose, UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate, CDP-choline, GDP-ethanolamine, N-Succinyladenosine, NADP, and NADPH.

The results of the analyses are tabulated in Tables I and II. All of the other nucleotides were also subjected to enzymatic hydrolysis to see whether they would liberate reducing sugar. No reducing sugar was obtained in these experiments, revealing the absence of detectable amounts of sugar nucleotides other than those listed in Table II. However, there were several compounds that were not identified beyond determination of the specific test.

### Table I

#### Separation and identification of nucleotides from hen oviduct

<table>
<thead>
<tr>
<th>Compound</th>
<th>Position on ion exchange chromatogram</th>
<th>$R_{UMP}^a$</th>
<th>Composition</th>
<th>Specific test</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP-mannose</td>
<td>I-12, U-13</td>
<td>0.83</td>
<td>0.55</td>
<td>1.20</td>
</tr>
<tr>
<td>GDP-fucose</td>
<td>I-12</td>
<td>1.12</td>
<td>0.09</td>
<td>1.20</td>
</tr>
<tr>
<td>UDP-glucose</td>
<td>I-11</td>
<td>1.27</td>
<td>0.50</td>
<td>1.40</td>
</tr>
<tr>
<td>UDP-galactose</td>
<td>I-11</td>
<td>1.27</td>
<td>0.50</td>
<td>1.40</td>
</tr>
<tr>
<td>UDP-2-acetamido-2-deoxy-n-glucose</td>
<td>M-0, I-11, U-12</td>
<td>1.36</td>
<td>0.60</td>
<td>1.32</td>
</tr>
<tr>
<td>UDP-2-acetamido-2-deoxy-n-galactose</td>
<td>M-9, I-11, U-12</td>
<td>1.36</td>
<td>0.69</td>
<td>1.33</td>
</tr>
<tr>
<td>UDP-glucuronic acid</td>
<td>I-12, U-13</td>
<td>0.69</td>
<td>0.40</td>
<td>1.63</td>
</tr>
<tr>
<td>UDP-2-acetamido-2-deoxy-n-glucose-4-phosphate</td>
<td>I-10</td>
<td>1.32</td>
<td>0.64</td>
<td>1.17</td>
</tr>
<tr>
<td>UDP-2-acetamido-2-deoxy-n-glucose-6-phosphogalactose</td>
<td>M-10, I-12, U-13</td>
<td>0.83</td>
<td>0.37</td>
<td>1.50</td>
</tr>
<tr>
<td>UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate</td>
<td>M-11 (trace), I-13, U-15</td>
<td>1.27</td>
<td>0.42</td>
<td>1.61</td>
</tr>
<tr>
<td>CDP-choline</td>
<td>M-2, I-3, U-1</td>
<td>1.98</td>
<td>1.43</td>
<td>0.60</td>
</tr>
<tr>
<td>CDP-ethanolamine</td>
<td>M-2, I-3, U-2</td>
<td>1.10</td>
<td>1.28</td>
<td>0.76</td>
</tr>
<tr>
<td>N-Succinyladenosine</td>
<td>M-9, I-12</td>
<td>1.24</td>
<td>1.47</td>
<td>1.31</td>
</tr>
<tr>
<td>α,β-NADP</td>
<td>I-8</td>
<td>0.18</td>
<td>1.00</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* I, isthmus; U, uterus; M, magnum. The numbers refer to the peak numbers in Fig. 1.

$^a$ Mobility of sample (in centimeters)/mobility of UMP (in centimeters).

$^b$ Based on millimolar absorbance ($\lambda_{max}$) at pH 2.0 of 12.2, 10.9, 13.0, 16.9, and 19.0, for guanosine ($\lambda_{max}$ 257 mr), uridine ($\lambda_{max}$ 262 mr), cytidine ($\lambda_{max}$ 231 mr), N-succinyladenosine ($\lambda_{max}$ 267 mr), and α,β-NADP ($\lambda_{max}$ 258 mr), respectively. The data represent the values of analysis of isthmus nucleotides. The values of other preparations were essentially the same as those presented here.

$^c$ Phosphate was liberated by 15 min of hydrolysis in 1 N H$_2$SO$_4$ at 100°.

$^d$ UDP-galactose and UDP-2-acetamido-2-deoxy-n-galactose were separated from UDP-glucose and UDP-2-acetamido-2-deoxy-n-glucose, respectively, according to the method of Carminatti et al. (35). UDP-galactose and UDP-2-acetamido-2-deoxy-n-glucose were not detected in the preparations.

$^e$ Aspartic acid was liberated by hydrolysis in saturated Ba(OH)$_2$ at 100° for 20 hours and identified by means of an amino acid analyzer. The analysis also showed occurrence of glutamic acid (about 10% of aspartic acid), suggesting the presence of N-glu-taryladenosine.

$^f$ α,β-NADP in the mixture was hydrolyzed by Nocardiophora sp. NAD glycohydrolase to give nicotinamide mononucleotide and 2',5'-ADP. α-NADP was separated from the reaction mixture by paper electrophoresis at pH 5.0 and identified by its characteristic absorption spectrum as a cyanide complex (38).
The ratio of uridine to total phosphate to labile phosphate to pentose (as ribose) was 1.00:2.00:0.99:1.10 (Table I). The reducing value, determined by the ferriyanide reduction (27) after hydrolysis with 0.1 N HCl for 15 min at 100°C and expressed relative to a glucose standard, was 1.40. With this method, the reducing values of authentic 2-acetamido-2-deoxy-D-glucose and D-fucose are 0.93 and 0.85, respectively, relative to glucose.

2. Enzymatic hydrolysis of the nucleotide with abalone α-L-fucosidase, which is devoid of activity on β-L-fucoside, yielded two products, which were separated from the intact nucleotide and from each other by paper electrophoresis at pH 5.0.

The fast spot, UDP-GlcNAc, was easily located by quenching of ultraviolet light (Table III). UDP-GlcNAc so prepared had electrophoretic and chromatographic mobilities identical with those of a UDP-2-acetamido-2-deoxy-D-glucose standard synthesized from UTP and 2-acetamido-2-deoxy-α-D-glucose-1-P by UDP-2-acetamido-2-deoxy-D-glucose pyrophosphorylase (Table III). The identity of UDP-GlcNAc and UDP-2-acetamido-2-deoxy-D-glucose was also affirmed by the finding that UDP-2-acetamido-2-deoxy-D-glucose pyrophosphorylase, purified 50-fold from A. vinelandii, converted both samples to UDP and 2-acetamido-2-deoxy-D-glucose-1-P (Table III) at the same rate (Fig. 4).

The slow spot, fucose, was located at the origin by staining for reducing sugar, but not by quenching of ultraviolet light. Further paper chromatography of the sample eluted from the electrophoretic strip showed a single spot, the mobility of which spectrum and phosphate content, since in most cases they were present in amounts too small for further analysis.

Structure of Uridine Nucleotide Containing 2-Acetamido-2-deoxy-D-glucose and Fucose.—As shown in Table II, about 21 moles of this nucleotide were obtained from 10 kg of isthmus. This nucleotide is completely missing in the magnum and uterus. A possible structure of this nucleotide is shown in Fig. 3. Evidence to support this structure may be summarized as follows.

1. The nucleotide exhibited an ultraviolet light absorption spectrum typical of a uridine derivative. The ratio of uridine to total phosphate to labile phosphate to pentose (as ribose) was 1.00:2.00:0.99:1.10 (Table I). The reducing value, determined by the ferriyanide reduction (27) after hydrolysis with 0.1 N HCl for 15 min at 100°C and expressed relative to a glucose standard, was 1.40. With this method, the reducing values of authentic 2-acetamido-2-deoxy-D-glucose and L-fucose are 0.93 and 0.85, respectively, relative to glucose.

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1. The nucleotide exhibited an ultraviolet light absorption spectrum typical of a uridine derivative. The ratio of uridine to total phosphate to labile phosphate to pentose (as ribose) was 1.00:2.00:0.99:1.10 (Table I). The reducing value, determined by the ferriyanide reduction (27) after hydrolysis with 0.1 N HCl for 15 min at 100°C and expressed relative to a glucose standard, was 1.40. With this method, the reducing values of authen-
was the same as that of authentic L-fucose (Table IV). The fucose was also identified as L-fucose with L-fucose isomerase; i.e. both authentic L-fucose and the fucose from the nucleotide gave the same absorption spectrum in the cysteine-carbazole reaction (40) after incubation with L-fucose isomerase, while D-fucose did not form any colored product under the same condition.

3. Digestion of the nucleotide with nucleotide pyrophosphatase liberated 5'-UMP, identified by paper chromatography (Table III) and by its conversion to uridine on treatment with 5'-nucleotidase, and a new phosphate ester (P-GlcNAc-Fuc) with a slower mobility than the intact nucleotide on paper electrophoresis at pH 5.0 (Table III). The spot of P-GlcNAc-Fuc was located by staining for acid-labile phosphate, but not by quenching of ultraviolet light. The electrophoretically isolated sample showed that the ratio of acid-labile phosphate to 2-acetamido-2-deoxy-D-glucose to L-fucose was 1.00:1.10:0.92. The phosphate group was easily liberated from P-GlcNAc-Fuc by digestion with alkaline phosphatase. The reaction mixture was passed, successively, through a column of IRC-50 resin (H+ form) and a column of Dowex 1 resin (bicarbonate form) at 3°C to remove the salts. Paper chromatography of the sample eluted from the column showed a single spot (Table V, GlcNAc-Fuc), the mobility of which was the same as that of the disaccharide fragment obtained from the nucleotide by mild acid hydrolysis. The sample thus prepared, GlcNAc-Fuc, was decomposed with 0.1 N HCl for 15 min to give two reducing sugars with the chromatographic mobilities of L-fucose and 2-acetamido-2-deoxy-D-glucose, respectively, as already reported in our preliminary paper (10).

4. Evidence that the fucose molecule, in pyranose ring form, is attached to position 4 of 2-acetamido-2-deoxy-D-glucopyranose was provided by periodate oxidation of the P-GlcNAc-Fuc fragment and a reduced disaccharide derived from the GlcNAc-Fuc fragment. The reduced disaccharide was prepared as follows. A 10-fold molar excess of a fresh sodium borohydride solution was added to the solution containing GlcNAc-Fuc. The mixture was kept in an ice bath for 30 min and at room temperature for 2 hours (41), and was then brought to pH 4.0 with acetic acid. The reduced material was separated by paper chromatography in Solvent F, 1-butanol-acetic acid-water (3:1:1). The guide strips of the chromatogram were stained with the periodate-benzidine reagent, and the corresponding zone was cut out and chuted with water (for \( R_{f \text{GlcNAc-Fuc}} \), see Table V).

Neither the reducing power nor the Morgan-Elson color reaction (see below) of GlcNAc-Fuc was retained with this preparation, while the fucose was recovered in over 90% yield, as measured by the cysteine-H\(_2\)SO\(_4\) reaction of the product.

P-GlcNAc-Fuc slowly consumed 1.9 moles of periodate with formation of 0.89 mole of formic acid within 10 hours. No formaldehyde was detected in the reaction mixtures. If P-GlcNAc-Fuc was treated for 20 hours with periodate, the fucose moiety (as measured by the cysteine-H\(_2\)SO\(_4\) reaction of the product) was consumed as expected (Table IV).

### Table IV

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_{f \text{GlcNAc-Fuc}}^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample, &quot;Fucose&quot; from UDP-GlcNAc-Fuc. Standards</td>
<td></td>
</tr>
<tr>
<td>L-Fucose (6-deoxy-L-galactose)</td>
<td>1.96</td>
</tr>
<tr>
<td>6-Deoxy-D-glucose</td>
<td>2.46</td>
</tr>
<tr>
<td>6-Deoxy-L-mannose</td>
<td>2.57</td>
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</tbody>
</table>

\( a \ R_{f \text{GlcNAc-Fuc}}, \) mobility of sample (in centimeters)/mobility of glucose standard (in centimeters).

### Table V

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_{f \text{GlcNAc-Fuc}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disaccharide, GlcNAc-Fuc.</td>
<td>0.81</td>
</tr>
<tr>
<td>Reduced GlcNAc-Fuc.</td>
<td>0.99</td>
</tr>
</tbody>
</table>

### Standards

<table>
<thead>
<tr>
<th>Compound</th>
<th>( R_{f \text{GlcNAc-Fuc}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Acetamido-2-deoxy-D-glucose</td>
<td>1.72</td>
</tr>
<tr>
<td>2-Acetamido-2-deoxy-D-glucitol</td>
<td>1.68</td>
</tr>
<tr>
<td>L-Fucose</td>
<td>2.21</td>
</tr>
</tbody>
</table>
PERIODATE CONSUMPTION

FORMALDEHYDE PRODUCTION

FORMIC ACID PRODUCTION

FIG. 5. Periodate oxidation of reduced disaccharide. The oxidation was carried out in 1.25 mM periodate containing 0.05 M acetate, pH 4.8. At the indicated times, aliquots were removed for the determination of formic acid, formaldehyde, and residual periodate.

the reaction mixture) was completely destroyed while the 2-acetamido-2-deoxy-α-glucose moiety (as measured by the Morgan-Elson reaction of the products on treatment of the reaction mixture with 0.1 N HCl at 100° for 15 min) was recovered in over 80% yield. The only conclusion to be drawn from these data is that an L-fucopyranose moiety is attached to either position 3 or position 4 of a 2-acetamido-2-deoxy-α-glucopyranose moiety in I'-GlcNAc-Fuc.

Evidence which supports the above interpretation and, furthermore, eliminates the possibility of a 1,3 linkage comes from the fact that the reduced disaccharide first consumed rapidly 1 mole of periodate with formation of formaldehyde and second consumed slowly 2 moles of periodate with formation of formic acid (Fig. 5). These data are compatible only with a 1,4 linkage since with a 1,3 linkage the first, fast 2 moles of periodate uptake, accompanied by formation of 1 mole of formic acid and 1 mole of formaldehyde would occur at C₄−C₅ and C₆−C₇ in the 2-acetamido-2-deoxy-α-glucitol moiety, and the second, slow 2 moles of periodate uptake with formation of 1 mole of formic acid would occur at C₂−C₃ and C₅′−C₆ in the fucose moiety.

Morgan-Elson Color Reaction of O-α-L-Fucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-α-glucopyranose—The disaccharide, GlcNAc-Fuc, gave a positive color in the standard versions of the borate-catalyzed Morgan-Elson reaction after 7 min of heating. The millimolar extinction coefficient of the disaccharide was found to be 220, the same value as that of unsubstituted 2-acetamido-2-deoxy-α-glucose. Since it had been shown that substitution with a methyl group at position 4 of 2-acetamido-2-deoxy-α-glucose abolishes the Morgan-Elson reaction whereas substitution at position 6 has no effect on the color reaction (42, 43), a 1,6 linkage was suggested for the disaccharide in our preliminary paper (10). A positive color has also been observed when a 1,4-linked disaccharide from staphylococcal cell walls, O-2-acetamido-2-deoxy-α-glucosyl-(1 → 4)-2-acetamido-3-O-(α-1-carboxyethyl)-2-deoxy-α-glucose, was subjected to the borate-catalyzed Morgan-Elson reaction (44), but Tipper, Ghuyesen, and Strominger (45) have lately shown that the positive reaction is due to the susceptibility of this disaccharide to hydrolysis at alkaline pH. These properties are consistent with the observation of Sharon, Osawa, Flowers, and Jeanloz (46), who first suggested that the disaccharide from micrococcal cell walls was 1,4 linked.

It was of interest therefore to test the susceptibility of GlcNAc-Fuc to hydrolysis at alkaline pH and the effect of the hydrolysis on the Morgan-Elson color reaction.

The susceptibility of GlcNAc-Fuc was indicated by the experiments in which the release of fucose from the disaccharide was followed by a modification of the method of Tipper, Ghuyesen, and Strominger (45). Samples of GlcNAc-Fuc (18 mmoles) were placed in tubes with 100 µl of 2% borate (pH 9.08), used in measurement of the Morgan-Elson reaction. These tubes were heated for various times at 100°, cooled, acidified with 2 N HCl, and, after 10 min at 0°, made alkaline again by the addition of 2 N NaOH. Fresh NaBH₄ solution (0.26 µ, 12 µl) was then added to each solution, and after 2 hours at room temperature the solution was acidified with 10% acetic acid (15 µl) and dried. After alternate addition of methanol and evaporation (two cycles), the residues were analyzed for fucose by the cysteine-H₂SO₄ reaction. The data indicate that rapid hydrolysis of the disaccharide was occurring, thus exposing fucose residues for reduction by NaBH₄ (Fig. 6). The hydrolysis appears to reach a maximum of only 77% even on prolonged heating.

When samples of 2-acetamido-2-deoxy-α-glucose (18 mmoles) and of the amino sugar plus fucose (18 mmoles) were treated with borate and then NaBH₄ followed by the cysteine-H₂SO₄ reaction, a chromophore with an absorption maximum at 400 mp was formed from each sample, in 11 and 17% yield, respectively, of that obtained from 18 mmoles of GlcNAc-Fuc without borate-NaBH₄ pretreatment. It is apparent, therefore, that

FIG. 6. Alkaline hydrolysis of disaccharide, GlcNAc-Fuc. A value obtained with the untreated disaccharide is set as 100%.
GlCNac-Fuc is completely hydrolyzed within 4 min, but that a portion of the product is still able to give a positive cysteine-
H$_2$SO$_4$ reaction. These results suggest that the positive Morgan-
Elsion reaction of GlCNac-Fuc obtained after 7 min of heating at pH 9.08 in borate buffer has been caused by the removal of the fucose residue during the heating.

**DISCUSSION**

The most striking aspect of the oviduct nucleotides is that several sugar nucleotides are present in relatively high concentrations. This was first recognized by Strominger (12, 47), who analyzed the homogenate of whole oviduct and identified UDP-2-acetamido-2-deoxy-n-glucose, UDP-2-acetamido-2-deoxy-n-galactose, GDP-mannose, UDP-2-acetamido-2-deoxy-n-glucose-6-P, and UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate. Subsequently, UDP-2-acetamido-2-deoxy-n-glucose-6-phosphogalactose was added to the list by Suzuki (9) and by Gabriel and Ashwell (11). Following these observations, the present analysis, which was designed so as to provide a complete list of the sugar nucleotides occurring in each of the three regions of oviduct at concentrations higher than 0.5 μmole per 10 kg of tissue, has revealed a number of nucleotides, including a new oligosaccharide-carrying nucleotide, and others which had not been detected by the earlier analyses. The new nucleotide, UDP-2-acetamido-2-deoxy-n-glucose-(4 ← 1)-L-fucose, represents the second oligosaccharide-carrying nucleotide isolated from the oviduct. It should be noted, however, that the presence of oligosaccharide-carrying nucleotides is not confined to the oviduct. For example, UDP-2-acetamido-2-deoxy-n-glucose-(4 ← 1)-galactose sialic acid and UDP-2-acetamido-2-deoxy-n-glucose-(6 ← 1)-galactose sialic acid have been isolated from goat colostrum by Jourdian, Shimizu and Rosenman (48), and UDP-2-acetamido-2-deoxy-n-glucose-(4 ← 1)-n-galactose-(2 ← 1)-L-fucose from human colostrum and milk by Kobata (49).

In regard to the physiological function of these nucleotides, one might expect that certain saccharides are synthesized by the addition of the oligosaccharide moieties to give rise to polymers with the oligosaccharide units. Although the occurrence of such a polymer as one related chemically to O-α-n-fucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-n-glucopyranosyl has not been demonstrable with the oviduct, it is interesting that the same sequence of sugars has been found by Vajayanti, Painter, Watkins, and Morgan (50) in a trisaccharide unit isolated from the blood group glycoprotein, Le$^b$ substance, in which the α-L-fucose residues are joined as branching units at position 4 to 2-acetamido 2-deoxy n-glucose residues in the group-specific determinant structure. We wonder whether there might be an enzyme responsible for a single step addition of the O-α-L-fucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-n-glucose moiety to a branching point in a growing polymer chain.

The central role played by sugar nucleotides in polysaccharide synthesis and especially in its regulation suggests that the sugar nucleotide pattern of a tissue provides an index to the nature and extent of the biosynthetic processes in that tissue. As shown in Table II, the isthmus region is much higher in content than other regions with respect to sugar nucleotides. It seems less than adequate, however, to interpret these differences as reflecting only differences in the type of metabolism occurring in the three regions. At least a part of the differences may be explained by the assumption that the amounts of connective tissue, fat, water, and other non-nucleotide materials are lower in the isthmus region. It should also be pointed out that large variations are commonly found in different preparations from the same regions (see Table II, footnotes a and b). Such variations could be partially accounted for by the adaptation of oviduct to the periodic egg formation, but interpretation of the results of a comparative study like this could not be more than speculation.

Nevertheless, the extremely high concentration of UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate in the isthmus region is noteworthy, since this nucleotide does not accumulate in the magnum region in more than trace amounts (Table II). We interpret the failure of the magnum to accumulate the nucleotide as a reflection of the presence of an effective system which converts UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate to some other form or forms. Recent work done in this laboratory has indicated that in the magnum an enzyme exists which catalyzes the transfer of the sulfate group from 3'-phosphoadenosine-5'-phosphosulfate to UDP-2-acetamido-2-deoxy-n-galactose-4-sulfate to give rise to UDP-2-acetamido-2-deoxy-n-galactose-4,6-disulfate. This enzyme exhibits a high degree of selectivity for the nucleotide acceptor and, according to our preliminary analyses, tends to be much lower in content in the isthmus region. The details of these experiments will be discussed elsewhere.

A few comments are appropriate in relation to the occurrence of N-succinyladenosine, α-NADP, and a group of oligonucleotides (Table II).

The occurrence of N-succinyladenosine is reminiscent of the observations by Joklik (51) that N-succinyladenine and N-succinyl-AMP occur in acid extracts of mammalian liver, while N-succinyladenosine has never been detected in the extracts. N-succinyl-AMP was first found by Carter and Cohen (52) to be formed from 5'-AMP and fumaric acid in yeast autolysates, and was shown by Lieberman (53) to be an intermediate in the transformation of 5'-IMP to 5'-AMP in extracts of Escherichia coli. Although one cannot eliminate a possibility that N-succinyladenine and N-succinyladenosine represent intermediates between hypoxanthine and adenine and between inosine and adenosine, respectively, as does N-succinyl-AMP in the case of nucleotide, it is a fair assumption that the base and nucleotide forms are merely breakdown products of N-succinyl-AMP.$^3$

Previously, we separated from extracts of Azotobacter vinelandii four unusual pyridine nucleotides and established that they are α-nicotinic acid mononucleotide, α-nicotinic acid adenine dinucleotide, α-NAD, and α-NADP (α-NAD was previously isolated from yeast by Kaplan, Ciotti, Stolzenbach, and Bachur (54)), which differ from the respective β isomers with respect to the pyridine riboside linkage. We have also shown that an enzyme system in A. vinelandii carries out the following reaction.

α-NAMN $\rightarrow$ α-NAD $\rightarrow$ α-NADP

$\rightarrow$ ATP $\rightarrow$ α-NADP

where α-NAMN represents α-nicotinic acid mononucleotide, and α-NAD, α-nicotinic acid adenine dinucleotide. These

$^3$ N-Succinyladenosine has also been found in extracts of bakers' yeast (S. Okada, N. Suzuki, and S. Suzuki, unpublished observation).
findings have raised the question of the function of this new class of nucleotides in cellular metabolism, e.g., the existence of an α-NADP-linked dehydrogenase system could be expected in analogy with the well known role of β-NADP. An interesting point in this respect is the relative amounts of α-NADP and β-NADP found in the microorganism and the isthmus. In the former, these two compounds occur in the molar ratio 5:1, while they occur in nearly equal concentration in the latter. Further investigations of the function of these nucleotides in the oviduct are currently in progress.

It is remarkable that the oviduct contains a large amount of oligonucleotides. Since the compounds were indicated to be breakdown products of RNA, they occur in nearly equal concentration in the latter.

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Structure and Distribution of a Disaccharide-carrying Nucleotide and Related Nucleotides in Hen Oviduct
Yasuo Nakanishi, Satoru Shimizu, Noriko Takahashi, Minekazu Sugiyama and Sakaru Suzuki


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