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1,5-Anhydroglucitol Promotes Glycogenolysis in *Escherichia coli*

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Glycogen is a storage compound that provides both carbon and energy, but the mechanism for the regulation of its metabolism has not been fully clarified. Recently, we found a new glycogenolytic pathway in rat liver in which glycogen is first metabolized to 1,5-anhydrofructose (AnFru) and then to 1,5-anhydroglucitol (AnGlc-ol). Because the amounts of glycogen and AnFru are closely related in various rat organs and the second reaction, AnFru to AnGlc-ol, is strongly inhibited in the presence of glucose, we expected that this pathway might play a regulatory role in glycogen metabolism. Here we evaluate the expected involvement of AnGlc-ol and AnFru in the regulatory mechanism in *Escherichia coli* C600. Having established the presence of this new glycogenolytic pathway in *E. coli* C600, we further show that the conversion of AnFru to AnGlc-ol is activated only after the exhaustion of glucose in the medium, and that as little as 5 μM AnGlc-ol in the medium acutely accelerates the degradation of glycogen by 40%. We consider the role of AnGlc-ol in the mechanism that controls glycogen metabolism in *E. coli* to be as follows. When glucose is abundant, *E. coli* accumulate glycogen, a fraction of which is steadily degraded so that the amount of AnFru is about 1/1,000 of glycogen on a weight basis. When glucose is depleted and the demand for glycogen utilization is elevated, AnFru, which has accumulated mostly in the medium, is promptly taken up and converted to AnGlc-ol, which accelerates glycogen degradation. We also suggest the possibility that AnGlc-ol is one of the extracellular signaling molecules for bacteria.

Key words: 1,5-anhydro-D-fructose, 1,5-anhydro-D-glucitol, *E. coli*, glycogenolytic pathway, regulation of glycogen metabolism.

Glycogenolysis through phosphorolysis and hydrolysis are the two well known pathways of glycogen utilization. We recently discovered a new glycogenolytic pathway in rat liver (Fig. 1) that consists of two steps, glycogen to 1,5-anhydrofructose (AnFru), and AnFru to 1,5-anhydroglucitol (AnGlc-ol) (*1*). The two liver enzymes, α-1,4-glucan lyase and AnFru reductase, that catalyze the two steps have been identified (*1, 2*); the reductase has been isolated (*2*) while the lyase has been partially purified. Seaweed (*3*) and fungal (*4*) α-1,4-glucan lyases have already been fully purified. The fact that the final product of this pathway, AnGlc-ol, is found in wide variety of organisms including animals, plants, and bacteria (*5*) suggests a universal and significant role of this pathway in nutritional physiology, but nothing concerning its role has so far been reported. Our recent findings that the distributions of AnFru and glycogen in rat organs are closely related (*1*), and that the AnFru to AnGlc-ol reaction in mammalian cells is strongly inhibited in the presence of glucose (*6*) indicate that this pathway may play an important role in the control of glycogen metabolism.

The control of glycogen metabolism is not yet fully understood. For example, the established mechanisms do not appear to apply to the effect of insulin on glycogen synthesis, which may play a central role in alleviating the postprandial blood glucose elevation. We expect there are still missing regulatory modules in this system. The products of lyase-catalyzed glycogenolysis, AnGlc-ol and AnFru, appear to be possible candidate effectors in such putative modules. We have assumed bacterial glycogenolysis as the primordial system for mammalian glycogenolysis and employed as the first step of such assessment; the known mechanism for the control of glycogen metabolism seems much simpler in bacteria. Therefore, we examined the effects of AnGlc-ol and AnFru on glycogenolysis in *Escherichia coli* and found that AnGlc-ol exerts a considerable effect. Here we describe a possible mechanism for the control of bacterial glycogen metabolism.

MATERIALS AND METHODS

Materials—Natural D-glucose and [*U*-^{13}C]glucose were the products of Wako Pure Chemical Industries (Osaka) and Isotec Inc. (Miamisburg, Ohio), respectively. They 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. AnGlc-ol was obtained

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Abbreviations: AnFru, 1,5-anhydro-D-fructose; AnGlc-ol, 1,5-anhydro-D-glucitol; AnGlc-ol6P, 1,5-anhydro-D-glucitol 6-phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

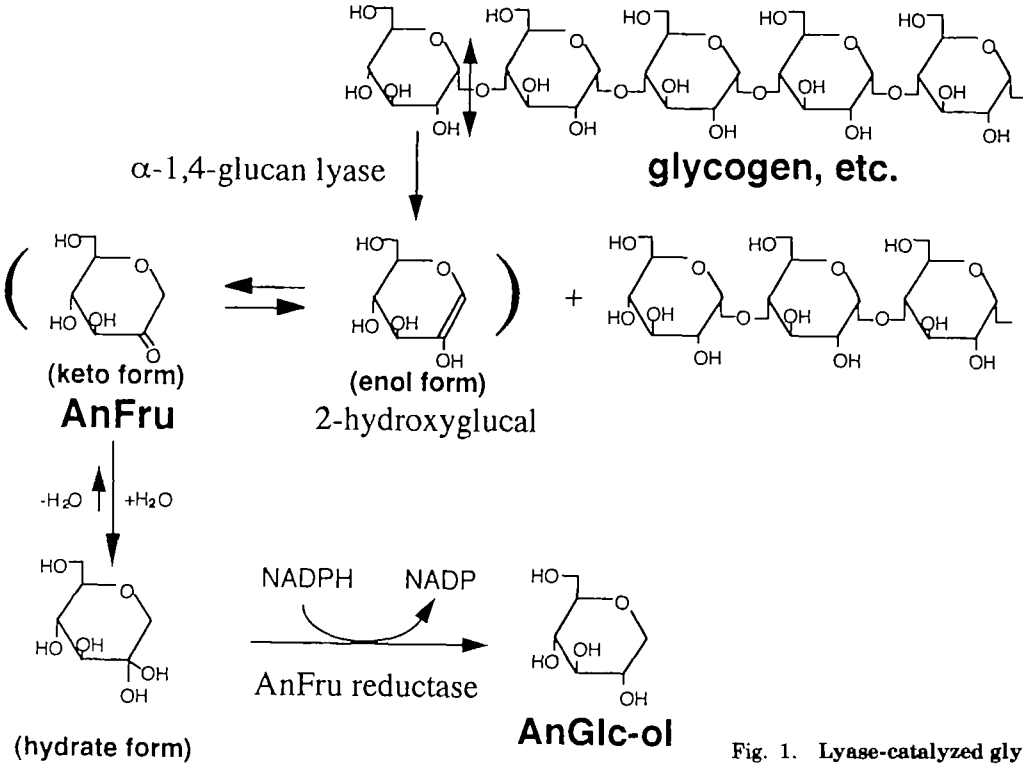

Fig. 1. **Lyase-catalyzed glycogenolytic pathway.**

from Nippon Kayaku (Tokyo) and [*U*-^{13}C]AnGlc-ol was prepared from [*U*-^{13}C]glucose according to an established method (*7*). AnFru, [*U*-^{13}C]AnFru, and [*U*-^{13}C]1,5-anhydroglucitol 6-phosphate (AnGlc-ol6P) were prepared according to the method described in our previous papers (*6, 8*). Casamino Acids, Bactotryptone, and Bacto yeast extract were purchased from Difco Laboratories (Detroit, MI), and amyloglucosidase from *Aspergillus niger* was the product of Boehringer Mannheim (Germany). Ethylhydroxylamine hydrochloride was obtained from Wako and its aqueous solution was treated on a Sep-Pak C_{18} cartridge (Waters) before use. Glucose concentration in the medium was determined with a kit (TIDEX, Sankyo, Tokyo). An anion exchange resin, AG1-X8, and a cation-exchange resin, AG50W-X8, were the products of Bio-Rad Laboratories (Richmond, California). All other chemicals were from Wako and used without further purification. *E. coli* C600 was a kind gift from Dr. Isao Katsura of the National Institute of Genetics (Shizuoka).

Cultivation of E. coli C600—Cultivation was carried out in M9 medium supplemented with Casamino Acids (0.2%) and glucose (0.2%) at 37°C with or without other additives indicated; occasionally the absorbance at 600 nm and the glucose concentration in the medium were measured. At the indicated times of incubation, appropriate amounts of the culture were removed for the measurements of glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P.

Determination of Glycogen—Glycogen was extracted from the cells according to a reported method (*9*) with slight modifications. A portion (0.1 ml) of the sampled culture was mixed with 0.4 ml of ethanol and the mixture was dried in a centrifugal evaporator (CC100; Tomy, Tokyo); 30 μl of 5.34 M KOH was added and the solution was heated at 100°C for 30 min. Then 70 μl of water, 100 μl of 0.14 M Na_2SO_4, and 800 μl of ethanol were added, and the mixture was left to stand for at least 12 h at 4°C. After centrifugation at 15,000 × *g* for 20 min, the precipitate was collected and dissolved in 500 μl of water, and a 50 μl portion was placed in a plastic tube. To the tube were added 100 μl of 0.2 M sodium acetate buffer (pH 4.8), 100 ng of [*U*-^{13}C]glucose in 50 μl of water as an internal standard, and 1.4 units of amyloglucosidase (10 μl), and the mixture was incubated at 37°C for 2 h to fully digest the glycogen (*10*). At the end of the incubation, 800 μl of ethanol was added, the mixture was centrifuged and the resulting supernatant was dried. The glucose produced by amyloglucosidase and [*U*-^{13}C]glucose were co-purified by HPLC on a TSK gel Amide-80 column. The eluate was monitored with a refractive index detector (RI-detector, RE-61; Showadenko, Tokyo). The fraction corresponding to glucose was dried and the resulting residue was subjected to acetylation in 75 μl of acetic anhydride/pyridine (1:2) at 80°C for 15 min. The acetylated sample was dried and the residue was dissolved in 250 μl of ethyl acetate. A 1-μl portion was injected onto a GC-MS (Hewlett Packard HP 6890 GC System and 5973 Mass Selective Detector) 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 glucose (m/z = 157 and 200), and to [*U*-^{13}C]glucose (m/z = 160 and 205) were monitored. The amount of glucose in the sample was calculated by comparing the peak area (m/z = 157 or 200) with that of the corresponding fragment of [*U*-^{13}C]glucose (m/z = 160 or 205). In separate measurements, the culture medium was confirmed to show a null background in glycogen measurement.

AnGlc-ol and AnGlc-ol6P Analyses—AnGlc-ol and AnGlc-ol6P in the cells and medium were measured accord-

ing to the methods described in our previous papers (*11, 12*). One milliliter of culture was centrifuged at 15,000×*g* for 2.5 min at 4°C to separate the medium and cells. Fixed amounts of authentic [*U*-^{13}C]AnGlc-ol and [*U*-^{13}C]AnGlc-ol6P (usually 20 ng each) were added to the supernatant as internal standards. The supernatant was then dried and the residue was dissolved in a small amount of water and applied to an anion exchange column (5.5 mm ID, 0.5 ml) in the OH^- form. The charged column was washed with 5 ml of distilled water and all the effluent was collected and saved for AnGlc-ol analysis. The trapped substances were then eluted from the column with 1 M ammonium acetate. The first 1 ml was collected and saved for AnGlc-ol6P analysis.

The water-eluted fraction was dried and the residue was dissolved in a small amount of water and applied to a cation exchange column (5.5 mm ID, 0.5 ml) in the H^+ form to remove cationic materials. The deionized fraction was collected by washing the column with 2 ml of distilled water, dried, and subjected further to HPLC on the TSK gel Amide-80 column using acetonitrile/water (80:20) as the eluent. Elution was monitored with the RI-detector. The fraction corresponding to AnGlc-ol was dried, acetylated, and injected onto the GC-MS. The ion fragments unique to AnGlc-ol (m/z=170) and [*U*-^{13}C]AnGlc-ol (m/z=176) were monitored, and the amount of AnGlc-ol in the sample was calculated by comparing its peak area with that of the corresponding fragment of [*U*-^{13}C]AnGlc-ol.

For AnGlc-ol6P assays, the 1 M ammonium acetate-eluted fraction was dried, and the residue was subjected to the alkaline phosphatase treatment. The liberated AnGlc-ol was then subjected to HPLC and GC-MS as described above.

To determine cellular levels of AnGlc-ol and AnGlc-ol6P, the cell pellet was washed once with cold saline, and authentic [*U*-^{13}C]AnGlc-ol and [*U*-^{13}C]AnGlc-ol6P were added as internal standards. The cells were disrupted and the enzymes denatured by suspending the cell pellet in a cold mixture of water, methanol, and chloroform (0.2, 0.5, and 0.25 ml, respectively) for 1 h on ice. 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 disc by brief centrifugation at 1,400×*g* [Folch's partitioning (*13*)]. The upper layer was collected and dried and the residue was treated as described above for the determination of AnGlc-ol and AnGlc-ol6P.

AnFru Analysis—AnFru levels in the cells and medium were measured according to the methods described in our previous papers (*1, 14*). One milliliter of culture was separated into medium and cells as described above, and 20 ng of [*U*-^{13}C]AnFru was added to each fraction. A 4-fold volume of ethanol was added to the medium and the mixture was centrifuged at 5,000×*g* for 5 min. The resulting supernatant was dried, and the residue was dissolved in 160 μl of water to which 40 μl of 10% *O*-ethylhydroxylamine/HCl and 20 μl of 0.5 M Tris/HCl (pH 8.5) containing 1 mM EDTA were added. The mixture was heated at 80°C for 30 min, applied to a reverse-phase column (DIACHROMA ODS, 4.6 mm×15 cm; Kakokishoji, Kawasaki), and developed with a linearly increasing gradient of acetonitrile (0–16% in 10 min) in water at a flow rate of 1.0 ml/min; elution was monitored by measuring the ultraviolet absorbance at 210 nm. The peak for AnFru ethyloxime was collected, dried, acetylated, and dried again. The dried sample was dissolved in 250 μl of ethyl acetate and a 1-μl portion was injected onto the GC-MS. The amount of AnFru in the sample was calculated by comparing the peak area of the fragment ion of AnFru oxime (m/z=169) with that of the corresponding fragment ion of [*U*-^{13}C]AnFru oxime (m/z=175).

For the determination of cellular AnFru, the cells were disrupted and the AnFru was extracted as above described for the determination of AnGlc-ol and AnGlc-ol6P. The AnFru fraction was then treated as is described for AnFru in the medium.

Measurement of α-1,4-Glucan Lyase Activity—Glucan lyase activity was determined by measuring the AnFru liberated from exogenous glycogen or maltose. Early stationary phase cells were harvested, sonicated at 4°C in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7.0) containing 1 mM EDTA, 1.6 mM dithiothreitol, 0.5 μg/ml of leupeptin, and 0.7 μg/ml of pepstatin A with an ultrasonic disrupter (UD 201; Tomy, Tokyo) at 80 W for 2 min with a 0.6-s interval per second. The lysate was centrifuged at 15,000×*g* for 5 min to separate the supernatant and precipitate, and the latter was resuspended in the original volume of the same buffer. The incubation mixture for the lyase reaction contained 0.5% glycogen or 14.6 mM maltose, and 2–6 mg protein from the cell lysate, supernatant, or precipitate suspended in 1.2 ml of MOPS buffer. The mixture was incubated at 37°C for 2 h before the lyase reaction was terminated by adding 5 ml of ethanol.

Measurement of Glycogen Phosphorylase and Glucosidase Activities—Glycogen phosphorylase and glucosidase activities were measured as follows. The cell lysate, the suspended 15,000×*g* precipitate, and the supernatant were prepared as described above. These preparations (0.1–0.5 mg protein) were then incubated individually at 37°C with 0.13% glycogen in 0.3 ml of 50 mM phosphate buffer containing 1 mM EDTA, pH 7.0, in the absence or presence of 5 μM AnGlc-ol. At the beginning of the incubation and at 30 min, 50 μl of each mixture was taken, and 400 μl of ethanol was added to terminate the reaction. Then 200 ng of [*U*-^{13}C]glucose was added to each mixture and the mixtures were dried. The residue thus obtained was dissolved in 200 μl of water and divided into two 100 μl portions: one for the measurement of glucose to determine glucosidase activity, and the other for the measurement of glucose 1-phosphate to determine phosphorylase activity. One 100 μl portion was added to 500 μl of ethanol, the resulting insoluble material was removed by centrifugation, and the supernatant was dried. Glucose produced by glucosidase and [*U*-^{13}C]glucose in the residue were determined as described above. The other 100 μl portion was treated with alkaline phosphatase, and the newly liberated glucose, together with [*U*-^{13}C]glucose and the glucose produced by glucosidase, was extracted and analyzed as described above. The phosphorylase activity was determined by subtracting the amount of glucose detected without alkaline phosphatase treatment from the total amount after phosphatase treatment.

RESULTS AND DISCUSSION

The Synthesis of Glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P by E. coli C600 —*E. coli* C600 was cultivated, and glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P were measured during the course of cell proliferation (Fig. 2). Cellular glycogen accumulated with a lag time of about 3 h after cell inoculation, and increased almost linearly until the glucose in the medium was exhausted. Although the amount of AnFru in the cells was very small (less than 10 nmol/cells harvested from 1 liter of culture; the plots are omitted from the figure) throughout the incubation time, the amount of AnFru in the medium increased linearly and reached a fairly high level (more than 100 nM), with the maximum reached just before the total exhaustion of glucose in the medium; the accumulation curve mimicked that of glycogen but the accumulated amounts were about 1/1,000 of glycogen on a weight basis. Both the levels of glycogen in the cells and AnFru in the medium began to decrease when glucose became scarce, but AnFru decreased far more rapidly than glycogen and virtually disappeared within 1 h after the start of the decline. AnGlc-ol in the medium and cells appeared rather abruptly as if it took the place of AnFru, and then it also decreased over a short time, with AnGlc-ol6P finally appearing in the medium; most of the synthesized AnGlc-ol6P seemed to be excreted into the medium and the amount in the cells harvested from 1 liter of culture was below 10 nmol at most (plots omitted). Although small fluctuations were observed in the amounts accumulated and the timing of the peak tops of these substances in several repeated experiments, the relative timings were reproducible. It is intriguing that both AnFru and AnGlc-ol, which are synthesized in the cells, accumulate in the medium, and are then taken up, and that AnGlc-ol6P is mostly excreted into the medium. These conspicuous phenomena will be discussed below.

The New Glycogenolytic Pathway in E. coli C600—The above-mentioned temporal changes in glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P strongly suggest that *E. coli* C600 also has the pathway for glycogen degradation to AnGlc-ol *via* AnFru, and therefore we examined this new glycogenolysis. Because *E. coli* cells do not take up macromolecules such as glycogen, it was necessary to use cell lysates to detect the activity of the first step, *i.e.*, glycogen to AnFru. The whole cell lysate showed no noticeable glucan lyase activity toward exogenous glycogen, but did show a weak but distinct lyase activity toward maltose; 90% of this activity was attributed to the 15,000× *g*-precipitate (12.3 pmol AnFru synthesized/mg protein·h). While the supernatant showed AnFru producing activity only in the presence of exogenous maltose, the precipitate showed about half of the AnFru-producing activity even in the absence of exogenous substrates. These observations indicate that both the enzyme and its intracellular substrate co-precipitate with the sonicated cell debris; the enzyme may be associated with the cell membrane and the substrate may be associated with the enzyme.

The second step in the pathway was easily demonstrated by an *in vivo* experiment (Fig. 3). *E. coli* was cultivated with 18.5 μM AnFru in M9 medium, and AnFru and AnGlc-ol in the medium were measured at the indicated incubation times. Although the rate of decrease of AnFru in the medium was rather slow (about 0.4 μmol/liter·h) up to 8 h, which is the time when glucose was exhausted in the medium, it increased abruptly to more than 15.4 μmol/liter·h until AnFru was totally depleted within 1 h; about

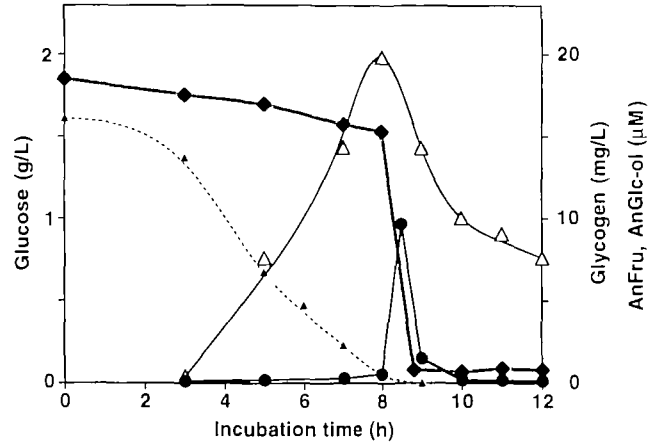

Fig. 3. **Uptake and metabolism of AnFru.** *E. coli* were cultivated with 18.5 μM AnFru in M9 medium, and then glucose, AnFru, and AnGlc-ol in the medium and glycogen in the cells were measured after the indicated incubation times. Symbols: ▲, ◆, ●, amounts of glucose, AnFru, and AnGlc-ol, respectively, in 1 liter of medium; △, amount of glycogen in the cells obtained from 1 liter of culture.

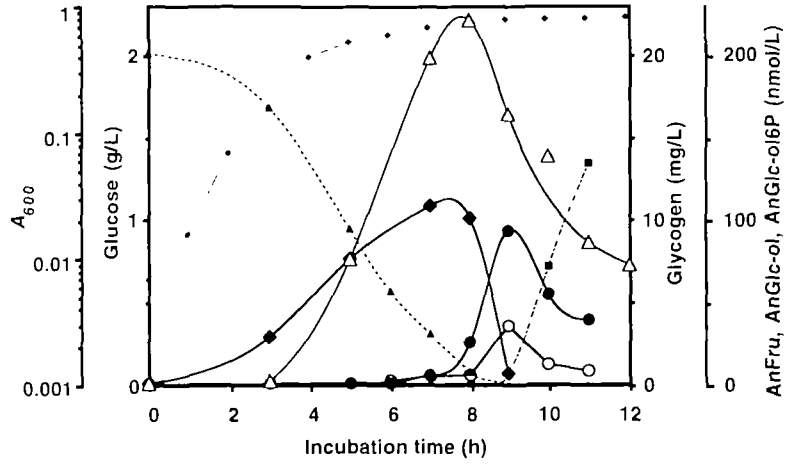

Fig. 2. **Time courses for the levels of glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P in *E. coli* C600 cultures.** *E. coli* cells were cultivated at 37°C in M9 medium. Aliquots were removed at the indicated times, and absorbance and the amounts of glucose, glycogen, AnFru, AnGlc-ol, and AnGlc-ol6P were measured as described in "MATERIALS AND METHODS." Symbols: ▲, ◆, ●, ■, amounts of glucose, AnFru, AnGlc-ol, and AnGlc-ol6P, respectively, in 1 liter of medium; ○, △, amounts of AnGlc-ol and glycogen, respectively, in the cells obtained from 1 liter of culture; ◇, absorbance at 600 nm.

half of the decreased amount was detected in the form of AnGlc-ol, which also vanished within 1 h. In separate experiments, the maximum speed of AnFru decrease in the absence of glucose was found to be about 54 μmol/liter·h (2×10^{12} cells/liter), which is more than one hundred times the speed in the presence of glucose. We have already demonstrated that erythroleukemia cells, K-562, take up AnFru (*6*), and although we did not demonstrate that *E. coli* cells also take it up, we consider that they do, since AnFru levels decreased in the medium and were changed to AnGlc-ol in this experiment.

Since we could not demonstrate lyase activity toward exogenous glycogen in the lysate, it is possible that the weak activity found with exogenous maltose was a lyase activity specific for low molecular weight glucans. This remains to be clarified. Still the above results and the results in rat liver (*1*) lead us to conclude that *E. coli* C600 degrades glycogen either directly or indirectly (*via* maltose or some other small α-1,4-glucan) to AnFru and then to AnGlc-ol, which is further phosphorylated to AnGlc-ol6P. This third step has already been demonstrated in our previous papers in *E. coli* C600 (*12*) and mammalian cells (*8*).

Effect of AnGlc-ol, AnFru, and AnGlc-ol6P on Glycogen Utilization in E. coli—In Figs. 2 and 3, it is notable that at the very same point when the rapid uptake of AnFru and the synthesis of AnGlc-ol begin, the curve of glycogen accumulation turns to a sharp decline. This suggests that these substances play some role in controlling glycogen metabolism. Accordingly, we examined the effects of AnFru, AnGlc-ol, and AnGlc-ol6P on glycogen utilization in *E. coli*. Cells were harvested during the early stationary phase when maximal amounts of glycogen are expected to have been accumulated, transferred to M9 medium without glucose but with AnFru, AnGlc-ol, or AnGlc-ol6P at various concentrations, and the effects of these compounds on glycogen utilization were examined. As Fig. 4A shows, AnGlc-ol accelerated glycogen utilization; during the first hour, a 30% acceleration was observed at an AnGlc-ol concentration as low as 5 μM, and 60% acceleration occurred at concentrations of 50 μM and over. At 30 min the effect was clearer: 40% acceleration at 5 μM, and 74% at 50 μM (Fig. 4A insert; the data are from a separate experiment). The accelerating effect lasted, although somewhat diminished, throughout incubation. AnFru also accelerated glycogen utilization (Fig. 4B), but at every concentration the effect was weaker and lasted a shorter time than in the case of AnGlc-ol. This tendency was clearer at lower initial concentrations; although AnFru showed 50% acceleration in the first 1 h at concentrations of 50 μM and over, the effect diminished markedly after 1 h at 50 μM or 2 h at 100 μM, and a slight effect was observed only at the beginning with the initial concentration of 5 μM. On the other hand, AnGlc-ol6P in the medium had no effect even at 100 μM (data not shown). Although we have not confirmed whether AnGlc-ol6P can enter cells across the cell membrane, we have already demonstrated in our previous paper that AnGlc-ol6P produced in *E. coli* cells is readily excreted into the medium (*12*). Accordingly, these observations suggest that the effect of AnGlc-ol on glycogenolysis is canceled by the conversion of AnGlc-ol to AnGlc-ol6P and/or its excretion.

AnGlc-ol as a Positive Effector in the Glycogen Utilization Mechanism in E. coli—As Fig. 4 shows, both AnFru and AnGlc-ol accelerate glycogen utilization in *E. coli* C600. However, this does not necessarily mean that they are both positive effectors of glycogenolysis. Even if AnGlc-ol is an effector while AnFru is not, AnFru could show an indirect effect because, as shown in Fig. 3, AnFru is readily converted to AnGlc-ol. The same might be applicable in the reverse case. Therefore we examined whether AnGlc-ol is converted to AnFru at a reasonable rate in bacterial cells and found it is not; when [*U*-^{13}C]AnGlc-ol was added to the medium in the absence of glucose at a concentration of 50 μM, which is enough to accelerate glycogen utilization, its level declined to virtually zero in 2 h, but no [*U*-^{13}C]AnFru was detected in either the cells or medium during that time (data not shown). This result confirms that AnGlc-ol rather

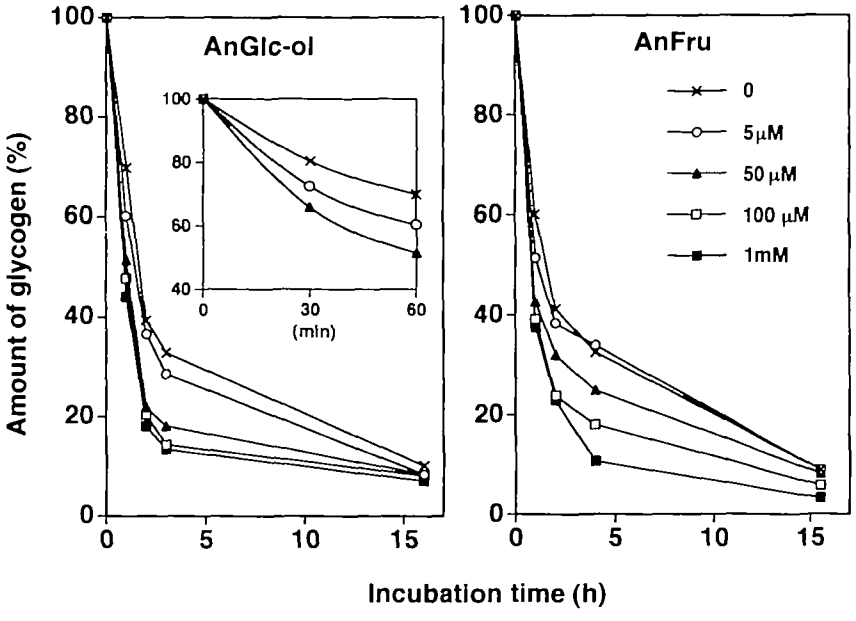

Fig. 4. **Effects of AnGlc-ol and AnFru on glycogen utilization.** *E. coli* were harvested in early stationary phase when maximal amounts of glycogen are expected to have been accumulated, and transferred to M9 medium without glucose but with the indicated concentrations of AnGlc-ol (A) or AnFru (B). Then glycogen in the cells was measured at the indicated incubation times. The concentrations of AnGlc-ol or AnFru were as follows: ×, 0 μM; ○, 5 μM; ▲, 50 μM; ⁻, 100 μM; ■, 1 mM. Up to 1 h, a separate experiment was carried out at 5 μM and 50 μM AnGlc-ol [Insert of (A)].

than AnFru is the direct effector in glycogenolysis. This conclusion is further supported by the following two findings. (i) The effect of AnGlc-ol is more pronounced compared with AnFru. (ii) As shown in Fig. 2, AnFru is present in the culture medium from 3 to 8 h when glycogen accumulates, but AnGlc-ol appears suddenly at 8 h, coincides with the beginning of the rapid utilization of glycogen.

If AnGlc-ol is the more direct effector of glycogenolysis, the observations shown in Fig. 4 can be reasonably explained as follows. The accelerating effect of AnFru is actually caused by AnGlc-ol, which is produced from AnFru in the absence of glucose with a production rate probably proportional to AnFru concentration. The faster decline in the glycogenolytic rate occurs due to the faster decrease in AnGlc-ol concentration which is resulted from the balance of ever-diminishing supply from AnFru and the conversion to AnGlc-ol6P, *etc.*

Glycogen phosphorylase and glucosidase are known to be the enzymes responsible for glycogenolysis, and it is likely that AnGlc-ol has an accelerating effect on them. Therefore, we examined the effect of AnGlc-ol on glycogenolysis *in vitro*, but 5 μM AnGlc-ol failed to increase the amount of either glucose 1-phosphate or glucose liberated from glycogen in 30 min (data not shown). This indicates that AnGlc-ol does not directly activate glycogen phosphorylase or glucosidase. The effect of AnGlc-ol on glycogenolysis must be indirect and the possible signal path from AnGlc-ol to glycogen metabolism may not be fully retained in the cell-free system.

A Possible Regulatory Mechanism of Glycogen Metabolism—Although we have not identified the receptor or target enzyme of AnGlc-ol, we can reasonably propose a regulatory mechanism for glycogen metabolism (Fig. 5). When glucose is abundant in the medium, bacteria synthesize and accumulate glycogen, and α-1,4-glucan lyase, which is seemingly constitutive, metabolizes its small portion to AnFru, most of which accumulates in the medium; the amount of accumulated AnFru is roughly 1/1,000 of intracellular glycogen on a weight basis. As long as enough glucose is present in the medium, the cells neither actively take up AnFru nor reduce it to AnGlc-ol. Thus, AnGlc-ol levels remain very low in the cells preventing glycogen from being wasted. When glucose in the medium becomes scarce and the cells need to utilize more glycogen as an energy source, the inhibition by glucose of AnFru reduction and/or uptake ceases, and the level of AnGlc-ol in the cells increases. This accelerates glycogenolysis, or may even trigger the glycogenolysis. Finally, the vigorous utilization of glycogen is stopped by the conversion of AnGlc-ol to AnGlc-ol6P, most of which is excreted so it has no effect on glycogenolysis.

There have been several agents reported to accelerate glycogenolysis in *E. coli, e.g.*, AMP (2 mM) (*15*), NaF (0.2 M) (*16*), and Na_2SO_4 (0.8 M) (*16*), but they are effective at concentrations much higher than are physiologically plausible. The idea that AnGlc-ol is a promoter of glycogenolysis is attractive in that (i) AnGlc-ol can exert its effect at very low concentration, (ii) it is a metabolite derived from glycogen, and (iii) the availability of ambient glucose is reflected in the level of AnGlc-ol present in the cell and medium.

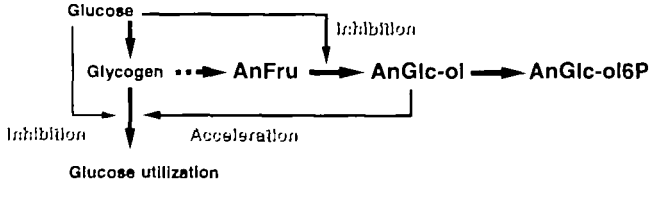

Fig. 5. **A possible mechanism controlling glycogen metabolism.**

Since AnGlc-ol is present in a wide variety of living organisms, it should be examined whether this mechanism also exists in other species. However, our preliminary observations of the effect of AnGlc-ol on Hep G2 cells were not clear; AnGlc-ol has no effect at concentrations below 1 mM. One probable reason for this noneffectiveness is that the mechanism for the activation of glycogenolysis in mammalian cells is different from that in bacteria. Typically, glycogen phosphorylase [EC 2.4.1.1], which catalyzes glycogen phosphorolysis and may play a central role in the regulation of glycogen metabolism, shows considerable differences in its regulatory properties. The mammalian enzymes are activated by covalent phosphorylation at a Ser residue near the N-terminal, and by the binding of intracellular ligands such as AMP and glycogen, while they are inhibited by glucose and glucose 6-phosphate. Although the bacterial enzymes are also inhibited by down-stream metabolites such as glucose and glucose 6-phosphate, they are active without phosphorylation or AMP binding (*17*). These regulatory properties have a structural basis as indicated by comparative sequence analyses of mammalian and non-mammalian phosphorylases including the enzyme from *Escherichia coli* (*18, 19*); in the bacterial phosphorylases, the glucose and glucose 6-phosphate binding residues are highly homologous to those in the mammalian enzymes, but the phosphorylation and AMP binding site residues are not. Another possible reason is that the control mechanism of glycogenolysis in Hep G2 cells may already be fully activated because Hep G2 cells are cancer cells.

An Extratercellular Signaling Molecule?—AnGlc-ol may play another role in unicellular organisms. It is usually assumed that most prokaryotes can grow and divide in the absence of sibling cells. However, recent studies have suggested that, for growth, prokaryotes need to communicate with each other, generally by means of self-produced signals (*20-23*). *Vibrio* sp. strain S14 produces extracellular signaling metabolites during carbon and energy starvation and these molecules play an important role in the expression of proteins critical to the development of starvation- and stress-resistant phenotypes (*22*). But such a signaling molecule(s) has not yet been identified. AnGlc-ol appears to be a candidate signaling molecule, because it seems to have every characteristic expected: (i) AnGlc-ol is synthesized specifically at the time of glucose exhaustion, (ii) AnGlc-ol is excreted into the medium and then taken back up again, and (iii) exogenous AnGlc-ol can accelerate glycogenolysis in *E. coli* C600.

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