Synthesis of 1,5-Anhydro-d-Glucitol from Glucose in Rat Hepatoma Cells

Miho Suzuki,* **, Hideaki Mizuno,* Yasuo Akanuma,** and Hiroshi Akanuma*

*Department of Chemistry, College of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo 153; and **Institute for Diabetes Care and Research, Asahi Life Foundation, Chiyoda-ku, Tokyo 100

Received for publication, August 2, 1993

A pyranoid polyol, 1,5-anhydroglucitol (AG), generally occurs in the human body as a humoral component. The plasma AG concentration in healthy individuals is maintained at a constant level, but it is markedly decreased in diabetes mellitus. This is due to hyperglycemia-dependent abolishment of renal AG retention. Hence, the plasma AG concentration has been established as a clinical marker for duration of hyperglycemia and since 1991 it has been practically applied to diabetic care in Japan. However, the details of the metabolism of AG and its physiological significance generally remain to be studied. In this study, we confirmed AG synthesis in cultured cells of a rat hepatoma line, Reuber H-35, in which AG was found to be derived from glucose, with retention of all six carbon atoms in the pyranoid structure. The fraction of the total glucose consumed by the cells, which was converted to AG (conversion efficiency) was at most 5×10⁻⁶. The conversion efficiency increased at higher glucose concentrations (mM orders) where the glucose consumption rate was saturated. Since the rate of the hexokinase reaction, one of the rate-limiting steps in glucose consumption, has been estimated to be saturated at μM orders of glucose concentration, this observation was interpreted as indicating that AG is synthesized through a pathway which does not share the hexokinase reaction with glucose utilization. The presence of precursors other than glucose was also indicated in the time-course study of AG synthesis. Further, the amount of AG synthesized daily in humans is significant in comparison with the amount obtained from the diet.

Key words: 1,5-anhydro-d-glucitol, biosynthesis, glucose metabolism.

A cyclic polyol, 1,5-anhydro-d-glucitol (AG) is the 1-deoxy form of d-glucopyranose. Its occurrence in humans was first demonstrated in the cerebrospinal fluid (1) and then in blood (2–4). The normal range of plasma AG concentration is very wide, with the average in the vicinity of 20 μg/ml (5), which is the second highest concentration, next to that of glucose, of sugars ordinarily observed in human plasma. The plasma AG concentration in individuals, however, is generally stable and scarcely affected by various metabolic effectors including ingestion of food, administration of most drugs, hormonal states, and the circadian rhythm (5–7). On the other hand, a remarkable decrease in the plasma AG concentration is observed specifically in diabetes mellitus (2, 3, 5–8); and the primary cause of this decrease has been proven to be enhanced excretion of AG into the urine in the hyperglycemic state (9–12). This hyperglycemia-related behavior of AG makes the plasma AG concentration useful as a clinical index of glycemic control in diabetic care (5). The physiological significance of this AG depletion, however, remains to be elucidated.

A study of the AG content in various food ingredients (13) suggested that AG occurs ubiquitously in animals and plants. Accordingly, we postulate that AG is essential to every organism. Among vegetables, cereals and beans contain the largest amounts of AG (13), thus suggesting that seeds store AG in preparation for germination. The wide distribution of AG in vegetables indicates that AG is synthesized in these autotrophs. Mammals are able to effectively obtain AG from foods through intestinal absorption (10, 14, 15), and they also efficiently retain it in their bodies through renal reabsorption (10–12). Since AG undergoes very slow metabolic turnover (10), it accumulates in animals' bodies. Most of the mammalian intracellular water spaces seem accessible to exogenous AG (10, 16, 17), and intracellular AG is washed out by perfusion (17). Furthermore, we have directly demonstrated rapid AG incorporation into cultured rat hepatoma cells, Reuber H-35 (18). Our last study indicated that a transporter specific to AG is located on the plasma membranes of Reuber H-35. Natural occurrence of AG inside the cells is another indication of AG being essential to cellular activity. It is highly plausible, therefore, that such an indispensable substance is endogenously supplied even in heterotrophs. In this report, we demonstrate AG synthesis in mammalian...
cells using Reuber H-35 cells.

From the structural point of view, glucopyranose is the strongest candidate among possible precursors of AG. The rate of AG synthesis should be very slow because AG undergoes very slow turnover (10); and this requires the use of a highly sensitive method for AG analysis. The present study demonstrated the synthesis of \([U^{-13}C]\)AG from \([U^{-13}C]\)glucose in cultured cells by means of gas-liquid chromatography/mass spectroscopy, one of the most sensitive methods available for carbohydrate analysis.

**EXPERIMENTAL PROCEDURES**

Materials—\([U^{-13}C]\)glucose (at least 99% pure) was purchased from Isotec (Miamisburg, OH). \([6,6^{-2}H]\)AG and \([U^{-13}C]\)AG were prepared in our laboratory according to established methods (19, 20). Eagle’s minimum essential medium and Dulbecco’s phosphate-buffered saline (PBS) were obtained from Nissui Pharmaceuticals (Tokyo). An anion exchange resin (AG1-X8) and a cation exchange resin (AG50W-X8) were purchased from Bio-Rad Laboratories (Richmond, CA). Prepacked columns for normal phase high performance liquid chromatography (HPLC), Amide-80, 4 mm ID × 25 cm, and a cation exchange column, TSK-SCX, 6 mm ID × 15 cm, were obtained from Tosoh (Tokyo). All other reagents were purchased from Wako Pure Chemical (Osaka).

Methods—Purification of \([U^{-13}C]\)glucose: Since \([U^{-13}C]\)glucose from commercial source contains about 1 ppm of AG, \([U^{-13}C]\)glucose was purified by normal phase HPLC. The column and eluent used for purification were Amide-80 and acetonitrile/water (72:28), respectively. \([U^{-13}C]\)Glucose in the eluate was monitored using a differential refractometer, R401 (Waters).

Culture and cell handling: All cell cultures were carried out in T-25 Corning culture bottles, using four types of culture media. Two of them were Eagle’s minimum essential medium with and without 6% fetal bovine serum, denoted as MEM(+) and MEM(−), respectively. The two other were MEM(+) and MEM(−) which contained \([U^{-13}C]\)-glucose instead of natural glucose, and abbreviated as \(\text{MEM}(+)\) and \(\text{MEM}(-)\). They were prepared by addition of \([U^{-13}C]\)glucose to the corresponding glucose-free media. All the media contained 1 mg/mL of glucose except in the experiment examining AG biosynthesis for glucose dose-dependency.

In the routine culture, Reuber H-35 cells, which had been derived from rat hepatoma cells, were grown to confluence at 37°C in a 95% air and 5% CO\(_2\) atmosphere in a 25 cm\(^2\) Corning culture bottle containing 8 ml of MEM(+) for 36-h incubation of the cells in \(13\text{C}-\text{MEM}(−)\). In other cases, the confluent cells were incubated in 4 ml of \(13\text{C}-\text{MEM}(−)\). In other cases, the confluent cells were incubated in 4 ml of either \(13\text{C}-\text{MEM}(+)\) or \(13\text{C}-\text{MEM}(−)\) for an indicated time.

Identification of \([U^{-13}C]\)AG in culture medium: After 36-h incubation of the cells in \(13\text{C}-\text{MEM}(−)\), 12 ml of the culture medium was collected from triplicate bottles and dried in a centrifugal evaporator (Model CC100; Tomy, Tokyo). The residue was dissolved in 12 ml of methanol, and the insoluble materials were removed by brief centrifugation. The clear supernatant thus obtained was dried again in the centrifugal evaporator. The resulting residue was dissolved in 1 ml of water, then charged onto a column packed with 6 ml of the H\(^+\)-form of AG50W-X8 and eluted with 30 ml of water. All of the eluate was dried, and the resulting residue was similarly charged onto a column packed with 6 ml of the OH\(^−\)-form of AG1-X8, eluted again with 30 ml of water and dried. The resulting residue was further fractionated by normal phase HPLC on a TSK-SCX column which was in the H\(^+\)-form. The eluent for this chromatography was acetonitrile/water (80:20). Non-volatile matter in the eluate whose retention time corresponded to that of authentic natural AG was subjected to analysis for \([U^{-13}C]\)AG by gas chromatography mass spectrometry (GC/MS) using a JEOL DX-300. The details of this method were described elsewhere (10).

Quantification of synthesized AG: After incubation of the cells in 4 ml of \(13\text{C}-\text{MEM}(+)\) or \(13\text{C}-\text{MEM}(−)\), 20 ng of \([6,6^{-2}H]\)AG, which served as the internal standard, was added to the culture medium. The AG in these media was pre-purified by two-step column treatment as described above except that the scale was reduced to one-third. AG in the eluate was further purified by normal phase HPLC on an Amide-80 column. The elution was performed isocratically with acetonitrile/water (80:20). The fraction eluted at the retention time determined for authentic natural AG was collected. The AG fraction was dried, and the resulting residue was treated with 100 μl of 6 N HCl at 110°C for 12 h to degrade any contaminants. Then the acid-treated sample was dried in vacuo over granulated NaOH, and the residue was again subjected to normal phase HPLC under the same conditions as before. The fraction corresponding to AG was collected again on the basis of the retention time. The eluate was dried and the residue was subjected to acetylation in 75 μl of acetic anhydride/pyridine (1:2) at 110°C for 15 min. The acetylated sample was dried again and dissolved in 5 μl of p-xylene and a 1-μl portion was injected into a capillary column (HiCap CBP 1-M25; Shimadzu, Kyoto) in a gas chromatograph-mass spectrometer (QP-2000; Shimadzu) at 120°C. For separation, we employed a temperature gradient rising from 180 to 182°C in 4 min. The peracetylated derivatives of natural AG and two isotope-labeled AGs, \([U^{-13}C]\)AG and \([6,6^{-2}H]\)AG, each yielded a respective unique ion fragment, i.e., m/z = 172 for [U-13C]AG, m/z = 176 for [6,6-2H]AG, which served as the internal standard, was added to the culture medium. The AG in these media was pre-purified by two-step column treatment as described above except that the scale was reduced to one-third. AG in the eluate was further purified by normal phase HPLC on an Amide-80 column. The elution was performed isocratically with acetonitrile/water (80:20). The fraction eluted at the retention time determined for authentic natural AG was collected. The AG fraction was dried, and the resulting residue was treated with 100 μl of 6 N HCl at 110°C for 12 h to degrade any contaminants. Then the acid-treated sample was dried in vacuo over granulated NaOH, and the residue was again subjected to normal phase HPLC under the same conditions as before. The fraction corresponding to AG was collected again on the basis of the retention time. The eluate was dried and the residue was subjected to acetylation in 75 μl of acetic anhydride-pyridine (1:2) at 110°C for 15 min. The acetylated sample was dried again and dissolved in 5 μl of p-xylene and a 1-μl portion was injected into a capillary column (HiCap CBP 1-M25; Shimadzu, Kyoto) in a gas chromatograph-mass spectrometer (QP-2000; Shimadzu) at 120°C. For separation, we employed a temperature gradient rising from 180 to 182°C in 4 min. The peracetylated derivatives of natural AG and two isotope-labeled AGs, \([U^{-13}C]\)AG and \([6,6^{-2}H]\)AG, each yielded a respective unique ion fragment, i.e., m/z = 172 for natural AG, m/z = 172 for [6,6-2H]AG, and m/z = 176 for [U-13C]AG, in their fragmentograms. The relative amounts of the natural AG and two isotope-labeled AGs were calculated from the peak heights of the respective fragments in the selected ion chromatography monitoring of these characteristic fragments. The natural AG showed a minor fragment with m/z = 172 due to a natural abundance of the heavier isotope in the carbon atoms and limited resolution of the spectrometer used. We therefore calculated the net intensity of the 172 fragment attributable to [6,6-2H]AG by subtracting the contribution of the natural AG to the intensity at m/z = 172 from the total intensity of the fragments showing m/z = 172. The contribution in each run was estimated from the intensity of m/z = 170, which was attributable solely to natural AG, using the following calculation formula:

\[
I(172,d2) = I(172,\text{obsvd}) - I(170,\text{obsvd}) \times I(172,\text{ntrl})/I(170,\text{ntrl})
\]

where \(I(172,d2)\) is the intensity attributable to the frag-
ment \( m/z = 172 \) derived from \([6,6-^2H]AG\), \( I(172, \text{obsvd}) \) and \( I(170, \text{obsvd}) \) are the observed intensities at \( m/z = 172 \) and 170, respectively, and \( I(172, \text{ntrl})/I(170, \text{ntrl}) \) stands for the intensity ratio of \( m/z = 172 \) and 170 determined in the control run using natural AG.

**Measurement of glucose concentration:** The glucose concentration in each culture medium was enzymatically determined with a glucose-measurement kit (Glucose B-test, Wako Pure Chemical).

![Figure 1. Total ion chromatograms and selected ion chromatograms for the two major fragments, \( m/z = 176 \) and 218, of the authentic \([U-^{13}C]AG\) and the sample partially purified from the culture supernatant. The authentic \([U-^{13}C]AG\) (panels a) was directly peracetylated and the sample from the culture supernatant (panels b) was peracetylated after the treatments described in the text. GC/MS analysis was performed using a JEOL, Model DX-300.](image)

**TABLE I.** Intensity and assignment of major fragments detected in gas chromatographic peak corresponding to AG. The intensity and the mass number of the major fragments detected in the fragmentograms for the 5.3-min peaks in Fig. 1 are summarized and each fragment is assigned to the one expected to arise when the peracetylated derivative of AG loses first an acetoxy group and then one or more of its acetic acids, ketenes, acetoxymethylenes.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Authentic ([U-^{13}C]AG)</th>
<th>Culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>( M^+ )</td>
<td>338 (0.0)</td>
<td>338 (0.0)</td>
</tr>
<tr>
<td>( M^+ - \text{AcO} )</td>
<td>279 (3.7)</td>
<td>279 (2.0)</td>
</tr>
<tr>
<td>( M^+ - \text{AcOCH} )</td>
<td>264 (3.8)</td>
<td>264 (3.9)</td>
</tr>
<tr>
<td>( M^+ - \text{AcOCH}_2 - \text{C-O} )</td>
<td>236 (2.3)</td>
<td>236 (3.0)</td>
</tr>
<tr>
<td>( M^+ - 2\text{AcO} )</td>
<td>218 (45.7)</td>
<td>218 (44.2)</td>
</tr>
<tr>
<td>( M^+ - 2\text{AcOCH}_2 - \text{C-O} )</td>
<td>176 (100.0)</td>
<td>176 (100.0)</td>
</tr>
<tr>
<td>( M^+ - \text{AcOCH} + \text{CH}_2 = \text{C-O} )</td>
<td>162 (22.7)</td>
<td>162 (20.8)</td>
</tr>
<tr>
<td>- \text{AcOCH} )</td>
<td>144 (46.0)</td>
<td>144 (36.1)</td>
</tr>
<tr>
<td>( M^+ - 2\text{AcOCH}_2 - \text{C-O} )</td>
<td>116 (91.6)</td>
<td>116 (60.8)</td>
</tr>
<tr>
<td>( M^+ - 2\text{AcOCH}_2 - \text{C-O} )</td>
<td>102 (127.1)</td>
<td>102 (81.6)</td>
</tr>
</tbody>
</table>

**RESULTS**

**Identification of AG Produced in Culture Medium**—Figure 1 shows the total ion chromatogram (TIC) and the selected ion chromatograms (SICs) of the major fragments, \( m/z = 176 \) and 218, for the authentic \([U-^{13}C]AG\) (a-panels) and for the sample partially purified from the culture supernatant obtained after 36-h incubation of the cells with \(^{13}C\)-MEM(-) (b-panels). The retention times of the single peaks in the run for the authentic sample were all 5.3 min. The TIC in the b-panels shows that the sample had many peaks derived from a large number and amount of impurities, while one of the major peaks in both SICs in panel b was observed at the retention time for AG, i.e., 5.3 min, at which the TIC showed a minor but distinctly isolated peak. No such peaks in the TIC or SICs were observed when the cells were instantaneously exposed to \(^{13}C\)-MEM(-) (data not shown), thus indicating that the substance responsible for the peaks at 5.3 min was produced only by a prolonged incubation of the cells with the medium. The relative intensities of the peaks for \( m/z = 176 \) and \( m/z = 218 \) at 5.3 min in the SICs, based on the total intensity of the peak at the same retention time in the TIC, were 7.7 and 3.5% for the authentic \([U-^{13}C]AG\) and 7.3 and 3.2% for the sample, respectively (note the difference in the scale for the ordinate among the figures for the SICs and TICs). The relative intensities for the sample thus almost agreed with those for the authentic \([U-^{13}C]AG\). This agreement indi-

![Figure 2. Time courses of glucose consumption (a) and AG accumulation (b). Confluent cells were cultured either in \(^{13}C\)-MEM(+) (●) or \(^{13}C\)-MEM(-) (■) for the indicated times. The glucose concentration in small aliquots of each culture supernatant was measured using the Glucose B-test kit. The AG in the remaining culture supernatant was measured as described in the text using a GC/MS QP-2000.](image)
cells at any time point in these incubations. Since \( \text{[U-13C]} \)AG contained approximately 90 ng/ml of natural AG was detected within the about 12 ng in either cases. On the other hand, only a produced in 4 ml of the medium after 48 h of incubation was incubation time in both media. The amount of \( \text{[U-13C]} \)AG the progress of incubation time. Third, the rate of AG accumulation is more closely related to the initial glucose medium. Figure 3a shows the relationship between the initial glucose concentration and the amount of glucose consumed in 48 h of incubation. The consumption increased linearly with the initial glucose concentration up to around 1 mg/ml, then abruptly became constant above this concentration. Thus, glucose utilization and AG synthesis from glucose showed markedly different profiles in their glucose concentration dependency.

**Fig. 3. Dependencies of glucose consumption (a) and AG accumulation (b) on initial glucose concentration.** Confluent cells were cultured in \(^{13}\text{C-MEM}(+)\) containing the indicated amounts of \(^{13}\text{C}\)-glucose. The culture supernatant was treated and analyzed for glucose and AG as described in the legend for Fig. 2.

cates that the 5.3-min peaks for the sample arose mostly from \( \text{[U-13C]} \)AG. Table I lists the relative intensities taking the 176 fragment as the base. The table also shows the assignments of all the major fragments which arose from peracetylated \( \text{[U-13C]} \)AG by loss of an acetoxo group and one or more acetic acids, ketenes, or acetoxymethylenes. The identity as AG of the 5.3-min peak in the TIC of the purified sample was thus confirmed by the close agreement of the relative intensities of all the major fragments with the intensities of the corresponding fragments for the authentic \( \text{[U-13C]} \)AG.

**Time Course of AG Synthesis and Glucose Consumption**—Figure 2a shows the amount of glucose consumed the incubation of the confluent cells in media in the presence or the absence of serum. In both media, the rate of glucose consumption was rather constant throughout the incubation, and glucose was still present in the medium 48 h after the start of the incubation when the initial glucose concentration was as much as 1 mg/ml. Only a minor effect of serum was observed in the glucose consumption under the present experimental conditions. Figure 2b shows the time course of \( \text{[U-13C]} \)AG accumulation in the culture media with or without serum, as a function of the incubation time. The rate of accumulation seemed to increase with incubation time in both media. The amount of \( \text{[U-13C]} \)AG produced in 4 ml of the medium after 48 h of incubation was about 12 ng in either cases. On the other hand, only a negligible amount of \( \text{[U-13C]} \)AG was detected within the cells at any time point in these incubations. Since \(^{13}\text{C-MEM}(+)\) contained approximately 90 ng/ml of natural AG originating from fetal bovine serum, we also studied the effect of added natural AG on the accumulation of \( \text{[U-13C]} \)AG, but found no notable effect (less than 3% of suppression).

**Glucose Dose Dependency of AG Synthesis**—The amounts of accumulated AG and consumed glucose in 48 h of incubation were measured for various initial concentrations of \( \text{[U-13C]} \)glucose ranging from 0.2 to 2.4 mg/ml. Since each incubation medium also contained ca. 0.032 mg/ml of natural glucose originating from the serum, the actual amount of glucose should be the sum of the two types of glucose, and the amount of accumulated AG was calibrated for that sum. Figure 3b shows the relationship between the amount of accumulated AG and the initial glucose concentration. The higher the initial glucose concentration was in the culture medium, the more AG was accumulated in the medium. Figure 3a shows the relationship between the initial glucose concentration and the amount of glucose consumed in 48 h of incubation. The consumption increased linearly with the initial glucose concentration up to around 1 mg/ml, then abruptly became constant above this concentration. Thus, glucose utilization and AG synthesis from glucose showed markedly different profiles in their glucose concentration dependency.

**DISCUSSION**

The present study demonstrated AG synthesis in cultured rat hepatoma cells. Several characteristic aspects of this synthesis were observed. First, AG is formed from glucose with retention of all six carbon atoms in the pyranose ring. Second, the synthesized AG accumulated in the culture medium and the accumulation rate appeared to accelerate with the progress of incubation time. Third, the rate of AG accumulation is more closely related to the initial glucose concentration in the medium than to the glucose consumption by the cells. And lastly, the observed conversion ratio is at most 5 x 10\(^{-4}\) at an initial glucose concentration of 1 mg/ml under the present experimental conditions.

Since the fraction of glucose converted to AG was very small and the AG accumulation detected in the 4 ml of medium was in the order of 10–20 ng, the mass spectrometry was considered the best method to confirm the chemical identity of AG newly synthesized in the cells. For clarity's sake, its identity was confirmed in the culture medium without serum, since serum would introduce 360 ng of natural AG into the experimental system (4 ml in volume), and this would considerably mask the 10–20 ng of AG expected to be newly synthesized by the cells. Other components in serum also might hamper the interpretation of the results of GC/MS analysis. The use of isotope-labeled glucose as the starting material in AG synthesis further confirmed the chemical identity of the product; the synthesized AG contained all of the heavier carbon atoms and produced unique fragments (e.g., 176 and 218) which were much less superimposed than those from the natural AG (e.g., 170 and 212) by the fragments derived from the contaminants in the AG peak in GC/MS analysis. Furthermore, the use of the universally-labeled glucose revealed that AG was rather directly derived from glucose: the conservation of all six carbons of glucose in the pyranoid ring of AG rules out the possibility that endogenous substrates arising from other nutrients in the medium and seeded cells are
Synthesis of 1,5-Anhydroglucitol in Rat Hepatoma Cells