JOURNAL OF BACTERIOLOGY, Nov. 1993, p. 7138–7141
0021-9193/93/227138-04$02.00/0

Vol. 175, No. 22

Conditional Synthesis and Utilization of 1,5-Anhydroglucitol in *Escherichia coli*

YOKO SHIGA, HIDEAKI MIZUNO, AND HIROSHI AKANUMA*

Department of Chemistry, College of Arts and Sciences, The University of Tokyo, Komaba, Meguro, Tokyo 153, Japan

Received 2 June 1993/Accepted 9 September 1993

A cyclic polyol, 1,5-anhydro-D-glucitol (AG), is widely detected in most organisms, although little is known about its metabolism and physiological roles. The present study demonstrates the synthesis of AG in *Escherichia coli* C600. The major portion of the synthesized AG was indicated to be derived from glucose retaining all the six carbon atoms, and only 5% was attributed to AG synthesized from C_3 compounds. AG synthesis is apparent in an early stage of the stationary phase, and accumulation is transient both in cells and in medium. Evidence is also presented for AG uptake and metabolism and for effects of cyclic AMP.

A polyol, 1,5-anhydro-D-glucitol (AG) (Fig. 1), is a 1-deoxy form of glucopyranose, which was first found in the polygala family (4) and is also present in human cerebrospinal fluid (10, 13) and blood (12). To date, it has been found in a wide range of animals and plants (17). The AG concentration in human plasma is usually maintained at a constant level independent of food intake (2, 14) and of changes in most other physiological conditions (14, 15). The concentration, however, specifically decreases in patients with diabetes mellitus (1, 7, 12, 14, 16, 18, 19); glucose at a high concentration in blood prevents AG from being reabsorbed in the renal tubules, and AG leaks into the urine (2). Recently, it was found that the AG concentration in human blood also decreased during the first 33 weeks of pregnancy and that it gradually returned to the normal level afterwards (5, 11). This observation implies an enhanced utilization of AG in fetuses, since we have already shown that AG undergoes very slow metabolic conversion in mature animals (6). Similarly, the largest AG stock in vegetables is observed in cereals and beans (17), thus raising the possibility that plant seeds store AG in preparation for germination. These observations indicate that AG might be indispensable, but virtually nothing is known about its roles in any organism.

The first stage of a metabolic study is often carried out most conveniently in well-established lines of bacteria. Only a limited number of reports have dealt with the metabolism of AG in microorganisms however (3, 8). We reported earlier that a strain of pseudomonas can grow on AG as a sole carbon source and that 1,5-anhydro-D-fructose is the intermediary metabolite in its catabolic degradation by the bacteria (8). We started a study of AG metabolism in *Escherichia coli* with [U-^{13}C]glucose as the major carbon source and observed a conditional synthesis and utilization of AG in this organism.

MATERIALS AND METHODS

Materials. AG and [U-^{13}C]AG were obtained from Nippon Kayaku Co., Ltd. (Tokyo, Japan). [6,6-^{2}H]AG was prepared from [6,6-^{2}H]glucose (MSD Isotopes, Montreal, Canada) in our laboratory according to an established method (9). Natural D-glucose and [U-^{13}C]glucose were the products of Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Isotec, Inc. (Miamisburg, Ohio), respectively, and they were further purified by high-pressure liquid chromatography on an Amide-80 column (4.6-mm internal diameter by 25 cm; Tosoh, Tokyo, Japan) with acetonitrile-water (72:28) as the eluent. The glucose B test (Wako) was used to determine the glucose concentration of the medium. Casamino Acids, Bacto tryptone, and Bacto yeast extract were purchased from Difco Laboratories (Detroit, Mich.). Cyclic AMP (cAMP) was obtained from Sigma Chemical Co. (St. Louis, Mo.); cAMP solutions were prepared fresh for each experiment. An anion exchange resin, AG1-X8, and a cation exchange resin, AG50W-X8, were the products of Bio-Rad Laboratories (Richmond, Calif.). All other chemicals were from Wako Pure Chemical Industries, Ltd., and were used without further purification. *E. coli* C600 was a kind gift from Isao Katsura of the National Institute of Genetics (Shizuoka, Japan).

Precultivation of *E. coli*. *E. coli* C600 was inoculated in 5 ml of Luria-Bertani medium (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl) and grown overnight at 37°C. The resulting cells were collected by a mild centrifugation (1,800 × *g* for 20 min), washed once with ice-cold saline, and then suspended in the original culture volume of saline.

Cultivation of *E. coli* with glucose. The suspended *E. coli* was added to the 100-fold volume of M9 medium (0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.024% $MgSO_4$, 0.001% $CaCl_2$ [pH 7.4]) supplemented with Casamino Acids (0.2%) and natural and/or [U-^{13}C]glucose (0.2% unless otherwise stated) with or without 5 mM cAMP. These culture mixtures were incubated at 37°C while shaking, and aliquots were taken at the indicated incubation times and poured into prechilled tubes kept on ice. A_{600} was first measured, and then a 0.1-ml portion was subjected to glucose determination and another 1-ml portion was subjected to AG analysis. The number of cells was calculated from the absorbance by using a calibration curve separately obtained by the colony counting method.

Measurement of AG uptake rate. *E. coli* was cultivated with natural glucose, and at various points in the course of proliferation, a 5-ml portion of the culture was placed in a separate tube and [U-^{13}C]AG (85 μg/ml) was added to a concentration of 170 ng/ml. After a further 1-h incubation at 37°C, the amounts of the isotopically labeled AG were measured both in the medium and in the cells.

AG analysis. One milliliter of the culture was centrifuged to separate the supernatant and the cells, and when needed, an appropriate amount of [6,6-^{2}H]AG (usually 10 ng) was added to each fraction as an internal standard. The supernatant was

* Corresponding author.

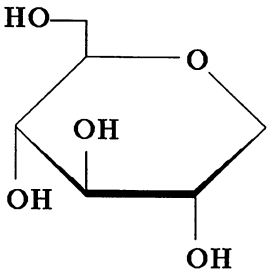

FIG. 1. AG structure.

first dried in a centrifugal evaporator (CC100; Tomy, Tokyo, Japan), and the residue was dissolved in a small amount of water and applied to a two-layer column (5.5 mm internal diameter) packed with, from the bottom to the top, 500 μl of the anion exchanger, in OH^- form, and 500 μl of the cation exchanger, in H^+ form. The charged column was washed with 5 ml of distilled water, and all the effluent was collected and then dried. The residue was further treated on a TSK gel SCX column (6.0-mm internal diameter by 15 cm; Tosoh) with acetonitrile-water (75:25) as the eluent. 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 and dissolved in 5 μl of *p*-xylene, and a 1-μl portion was injected into a capillary column (HiCap CBP1-M25; Shimadzu, Kyoto, Japan) 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. For quantitative analysis by selected ion chromatography, the ion fragments unique to [6,6-^{2}H]AG (*m/z* = 172 and 214) and to [U-^{13}C]AG (*m/z* = 176 and 218) were monitored. The amount of [U-^{13}C]AG in the sample was calculated by comparing its peak area (*m/z* = 176 or 218) with that of the corresponding fragment of [6,6-^{2}H]AG (*m/z* = 172 or 214).

For determination of AG in the cells, the cells were first sonicated in 1 ml of distilled water with an ultrasonic disrupter (UD-201; Tomy) at 80 W for 2 min with a 0.6-s interval every second. Then, the disrupted mixture was treated in the same way as the supernatant.

The isotopic composition of AGs synthesized in the culture with a mixture of natural and [U-^{13}C]glucose was determined from the relative peak areas of the fragments whose *m/z*s were 170, 171, 172, 173, 174, 175, and 176 obtained in the selected ion chromatography monitoring these fragments. These fragments are considered to retain all the six carbons in AGs because the major fragment (*m/z* = 170) produced from natural AG has been assigned to the fragment retaining all six carbon atoms in its structure (peracetylated AG minus two acetic acids and one ketene) (6). In this determination, the combined contribution of the natural occurrence of carbon 13

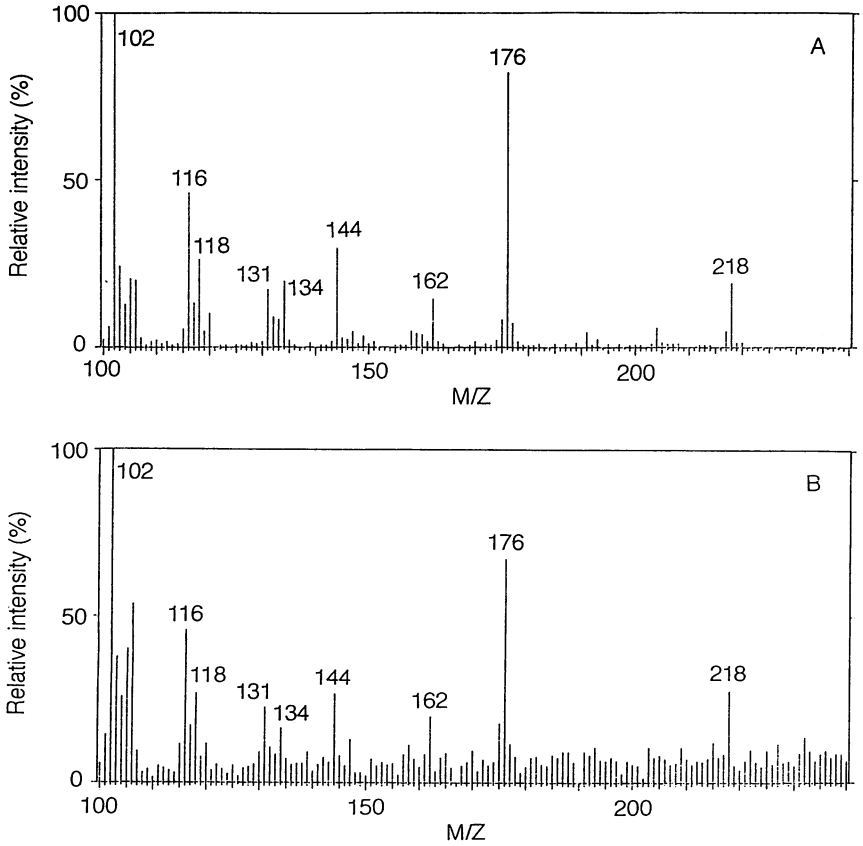

FIG. 2. Mass fragmentograms for the peak of peracetylated derivative of authentic [U-^{13}C]AG (A) and for the corresponding peak of the preparation made from the culture medium in which *E. coli* C600 had been grown on [U-^{13}C]glucose (B).

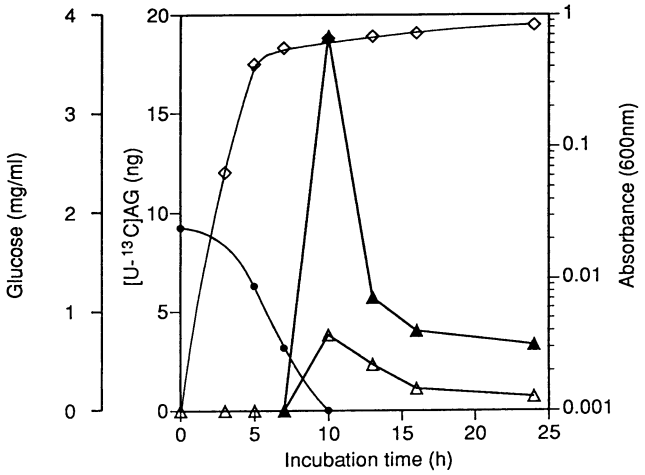

FIG. 3. Time course of AG synthesis by *E. coli* C600. *E. coli* cells were cultivated with [U-^{13}C]glucose, aliquots were taken at the indicated times, and absorbance, glucose concentration, and AG content were determined as described under Materials and Methods. Symbols: ▲, amount of AG in 1 ml of the medium; △, amount of AG in the cells obtained from 1 ml of the culture; ●, glucose concentration in the medium; ◇, A_{600}.

in natural glucose and the signal spillover from the fragment of *m/z* 170 to the observed signal intensity for that of *m/z* 171 were estimated from the relative signal intensities for these mass numbers obtained with authentic natural AG and subtracted from the observed relative intensity for the fragment of *m/z* 171; the resulting value was regarded as the relative amount of AG containing five carbons from natural substrates and one carbon from [U-^{13}C]glucose. The net signal intensity for the fragment of *m/z* 175 was similarly deduced by compensating the spillover from the fragment of *m/z* 175 into the signal for that of *m/z* 175 by using the relative signal intensities of *m/z* = 175 and 176 obtained with authentic [U-^{13}C]AG.

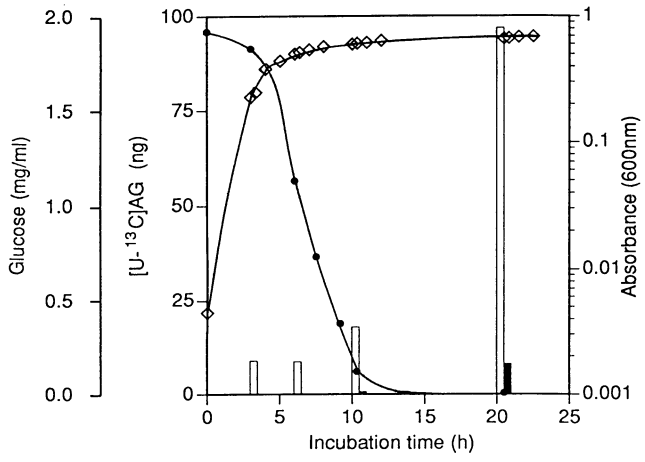

FIG. 4. AG uptake by *E. coli* in various growth phases. *E. coli* cells were cultivated in natural glucose, and at the indicated times portions were removed and incubated for 1 h with [U-^{13}C]AG (170 ng/ml). [U-^{13}C]AG was then measured in the medium and cells. Symbols: ●, glucose concentration in the medium; ◇, A_{600}; □, [U-^{13}C]AG disappearance from 1 ml of the medium in 1 h; ■, [U-^{13}C]AG in the cells obtained from 1 ml of the culture.

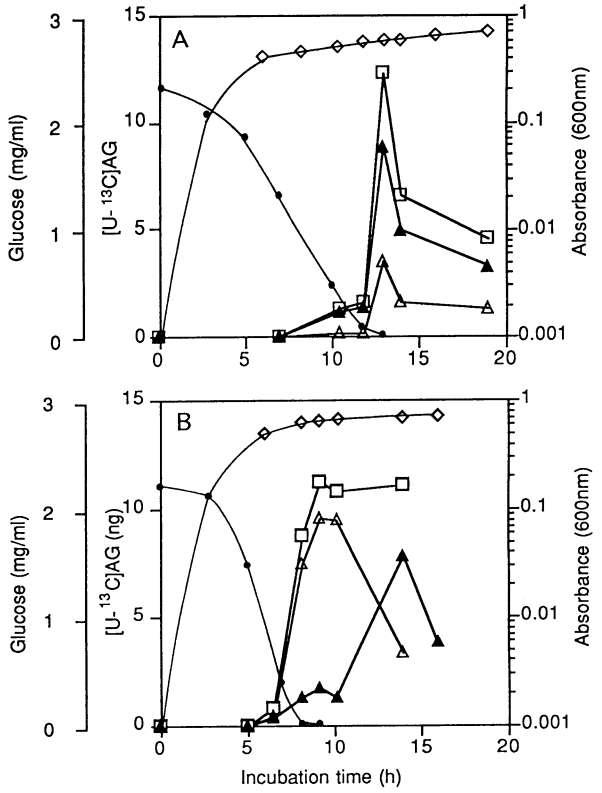

FIG. 5. Effect of cAMP on accumulation of AG. *E. coli* cells were cultivated in the medium in the absence (A) and presence (B) of 5 mM cAMP. Aliquots were taken from the culture at the indicated times and subjected to the subsequent determinations. ▲, amount of AG in 1 ml of the medium; △, amount of AG in the cells obtained from 1 ml of the culture; □, total amount of AG in 1 ml of the culture; ●, glucose concentration in the medium; ◇, A_{600}.

RESULTS AND DISCUSSION

AG synthesis by *E. coli* C600. The supernatant obtained from the [U-^{13}C]glucose culture showed in gas chromatography-mass spectrometry analysis a distinct gas chromatograph peak exactly coinciding with the single peak of authentic [U-^{13}C]AG. Figures 2A and B show their mass spectra, which bear a close resemblance to each other, thus demonstrating the presence of [U-^{13}C]AG in the supernatant. When *E. coli* was cultivated in the medium supplemented with 0.1% each of natural and [U-^{13}C]glucose instead of [U-^{13}C]glucose alone, the synthesized AG was found to be composed of natural AG (53.5%), [U-^{13}C]AG (43.9%), and a hybrid AG (2.6%) which has three atoms each of carbons from natural and [U-^{13}C]glucose in the molecule. Other molecular species of AG were virtually not detectable.

Figure 3 shows that [U-^{13}C]AG accumulated both in cells and in the medium in the [U-^{13}C]glucose culture and then declined. At the peak, there were 18.9 ng in the medium and 3.8 ng in the cell. The latter amount is calculated as a concentration factor of ca. 40-fold (considering, on the basis of wet weight of the cell pellet, that total cell volume was ca. 5 mm^3, about 1/200 of the volume of the medium). Analogous time courses—accumulation of AG after glucose exhaustion followed by decline—were observed for cultures with initial glucose concentrations of 1 to 5 mg/ml, with the amounts of

AG per single cell at the peak ranging from 3.5 to 9.6 ag per cell, respectively.

AG uptake. Since AG is stable in the medium, its decline after accumulation likely represents uptake and/or metabolism. As shown in Fig. 4, addition of [U-^{13}C]AG to stationary-phase cells resulted in substantial decline from the medium in 1 h (97 of 170 ng added) and 8 ng was recovered in the cells. The decline was less with earlier additions, and no AG was found in the cells.

Effect of cAMP. The inclusion of 5 mM cAMP altered the profile of AG appearance with a relatively larger accumulation in the cells and delayed appearance and decline in the medium (Fig. 5).

Comments. The use of [U-^{13}C]glucose allowed discrimination of AG fragments from contaminants of other natural compounds, hence aiding its positive identification. It also allowed, in the experiments employing [U-^{13}C] and natural glucoses together, demonstration of the fact that AG derives from the C_6 skeleton of glucose largely intact: 97.4% have *m/z*s of 170 and 176. (The remaining 2.6%, with an *m/z* of 173, would suggest a combination of two C_3 derivatives, perhaps related to gluconeogenesis.)

There is no knowledge of the pathways of synthesis or degradation. The material appears largely free as found in the cell; the possible existence of derivatives may be indicated by the observation of some increase in free AG in a homogenate incubated at room temperature (data not shown). In any case, this work demonstrates the existence of both biosynthetic and degradative pathways, as well as mechanisms for uptake and release. The actual amounts found in cells are extremely low (2×10^{-5} M at most), and the evidence for active metabolism (particularly in stationary phase) with some effects of cell density and data from the time courses suggest a possible role in metabolic signaling of nutritional conditions.

REFERENCES

1. **Akanuma, H., K. Ogawa, Y. Lee, and Y. Akanuma.** 1981. Reduced levels of plasma 1,5-anhydroglucitol in diabetic patients. J. Biochem. **90:**157–162.
2. **Akanuma, Y., M. Morita, N. Fukuzawa, T. Yamanouchi, and H. Akanuma.** 1988. Urinary excretion of 1,5-anhydro-D-glucitol accompanying glucose excretion in diabetic patients. Diabetologia **31:**831–835.
3. **Dozois, K. P., C. J. Carr, J. C. Krantz, Jr., F. Hachtel, and F. F. Beck.** 1936. Sugar alcohols. VI. The utilization of sugar alcohols and their anhydrides by various microorganisms. J. Bacteriol. **32:**499–503.
4. **Freudenberg, W., and E. F. Rogers.** 1937. The chemistry of naturally occurring monohydrohexitols. J. Am. Chem. Soc. **59:** 1602–1605.
5. **Hamada, T. M., K. Yoshimatsu, J. Ishimatsu, and T. Matsunaga.** 1990. Serum levels of 1,5-anhydro-D-glucitol during the normal and diabetic pregnancy and puerperium. Acta Obstet. Gynecol. Scand. **69:**479–485.
6. **Kametani, S., Y. Hashimoto, T. Yamanouchi, Y. Akanuma, and H. Akanuma.** 1987. Reduced renal reabsorption of 1,5-anhydro-D-glucitol in diabetic rats and mice. J. Biochem. **102:**1599–1607.
7. **Morita, M., and H. Akanuma.** 1992. Distribution of 1,5-anhydro-D-glucitol in normal, diabetic and perfused rat bodies. J. Biochem. **112:**385–388.
8. **Nakamura, T., A. Naito, Y. Takahashi, and H. Akanuma.** 1986. Oxidation of 1,5-anhydro-D-glucitol to 1,5-anhydro-D-fructose catalyzed by an enzyme from bacterial membranes. J. Biochem. **99:**607–613.
9. **Ness, R. K., H. G. Fletcher, and C. S. Hudson.** 1950. The reduction of acetylated glycopyranosyl bromides to 1,5-anhydroglucitols with lithium aluminum hydride: 1,5-anhydro-L-rhamnitol. J. Am. Chem. Soc. **72:**4547–4553.
10. **Pitkänen, E.** 1973. Occurrence of 1,5-anhydroglucitol in human cerebrospinal fluid. Clin. Chim. Acta **48:**159–166.
11. **Sanaka, M., Y. Omori, S. Minei, M. Shimizu, S. Saitou, and S. Yoshioka.** 1993. Serum 1,5-anhydroglucitol in pregnant diabetics. J. Jpn. Diabetes Soc. **36:**93–100.
12. **Servo, C., and E. Pitkänen.** 1975. Variation in polyol levels in cerebrospinal fluid and serum in diabetic patients. Diabetologia **11:**575–580.
13. **Smith, S. L., M. Novotny, and E. L. Weber.** 1978. Gas-chromatographic determination of polyol profiles in cerebrospinal fluid. Clin. Chem. **24:**545–548.
14. **Yamanouchi, T., H. Akanuma, T. Asano, C. Konishi, I. Akaoka, and Y. Akanuma.** 1987. Reduction and recovery of plasma 1,5-anhydro-D-glucitol level in diabetes mellitus. Diabetes **36:**709–715.
15. **Yamanouchi, T., H. Akanuma, T. Nakamura, I. Akaoka, and Y. Akanuma.** 1988. Reduction of plasma 1,5-anhydroglucitol (1-deoxyglucose) concentration in diabetic patients. Diabetologia **31:**41–45.
16. **Yamanouchi, T., H. Akanuma, F. Takaku, and Y. Akanuma.** 1986. Marked depletion of plasma 1,5-anhydroglucitol, a major polyol, in streptozocin-induced diabetes in rats and the effect of insulin treatment. Diabetes **35:**204–209.
17. **Yamanouchi, T., Y. Tachibana, H. Akanuma, S. Minoda, T. Shinohara, H. Moromizato, H. Miyashita, and I. Akaoka.** 1992. Origin and disposal of 1,5-anhydroglucitol, a major polyol in the human body. Am. J. Physiol. **263**(Endocrinol. Metab. 26):E268–E273.
18. **Yoshioka, S., S. Saitoh, T. Fujisawa, A. Fujimori, O. Takatani, and M. Funabashi.** 1982. Identification and metabolic implication of 1-deoxyglucose (1,5-anhydroglucitol) in human plasma. Clin. Chem. **28:**1283–1286.
19. **Yoshioka, S., S. Saitoh, C. Negishi, T. Fujisawa, A. Fujimori, O. Takatani, M. Imura, and M. Funabashi.** 1983. Variation of 1-deoxyglucose (1,5-anhydroglucitol) content in plasma from patients with insulin-dependent diabetes mellitus. Clin. Chem. **29:** 1396–1398.