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Escherichia coli Phosphorylates 1,5-Anhydroglucitol and Releases 1,5-Anhydroglucitol 6-Phosphate When Glucose Is Absent in the Medium

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The cyclic polyol 1,5-anhydro-D-glucitol (AG) is detected in most organisms, but little is known about its metabolism and physiological roles. Our previous study demonstrated that *Escherichia coli* C600 synthesizes AG when glucose is exhausted in the medium and that it temporarily releases AG into and then takes it back from the medium, thus forming a sharp peak in AG concentration in the medium a few hours after reaching stationary growth phase. The present study demonstrates that when glucose is absent in the culture medium, *E. coli* C600 takes up and phosphorylates AG and releases a large portion of it back into the medium in the form of a phosphate ester. [*U*-^{13}C]AG was added to the medium after the exhaustion of glucose and the resulting [*U*-^{13}C]AG phosphate was partially purified by several steps of anion exchange chromatography and identified as AG 6-phosphate by ^{13}C-NMR. The identity of the phosphate ester was also confirmed by GC-MS analysis after further purification.

Key words: 1,5-anhydro-D-glucitol, ^{13}C-NMR, *E. coli*, phosphorylation, sugar metabolism.

The cyclic polyol 1,5-anhydro-D-glucitol (AG) is the 1-deoxy form of glucopyranose. It was first found in the polygara family (*1*) and has also been shown to be present in human cerebrospinal fluid (*2, 3*) and blood (*4*). It is now known to be present in a wide variety of animals and plants (*5*). The plasma AG concentration in healthy humans is unusually stable and relatively unaffected by food intake (*6, 7*) and by changes in most other physiological states (*7, 8*). However, it specifically decreases in diabetes mellitus (*4, 7, 9-13*); glucose at a high concentration in blood prevents AG from being reabsorbed in the renal tubules and AG leaks into the urine (*6*). This characteristic behavior makes it possible for AG concentration in blood to serve as a clinical index for glycemic control (*14, 15*).

We have reported previously that *Escherichia coli* C600 and the hepatoma cells H-35 synthesize AG (*16, 17*), and much evidence is being accumulated for AG synthesis in other strains of animal cells (our unpublished observations). We have also found that the human chronic myelogenous leukemia cells K-562 phosphorylate AG, and that the phosphorylated AG (AGP) is present in various rat organs (*18*). The ubiquitous occurrence and self-provision of AG in a number of organisms belonging to various genera indicate that AG and/or AG-related compounds may be indispensable to them. Little is known about their roles or functions in any organism though.

In this study we cultured *E. coli* C600 in a medium containing [*U*-^{14}C]AG and [*U*-^{13}C]AG, and traced their metabolites in the medium and cells. We also partially purified the main metabolite of the latter and identified it as AG 6-phosphate (AG6P) by ^{13}C-NMR. We have not yet fully purified the AG phosphate due to its unexpected instability in the process of purification, but the present structural study has overcome this disadvantage by the use of AG universally labeled with carbon-13.

MATERIALS AND METHODS

Materials—Natural D-glucose, [6,6-^{2}H]glucose, [*U*-^{13}C]-glucose, and [*U*-^{14}C]glucose were products of Wako Pure Chemical Industries (Osaka), MSD isotopes (Montreal, Canada), Isotec (Miamisburg, OH), and Amersham (Buckinghamshire, England), respectively. The former three were further purified by HPLC on a TSK gel Amide-80 column (4.6 mm ID, 25 cm; Tosoh, Tokyo) using acetonitrile/water (72 : 28) as the eluent. AG was obtained from Nippon Kayaku (Tokyo). [*U*-^{14}C]AG was synthesized from [*U*-^{14}C]glucose (10.4 GBq/mmol) at Nippon Kayaku. [6,6-^{2}H]AG and [*U*-^{13}C]AG were prepared from [6,6-^{2}H]-glucose and [*U*-^{13}C]glucose, respectively, in our laboratory according to an established method (*19*). AG6P, [*U*-^{13}C]-AG6P, and [6,6-^{2}H]AG6P were prepared from corresponding AGs according to the method described in our previous paper (*18*). (Trimethylsilyl)diazomethane ($TMSCHN_2$) was from Aldrich (Wisconsin, USA). *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Pierce (Illinois,

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Abbreviations: AG, 1,5-anhydro-D-glucitol; AGP, phosphorylated 1,5-anhydro-D-glucitol; AG6P, 1,5-anhydro-D-glucitol 6-phosphate; AGXs, unknown metabolites of 1,5-anhydro-D-glucitol; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; RI-detector, refractive index detector; SIC, selected ion chromatogram; $TMSCHN_2$, (trimethylsilyl)diazomethane; TMSAGPDE, tri-*O*-trimethylsilyl derivative of AG phosphate dimethylester.

USA). Glucose B-test (Wako) was used to determine glucose concentration in the medium. Casamino Acids, Bacto tryptone, Bacto yeast extract were purchased from Difco Laboratories (Detroit, MI). Alkaline phosphatase from calf intestine was obtained from Boehringer Mannheim Biochemica (Mannheim, Germany). An anion exchange resin, AG1-X8, and a cation exchange resin, AG50W-X8, were the products of Bio-Rad Laboratories (Richmond, CA). A liquid scintillation cocktail, ACS II, was from Amersham. All other chemicals were from Wako Pure Chemical Industries, and used without further purification. *E. coli* C600 was a kind gift from Dr. Isao Katsura of the National Institute of Genetics (Shizuoka).

Precultivation and Cultivation with Glucose—*E. coli* C600 was first inoculated in 5 ml of Luria-Bertani medium (Bacto tryptone 1%, Bacto yeast extract 0.5%, NaCl 1%) and grown overnight at 37°C. The resulting cells were collected by a mild centrifugation (1,400×*g*, 20 min), washed once with ice-cold saline, and then suspended in the original culture volume of saline. An appropriate volume of the suspension was added to 100-fold volume of M9 medium (KH_2PO_4 0.3%, Na_2HPO_4 0.6%, NaCl 0.05%, NH_4Cl 0.1%, $MgSO_4$ 0.024%, $CaCl_2$ 0.001%, pH 7.4) supplemented with Casamino Acids (0.2%) and glucose (0.2%). The culture mixture was incubated at 37°C; occasionally the absorbance at 600 nm was measured, and portions of 0.01-0.1 ml were saved for determination of the glucose concentration. At indicated times of incubation, 3-ml portions of the culture were taken for further incubation with [*U*-^{13}C]AG or [*U*-^{14}C]AG.

Incubation of the Culture with [U-^{13}C]AG—Each 3-ml portion of the culture was placed in a separate tube, to which [*U*-^{13}C]AG was added as the exogenous substrate to a concentration of 1.3 nmol/ml. Immediately after the addition and after 30 min, 1 h, and 2 h of incubation at 37°C, aliquots were taken and the amounts of remaining [*U*-^{13}C]AG in the medium and [*U*-^{13}C]AG and [*U*-^{13}C]-AGP in the cells were measured. As a control experiment, the harvested *E. coli* culture was centrifuged and the resulting cell-free medium was also incubated similarly, and the absence of AG-converting activity was confirmed. All incubations were triplicated for each incubation time.

Incubation of the Culture with [U-^{14}C]AG—A similar experiment was carried out by adding [*U*-^{14}C]AG to a concentration of 1.46 nmol/ml (5,500 cpm/ml) instead of [*U*-^{13}C]AG, and the amounts of [*U*-^{14}C]AG, [*U*-^{14}C]AGP, and other products were measured in the medium and cells. This experiment was also carried out in triplicate.

AG and AGP Assays—One milliliter of the culture was centrifuged at 15,000×*g*, for 2.5 min, at 4°C to separate the medium and cells. Fixed amounts of authentic [6,6-^{2}H]AG and [6,6-^{2}H]AG6P (usually 20-50 ng each) were added to the supernatant as internal standards. The supernatant was then dried in a centrifugal evaporator (CC100; Tomy, Tokyo) and the residue was dissolved in a small amount of water and applied onto an anion exchange column (5.5 mm ID, 0.5 ml) in OH^- form. The charged column was washed with 5 ml of distilled water and all the effluent was collected. This fraction was saved for AG assay. The trapped substances were then eluted from the column with 1 M ammonium acetate. The first 1 ml was collected and saved for AGP analysis.

The water-eluted fraction was dried and the residue was dissolved in a small amount of water and applied onto a cation exchange column (5.5 mm ID, 0.5 ml) in H^+ form to remove the cationic materials. The deionized fraction was collected by washing the column with 2 ml of distilled water, then dried and further treated by HPLC on the TSK gel Amide-80 column using acetonitrile/water (80:20) as the eluent. The eluate was monitored with a refractive index detector (RI-detector, RE-61; Showadenko, Tokyo). The fraction corresponding to AG was dried and the resulting 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 up and the residue was dissolved in 5 μl of *p*-xylene. A 1-μl portion was injected onto a capillary column (HiCap CBP1-M25; Shimadzu, Kyoto) in a GC-MS (QP-2000; Shimadzu) at 130°C and the final separation was carried out at 230°C. For quantitative analysis by selected ion chromatography, the ion fragments unique to [6,6-^{2}H]AG (m/z=172) and to [*U*-^{13}C]AG (m/z=176) were monitored. The amount of [*U*-^{13}C]AG in the sample was calculated by comparing its peak area (m/z=176) with that of the corresponding fragment of [6,6-^{2}H]-AG (m/z=172).

For AGP assays, the 1 M ammonium acetate-eluted fraction was dried, and the residue was dissolved in a small amount of water and applied to the cation exchange column (5.5 mm ID, 0.5 ml) in H^+ form. The untrapped materials were collected with 2 ml of distilled water. The collected effluent was dried and applied to a TSK gel SAX column (6.0 mm ID, 25 cm; Tosoh) and eluted with 0.5 M ammonium acetate. Elution was also monitored with the RI-detector. The fraction corresponding to AG6P was dried, the residue was subjected to the alkaline phosphatase treatment described below, and the liberated AG was analyzed by GC-MS as stated above.

For determination of AG and AGP in the cells, the cell pellet was once washed with cold saline, and authentic [6,6-^{2}H]AG and [6,6-^{2}H]AG6P were added as internal standards. To disrupt the cells and denature the enzymes, the cell pellet was suspended in a cold mixture of water, methanol and chloroform (0.2, 0.5, and 0.25 ml, respectively), and the suspension was kept on ice for 1 h. Then, 0.1 ml of water and 0.75 ml of chloroform were further added to the suspension and the mixture was vigorously agitated. The suspension was then separated into three layers including the intervening solid layer by a brief centrifugation at 1,400×*g* [Folch's partitioning (*20*)], and the upper layer was collected. The upper layer was dried and the residue was treated as described above stated for AG and AGP determination.

Phosphatase Treatment—The fractions recovered in HPLC on the TSK gel SAX column were placed in pointed-tip plastic tubes, dried and further kept *in vacuo* for full removal of ammonium acetate by sublimation. Then 9 μl of dephosphorylation buffer (50 mM Tris-HCl, 0.1 mM EDTA, pH 8.5) and 1 μl of the alkaline phosphatase solution (1 unit) were added to each tube and the mixtures were incubated at 37°C. After 1 h of incubation, each reaction mixture was diluted with 0.5 ml of water and charged onto a two-layer column (5.5 mm ID) packed with 0.25 ml of the anion exchanger in OH^- form at the bottom and 0.25 ml of the cation exchanger in H^+ form at the top. Each charged column was washed with 2.5 ml of distilled water and all the effluent was collected and dried. Then free AG was

isolated on TSK gel Amide-80, acetylated and subjected to GC-MS analysis as above.

Assays of [U-^{14}C]AG, [U-^{14}C]AGP, and Related Metabolites—These assays were done by similar but simpler methods than AG and AGP analyses. One milliliter of the culture was centrifuged to get the supernatant and the cell pellet. One half of the supernatant was applied onto an anion exchange column (0.25 ml) without adding internal standards. The whole cell pellet, also without any internal standards, was treated with methanol and chloroform, and the upper layer obtained by Folch's partitioning was applied onto another anion exchange column (0.25 ml). They were eluted with water (2.5 ml) and then 1 M ammonium acetate (1 ml). Appropriate amounts (less than 1 ml) of the water- and ammonium acetate-eluted fractions were mixed with 5 ml of ACS II and the radioactivities were measured in a liquid scintillation counter (LS-1800; Beckman, California, USA). Radioactivity in the water-eluted fraction was assigned to [U-^{14}C]AG, and that in the 1 M ammonium acetate-eluted fraction to [U-^{14}C]AGP. The validity of this assignment was confirmed in separate experiments (*cf.* "RESULTS AND DISCUSSION"). The radioactivity remaining on the column after the elution with 1 M ammonium acetate was counted directly after transferring all the anion exchange resin from the column into a scintillation vial. This radioactivity was assigned to unknown anionic metabolites of [U-^{14}C]AG ([U-^{14}C]AGXs).

Partial Purification of AGP—E. coli cells were cultured in four separate bottles each containing 500 ml of M9 medium. After the culture had reached the stationary phase, [U-^{13}C]AG (1.5 mg) was added, and it was further incubated at 37°C for 2 h. The culture supernatant was separated from the cells by centrifugation at 4°C and applied onto an anion exchange column (8.0 cm ID, 250 ml) in OH^- form. The charged column was washed thoroughly with two liters of distilled water to remove the remaining free AG. Then the trapped substances were eluted with 1 M ammonium acetate. The first fraction of 200 ml was discarded and the next fraction (100 ml) was collected and lyophilized. The resulting residue was dissolved in a small amount of water and applied onto a cation exchange column (4.6 cm ID, 100 ml) in H^+ form to remove the cationic substances. The untrapped materials were recovered from the column in 200 ml of eluate, which was then lyophilized and the resulting residue was charged onto an anion exchange column (4.6 cm ID, 100 ml) in OH^- form. The column was washed with 220 ml of water, then the materials were eluted with 0.5 M ammonium acetate. The first 80 ml of eluate was discarded, and the next 20 ml was collected and lyophilized. The residue was dissolved in 1 ml of water, a 0.1-ml portion was applied onto the TSK gel SAX column, and the substances were eluted with 0.5 M ammonium acetate at a flow rate of 1 ml/min. This chromatography was repeated and the AGP fraction in each run was pooled. After the evaporation, the pooled materials were further purified by a single cycle of rechromatography under the same conditions, which was repeated two times. When necessary, the sample was further purified on the TSK gel Amide-80 column using acetonitrile/water (70 : 30) as the eluent at a flow rate of 0.8 ml/min.

AGP was also partially purified from the cells and subjected to qualitative analysis. For this purpose, the cell pellet was treated essentially by the same method as was employed for quantification, but on a larger scale. The upper layer in Folch's partitioning was treated similarly to the culture supernatant.

Derivatization of AGP and Analysis by GC-MS—The phosphate moiety of each AGP was first methylated, then the alcoholic OHs were trimethylsilylated. Thus rather stable tri-*O*-trimethylsilyl derivatives of AG phosphate dimethylesters (TMSAGPDEs) were synthesized. About 1 μg each of authentic [U-^{13}C]AG6P, [6,6-^{2}H]AG6P, or the above partially purified [U-^{13}C]AGP was dissolved in 100 μl of methanol, then 50 μl of 2 M $TMSCHN_2$ in hexane was added, and the mixture was left to stand at room temperature overnight in the dark. The solvent was removed under a nitrogen stream, the residue was dissolved in 20 μl of pyridine and 20 μl of BSTFA was added to the solution. After the mixture had stood for 30 min at room temperature, an aliquot was applied to GC-MS analysis using the same system employed for peracetylated AG analysis; a 2-μl portion was injected onto the column at 130°C and the final separation was carried out at 230°C. Three ion fragments were monitored. One was $[TMSi]^+$ ($m/z=73$), which is the most abundant ion and unique to every trimethylsilylated compound; the other two were [TMSiO-CH=CHOTMSi]$^+$ ($m/z=204$) and [TMSiO^{13}CH=^{13}CHO-TMSi]$^+$ ($m/z=206$), which are general predictors of the natural and [U-^{13}C]pyranose ring, respectively (*21*).

Identification of the Phosphorylation Site of the AGP Compound by ^{13}C-NMR—About 100 μg of [U-^{13}C]AGP purified from the supernatant, or about 200 μg of enzymatically phosphorylated [U-^{13}C]AG6P was dissolved in 0.5 ml of H_2O plus 0.1 ml of D_2O and submitted to ^{13}C-NMR analysis. The ^{13}C-NMR spectrum of each sample was obtained on an NMR spectrometer (JNM-A500; JEOL, Tokyo) operating for ^{13}C aquisition at 125.65 MHz and simultaneous broad-band decoupling at 500 MHz. The utilization of this technique eliminates all ^{13}C-^{1}H spin-spin splittings but retains the coupling patterns caused by ^{13}C-^{13}C and ^{13}C-^{32}P interactions. Data were accumulated employing 33,898 Hz sweep width in 32,768 points (resolution 1.03 Hz). The acute peak at 49.90 ppm of methanol was used as the external standard of chemical shifts.

RESULTS AND DISCUSSION

Time Courses of AG Uptake and Accumulations of AG and AGP in the Cells—Figure 1 shows the amounts of AG consumed in the medium and of AG and AGP in the cells as function of incubation time. As we reported previously (*16*), AG added to the medium was not taken up when glucose was present in the medium. But after its exhaustion, the exogenous AG in the medium decreased at a dramatically high rate in the first stage: the incorporation rate was so high that the concentrations of AG determined in the medium separated from the first aliquots of culture, which were removed immediately after the addition of AG, were already 0.2-0.4 nmol/ml lower than was expected for the initial concentration (1.3 nmol/ml). The cells must have incorporated such amounts in the few minutes needed for centrifugal separation of the medium and cells. Although the decrease in AG in the medium in 2 h was 0.9-1.1 nmol/ml, the total amount of AG and AGP concomitantly accumulated in the cells in 1 ml of culture was only 0.1-0.2 nmol, not enough to account for the decrease in the

medium.

Tracing the Missing AG Metabolites—To find the missing AG metabolites, we carried out a similar experiment using [*U*-^{14}C]AG as the exogenous substrate. Radioactivity was measured in the water-eluted and 1 M ammonium acetate-eluted fractions obtained from the cells and medium harvested 2 h after addition of [*U*-^{14}C]AG to the culture in the stationary phase. Of the original radioactivity, about 90% was recovered: about 20% in the cells (4% in the water-eluted fraction, 10% in the 1 M ammonium acetate-eluted fraction, and 6% remaining on the anion exchange column); and, unexpectedly, about 70% in the medium (24% in the water-eluted fraction, as much as 45% in the 1 M ammonium acetate-eluted fraction, and 1-2% remaining on the anion exchange column). Upon co-chromatography with authentic AG on TSK gel amide 80, all the radioactivity in the water-eluted fraction either from cells or medium was recovered in the AG peak. Upon phosphatase treatment followed by HPLC on TSK gel amide 80, most of the radioactivity in the 1 M ammonium acetate-eluted fraction from either cells or medium was also recovered in the AG fraction in the HPLC. These observations indicate that the AG derivative in the 1 M ammonium acetate-eluted fraction was AGP, and that most of the AG incorporated into the cells was phosphorylated and released back into the medium in the form of AGP. Although about 10% of the radioactivity was still missing, we decided to first purify and identify AGP, because this substance was shown to be the main metabolite of AG in the early stage.

Partial Purification of [U-^{13}C]AGP—From the following features, the AGPs in the cells and medium were both considered to be AG6P. (i) They were trapped in the anion exchange columns, and eluted with 1 M ammonium acetate: they are negatively charged. (ii) They were dephosphorylated by the intestinal alkaline phosphatase treatment and liberated free AG: they are phosphomonoesters and not di- or triesters. (iii) They showed the same retention time, which coincides with that of authentic AG6P in HPLC; when each AGP was mixed with authentic AG6P, charged on the SAX column and eluted with 0.5 M ammonium acetate, it was coeluted with the authentic AG6P at 6.8 min. They were also coeluted with AG6P at 6.2 min from the Amide-80 column with acetonitrile/water (70 : 30). AGP was partially purified to confirm its structural identity by GC-MS and NMR.

Every time the sample went through the TSK gel SAX column and Amide-80 column, free AG was observed in the corresponding fraction. This decomposition was more marked when Amide-80 column was used, thus indicating that AGP is dephosphorylated in the preparation process for column separation and/or on the column. Since AGP became more susceptible to dephosphorylation as the purification proceeded, we did not try full purification, in order to limit its loss. The fraction obtained after three passages through the SAX column was subjected to ^{13}C-NMR. The content of [*U*-^{13}C]AGP accounted for 3% of the weight of the dried-up preparation; about 100 μg of [*U*-^{13}C]AGP was obtained from 2 liters of the culture to which 6 mg of [*U*-^{13}C]AG was initially added. This very low yield was due not only to the decomposition of AGP through the purification process but also to the much higher concentration of AG employed in this preparative incubation: when AG was added to a rather low concentration [about 1.5 nmol (240 ng)/ml], as in the uptake experiment in Figs. 1 and 3, AG was taken up efficiently and 35-50% of the added amount was converted to AGP. However, our rough estimate of the K_m for AG in the overall phosphorylation reaction was such that when AG was added to the higher concentration of 18 nmol (3 μg)/ml, only 10% was expected to be converted to AGP (data not shown).

Mass Spectra of TMS[U-^{13}C]AGPDE, Authentic TMS-[U-^{13}C]AG6PDE, and Authentic TMS[6,6-^{2}H]AG6PDE—For GC-MS analysis, the preparation used for ^{13}C-NMR was further subjected to the HPLC on TSK gel amide 80 in order to fully remove ammonium acetate and other ionic materials. In the selected ion chromatogram (SIC) monitored at $m/z=206$ for the TMS derivative of the authentic [*U*-^{13}C]AG6P dimethylester, the peak eluted at 12.96 min was the only peak whose corresponding peak was absent in the blank runs [the fragment $m/z=206$ for [*U*-^{13}C]AG6P corresponds to the one with $m/z=204$ which is unique to the pyranose ring (*21*)]. Reasonably, a peak with almost the

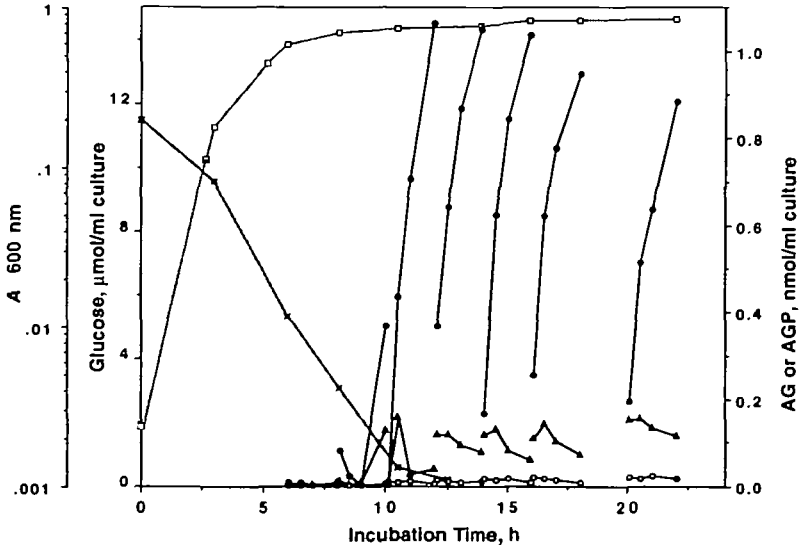

Fig. 1. **Time courses of AG uptake and accumulations of AG and AGP in *E. coli* cells.** *E. coli* cells were cultivated with glucose, and at the indicated time points aliquots were taken, to which [*U*-^{13}C]AG was added. After 0 min, 30 min, 1 h, and 2 h of further incubation, the amounts of [*U*-^{13}C]AG remaining in the medium, and [*U*-^{13}C]AG and [*U*-^{13}C]AGP in the cells were measured. The amount of [*U*-^{13}C]AG consumed in the medium was calculated by subtracting the remaining amount from the initial one, *i.e.*, 1.3 nmol/ml. The values are means of triplicate measurements. Absorbance and glucose concentration of the culture were also measured. Symbols: ●, amount of AG consumed in 1 ml of the medium; ○, △, amounts of AG and AGP, respectively, in the cells obtained from 1 ml of the culture; ×, glucose concentration in the medium; ⊡, absorbance of the culture at 600 nm.

same retention time was also observed in the chromatogram for the corresponding derivative of [6,6-^{2}H]AG6P, which was monitored at $m/z=204$. These observations show that 12.96 min was the elution time of TMSAG6PDE. The [U-^{13}C]AGPs purified from the medium and cells also showed an unambiguous peak at 12.96 min in SIC monitored at $m/z=206$, which suggests that both AGPs are AG6P. The fragmentograms of the peaks for [U-^{13}C]AGP purified from the medium, authentic [U-^{13}C]AG6P and [6,6-^{2}H]-AG6P are shown in Fig. 2 (the pattern for [U-^{13}C]AGP from cells is not shown in Fig. 2, but is essentially the same as the one from the medium). The main fragments were all assigned (details will be published elsewhere). The observation of fragment $m/z=368$, which retained all six carbons of the sugar skeleton, indicates that the phosphorylation site of [U-^{13}C]AGP is C6 and that this compound is [U-^{13}C]AG6P, because no major fragment retains C6 in the case of the derivatives having directly trimethylsililated hydroxymethyl groups at the C6 position. Since GC-MS has such a high sensitivity, its application to sugar phosphates will provide a valuable method for their quantitative analysis, as was demonstrated in this study.

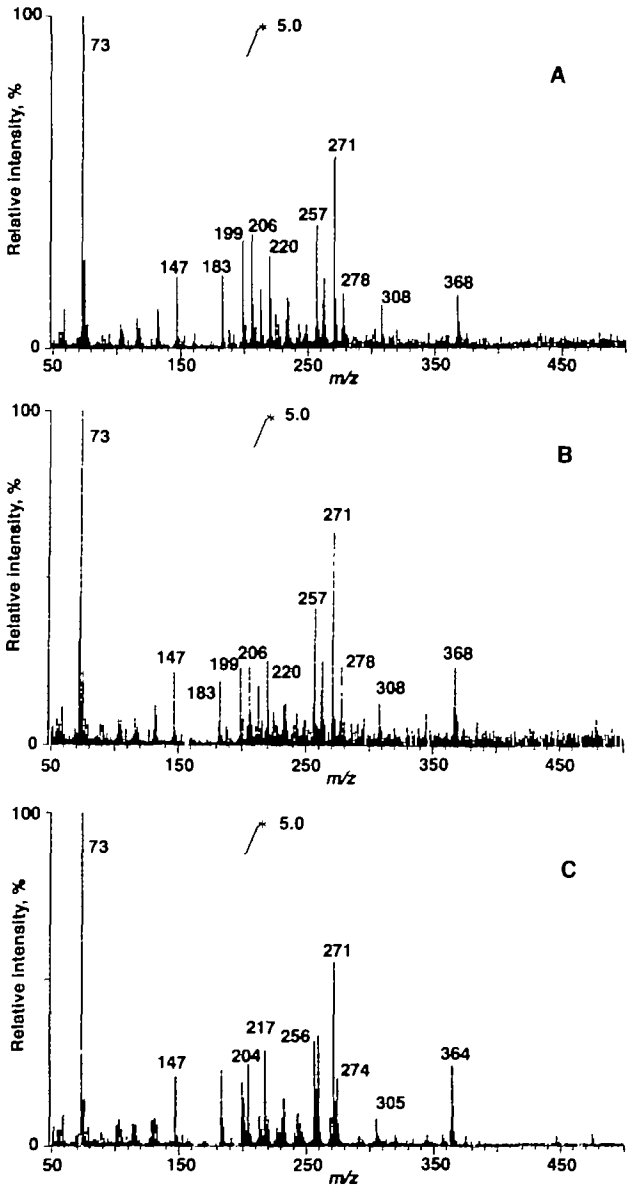

Fig. 2. **Mass fragmentograms for the peaks of tri-*O*-trimethylsilyl derivatives of AG phosphate dimethylesters.** The derivative of authentic [U-^{13}C]AG6P (A), that of [U-^{13}C]AGP in the preparation made from the culture medium in which *E. coli* C 600 had been given [U-^{13}C]AG in the stationary phase (B), and that of authentic [6,6-^{2}H]AG6P (C).

Determination of Phosphorylation Site of [U-^{13}C]AGP by ^{13}C-NMR—The Fourier transform ^{13}C magnetic resonance spectra of the partially purified [U-^{13}C]AGP from the medium (scans = 10,000 times) and authentic [U-^{13}C]-AG6P (scans = 13,000 times) were obtained. The resonance pattern of [U-^{13}C]AGP is almost identical to that of [U-^{13}C]AG6P. The resonances are readily assigned by comparison with the chemical shifts of AG reported in the accompanying paper (*22*) and they are listed in Table I. These assignments were also confirmed by the measurement in two dimensional "incredible natural abundance double quantum transfer experiment" mode, which is the technique for tracing out the carbon skeleton by the momentary creation of double-quantum coherence (*23, 24*). The chemical shifts of C1 through C5 carbons are almost identical in the purified [U-^{13}C]AGP and authentic [U-^{13}C]AG6P, as well as authentic [U-^{13}C]AG. Only those of C6 in the purified [U-^{13}C]AGP and authentic [U-^{13}C]-AG6P are shifted downfield by 3~4 ppm from that of C6 in authentic [U-^{13}C]AG, which indicates phosphorylation of C6 carbon atoms (*25*). The coupling constants of the purified [U-^{13}C]AGP, authentic [U-^{13}C]AG6P, and authentic [U-^{13}C]AG are also shown in Table II. The coupling constants of C6-P in the purified [U-^{13}C]AGP and authentic [U-^{13}C]AG6P are 4.1 and 4.2, while those of C5-P are 8.3 and 7.3, respectively. Phosphorus atoms in ester linkages have a larger effect on the coupling constant

TABLE I. **^{13}C-chemical shifts of purified [U-^{13}C]AGP, authentic [U-^{13}C]AG6P, and authentic [U-^{13}C]AG.**

Assignment	Chemical shift (ppm)		
	Purified [U-^{13}C]AGP	Authentic [U-^{13}C]AG6P	Authentic [U-^{13}C]AG[a]
C1	69.76	69.53	69.62
C2	70.37	70.15	70.06
C3	78.27	78.00	78.63
C4	70.15	69.89	70.46
C5	80.11	79.93	81.76
C6	64.97	64.69	61.63

[a]Values are from the Ref. *22*.

TABLE II. **Coupling constants of purified [U-^{13}C]AGP, authentic [U-^{13}C]AG6P, and authentic [U-^{13}C]AG.**

Assignment	Coupling constant (Hz)		
	Purified [U-^{13}C]AGP	Authentic [U-^{13}C]AG6P	Authentic [U-^{13}C]AG[a]
C1-C2	38.3	38.3	43
C2-C3	37.2	36.7	37
C3-C4	38.5	39.3	40
C4-C5	40.1	40.3	41
C5-C6	44.5	44.5	40
C5-P	8.3	7.3	–
C6-P	4.1	4.2	–

[a]Values are from the Ref. *22*.

of the second nearest carbon atom by a long-range coupling than on that of the nearest carbon atom (*25*). This is also evidence for phosphorylation at the C6 position. According ly, the ^{13}C-NMR demonstrates that the purified [*U*-^{13}C]-AGP was [*U*-^{13}C]AG6P. Although we did not take the NMR spectrum of [*U*-^{13}C]AGP from the cells because the purified sample was too scarce for NMR analysis, GC-MS study indicated that it is also [*U*-^{13}C]AG6P.

The [*U*-^{13}C]AGP sample used here was not thoroughly purified: it was only 3% pure on a weight basis. Since NMR study requires nearly milligram quantity, we could not proceed with further purification. Fortunately, however, the highly impure preparation was applicable to the ^{13}C-NMR study and we could obtain resonance spectra of satisfactory quality. This was because we used a substrate with all the carbons substituted with carbon-13 instead of relying on its natural abundance. In the ordinal ^{13}C-NMR, the weak ^{13}C-^{13}C coupling signals flanking the strong signals of isolated ^{13}C spins are difficult to identify with high sensitivity; but by using [*U*-^{13}C]compounds, the ^{13}C-^{13}C coupling signals were observed without interference from signals of isolated carbon-13s.

Qualitative and Quantitative Estimations of Other Metabolites—Figure 3 shows the time courses of exogenous AG uptake and the distribution of the resulting AG metabolites in the cells and medium. The behaviors of AG and AG6P in the cells observed in Fig.1 were reproduced in this experiment. Figure 3 illustrates the rapid accumulation of AG6P in the medium. The amount of AG6P in the medium corresponds to about 50% of the AG first incorporated into the cells. The temporal behavior of AG6P in the cells suggests that the phosphorylation of AG occurred without delay, and AG6P was released with a short lag time.

The sum of the radioactivities of AG and AG6P in the cells and AG6P in the medium is still smaller than the decrease of AG radioactivity in the medium: 18-22% of the total activity is missing. A portion (10-11% of the added amount) of the missing activity was trapped on the anion exchange columns as AGXs. The rest (less than 12% of the added amount) may have escaped into cell-washings and into the air as CO_2. Since AGXs in the cells increased with the time elapsed after the addition of AG, a part of AG and/or AG6P must have been rather constantly metabolized to more negatively charged substances. In contrast, the slow increase of AGXs in the medium indicated that they were retained in the cells more than AG6P. These substances are yet to be identified.

AG and AG6P Transport across Bacterial Plasma Membranes—AG behaved differently in *E. coli* cells from in mammalian cells. In mammalian cells, it rapidly establishes a concentration equilibrium across the plasma membrane, thus indicating passive AG transport system in the plasma membrane (*26-28*); AG6P is accumulated only in the cells at a much slower rate. By contrast, AG appeared to be concentrated in *E. coli* cells and instantaneously phosphorylated when glucose was absent in the medium, which indicates active transport systems in the bacterial plasma membranes. In this study we also found that a relatively large amount of AG6P was released outside the cells. Phosphorylated sugars are usually found inside the cell, and rarely in the medium. This is because phosphorylation of sugars occurs simultaneously with their transportation into the cells, and because phosphorylated sugars do not freely diffuse across the plasma membrane to the medium (*29*). There are three ideas to explain the existence of large amount of AG6P in the medium: (i) the culture medium has attained AG-phosphorylating activity, (ii) the cells are disrupted and the phosphorylated contents liberated into the medium, (iii) there is a specific transport system which pumps out AG6P. The first idea is ruled out, because the cell-free medium obtained from the culture at any time showed no AG-converting activity in the control incubation. The second idea is not likely either, because the amount of AG6P in the medium after 2 h of incubation with AG was about two times that in the cells, which means that about two thirds of the cells must have been lysed. The turbidity of the culture did not change so much during the

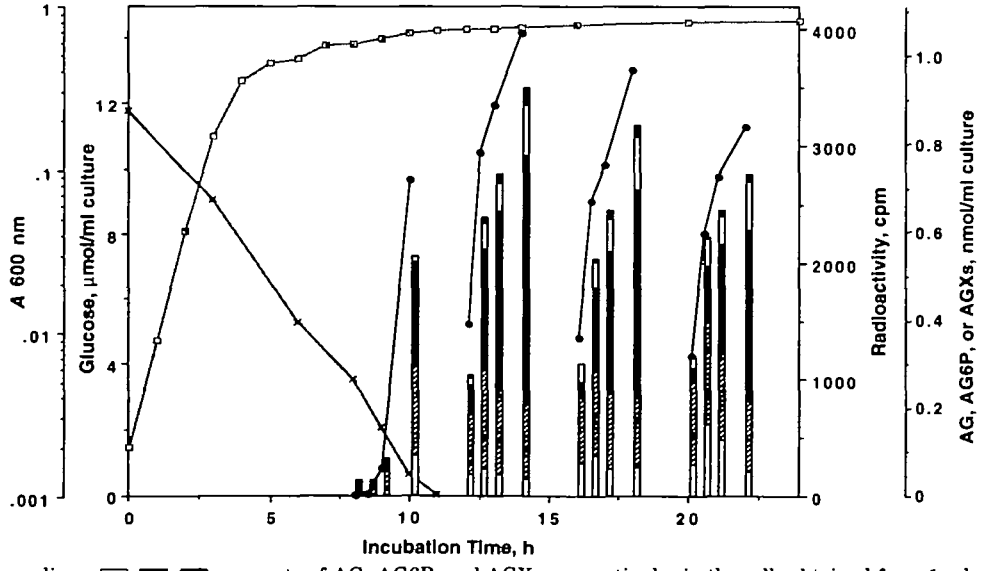

Fig. 3. **Time courses of accumulation of AG6P and AGXs in the medium and cells.** *E. coli* cells were cultivated with glucose, then aliquots of the culture were taken at the indicated time points, supplemented with [*U*-^{14}C]AG, and further incubated for the indicated times. The amounts of [*U*-^{14}C]AG, [*U*-^{14}C]AG6P, and [*U*-^{14}C]AGXs (on the AG content basis) in the medium and cells were determined by their radioactivity and the amount of [*U*-^{14}C]AG consumed in the medium was calculated. The values are means of triplicate measurements. Absorbance and glucose concentration of the culture were also measured. Symbols: ●, amount of AG consumed in 1 ml of the medium; ■, ■, amounts of AG6P and AGXs, respectively, in 1 ml of the medium; □, ▨, □, amounts of AG, AG6P, and AGXs, respectively, in the cells obtained from 1 ml of the culture; ×, glucose concentration in the medium; ⊟, absorbance of the culture at 600 nm.

incubation with AG, which indicated that disruption of so many cells had not occurred. The third idea seems likely, but we have not studied any properties of this putative transport system yet.

What Are the Roles of AG and/or AG6P?—There are noticeable characteristics in synthesis and metabolism of AG in *E. coli*. (i) When glucose is exhausted in the medium, *E. coli* synthesizes AG and immediately releases it to the medium (*16*). (ii) Then *E. coli* takes AG back from the medium and phosphorylates it to AG6P. (iii) *E. coli* again releases AG6P to the medium, and as a result the amounts of AG and AG6P in the cells become far smaller than those in the medium. The following is the most persistent question among many suggested to us by these seemingly unusual observations. Namely, if *E. coli* synthesizes AG and AG6P to meet the cell's demand, why does the cell immediately release AG and AG6P to the medium? Does AG and/or AG6P play any roles outside the cell? Although we have so far no evidence for such roles of AG and/or AG6P, the conditional AG and AG6P effluxes prompt us to the speculation that AG and/or AG6P may be a signal substance for cell-to-cell communication (if any such communications exist in the bacterial population), possibly for growth control.

The abilities of the cells to take up AG in terms of both rate and amount decreased with the time elapsed after reaching the stationary phase, concomitantly with the decrease in the amounts of AG6P both synthesized in the cells and released into the medium (Figs. 1 and 3), which were probably the results of the gradual reduction of general biochemical activities as cells entered the resting state in the stationary phase. On the other hand, the amounts of AG and AG6P remaining in the cells were rather constant throughout the stationary phase within the range of our examination. This observation suggests that AG and AG6P may play roles in maintaining the latent viability of the cells in the stationary phase. This will be our concern in future study.

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