STUDIES ON BIOSYNTHESIS OF 3-AMINO-3-DEOXY-D-GLUCOSE

SUMIO UMEZAWA, SEIJI SHIBAHARA, SHOJI OMOTO
Department of Applied Chemistry, Faculty of Engineering, University of Keio

TOMIO TAKEUCHI and HAMAO UMEZAWA
Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo

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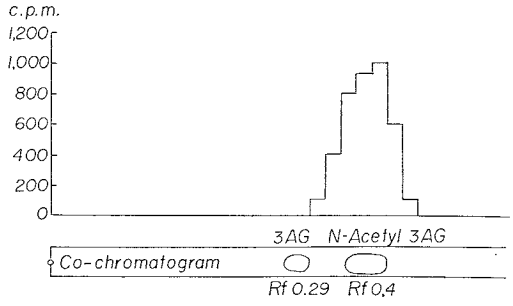
The biosynthesis of 3-amino-3-deoxy-D-glucose by *Bacillus aminoglucosidicus* was studied. Incorporation of C-1-, C-2-, C-6- and C-U-labeled glucoses into the aminosugar was proved. The aminosugar was synthesized in a cell-free system by a route which involved UDP-D-glucose. It was synthesized from enzyme solution, UDP-D-glucose, DPN and glutamine or ammonia.

3-Amino-3-deoxy-D-glucose was first shown to occur naturally as a constituent of kanamycin, and recently this aminosugar itself was found in a culture filtrate of *Bacillus aminoglucosidicus*[1]. In the present paper the biosynthesis of this aminosugar is reported.

As reported in a previous paper[1], more than 5 g of 3-amino-3-deoxy-D-glucose is produced in a liter of the cultured broth of *B. aminoglucosidicus* after 3-day shaking culture at 34°C. It is adsorbed on an Amberlite IRC-50 resin column in the ammonium salt form, and after washing with distilled water it is eluted with 1.0% aqueous ammonia. The lightly brownish colored powder obtained by the evaporation of the eluate under vacuum consists almost exclusively of 3-amino-3-deoxy-D-glucose. When D-glucose-U-^{14}C (5 μC) was added at 16 hours to the shaking culture and the fermentation was stopped 20 hours thereafter, with a specific activity of 1.2×10^4 cpm/mg, 96 mg of this aminosugar was obtained from 100 ml of the cultured broth. The aminosugar obtained by the addition of the same amount (5 μC) of sodium pyruvate-U-^{14}C, glycerol-1-^{14}C and sodium acetate-1-^{14}C showed less than 1/10 the radioactivity derived from D-glucose-U-^{14}C. To confirm the purity of 3-amino-3-deoxy-D-glucose produced by the addition of glucose-U-^{14}C, the aminosugar was N-acetylated and crystallized. As shown in Fig. 1, 3-acetamido-3-deoxy-D-glucose thus obtained showed an Rf of 0.4 by paper chromatography using *n*-butanol - pyridine - water - acetic acid (6 : 4 : 3 : 1) (Rf of 3-amino-3-deoxy-D-glucose was 0.29), and its specific activity was 1.0×10^4 cpm/mg. Thus, it was proved

Fig. 1. Paper chromatography of 3-acetamido-3-deoxy-D-glucose from D-glucoses-U-^{14}C.
Solvent system: *n*-BuOH - pyridine - water-AcOH (6 : 4 : 3 : 1)



that glucose was incorporated to a much greater extent into the aminosugar than glycerol, pyruvate and acetate.

D-Glucose-1-^{14}C, D-glucose-2-^{14}C and D-glucose-6-^{14}C were incorporated into 3-amino-3-deoxy-D-glucose similarly as D-glucose-U-^{14}C. They were added at 16 hours of the shaking culture and the aminosugar was isolated from the broth at 17, 19, 23, 25 and 40 hours. The incorporation of the radioactivity into the aminosugar is shown in Fig. 2. The distribution of the radioactivity in 3-amino-3-deoxy-D-glucose derived from D-glucose-6-^{14}C or D-glucose-1-^{14}C was determined by measuring the radioactivity of formaldehyde obtained by periodate oxidation of the N-acetylphenylosazone derivative of the aminosugar, formaldehyde being crystallized as methylenebismethone or by measuring the radioactivity of formic acid produced by periodate oxidation of the N-acetyl derivatives, formic acid being crystallized as S-benzylthiouronium salt. Fifty-eight percent of the radioactivity of the aminosugar was present in C-6 of the aminosugar biosynthesized from D-glucose-6-^{14}C and 73% of the radioactivity of the aminosugar obtained by addition of glucose-1-^{14}C was present in C-1. Thus, the whole carbon skelton of D-glucose is incorporated into 3-amino-3-deoxy-D-glucose.

Fig. 2. Incorporation rate of specifically labeled D-glucoses.

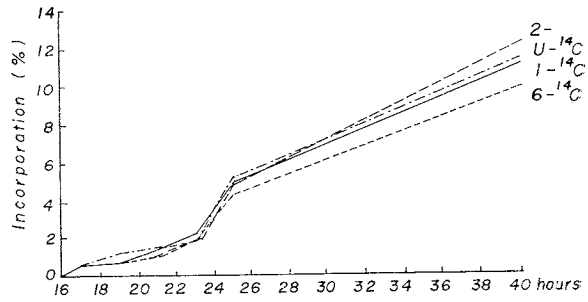


Table 1. Cofactor requirement for the synthesis of 3-amino-3-deoxy-D-glucose

Addition	Omission	Total activity of 3-amino-3-deoxy-D-glucose produced, c.p.m.	Percent incorporation
	ATP	959	0.04
	UTP	1,068	0.05
	Glutamine	479	0.02
	$MgSO_4$	483	0.02
	ATP, UTP	215	0.009
GTP	UTP	954	0.04
CTP	UTP	476	0.02
Complete system		2,800	0.13

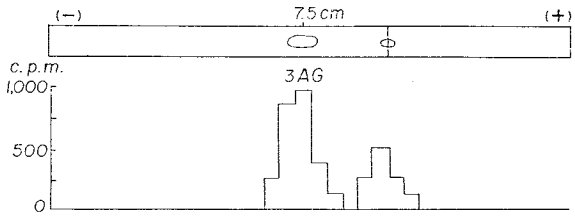
* The complete system consisted of the enzyme solution (6.9 mg as protein) 1.0 ml, D-glucose-U-^{14}C 1.0 μC, ATP 1.0 μmole, UTP 1.0 μmole, glutamine 5.0 μmoles, $MgSO_4$ 20 μmoles, DPN 5.0 μmoles and phosphate buffer ($\frac{1}{4}$ M, pH 6.8) 0.5 ml.

Fig. 3. Enzymatic synthesis of 3-amino-3-deoxy-D-glucose (3AG) from UDP-D-glucose-U-^{14}C.

Medium : UDP-^{14}C-D-glucose 15 mg (21.9 μmoles, 1.5×10^6 c.p.m.)

Enzyme solution	1 ml
Glutamine	5 mg (35.7 μmoles)
DPN	30 mg (50 μmoles)
Phosphate buffer	(0.5 M, pH 6.8) 0.5 ml
water	1 ml

High voltage electrophoresis.



A cell-free system of *B. aminoglucosidicus* where the aminosugar was synthesized by a series of enzyme reactions was prepared by disruption of the cells through a French pressure cell, centrifugation at $5,000\times g$ and $15,000\times g$ successively, and dialysis

of the supernatant. In a reaction mixture containing the enzyme solution, glucose-U-^{14}C, ATP, UTP, glutamine, $MgSO_4$, DPN and phosphate buffer of pH 6.0 at 28°C for 30 minutes, radioactive 3-amino-3-deoxy-D-glucose was produced. The aminosugar thus produced was isolated by the cation-exchange resin process followed by high voltage paper electrophoresis. It was further purified by preparing the N-acetyl derivative which was crystallized with non-radioactive material. The radioactivity of the crystals was not changed by repeated recrystallization.

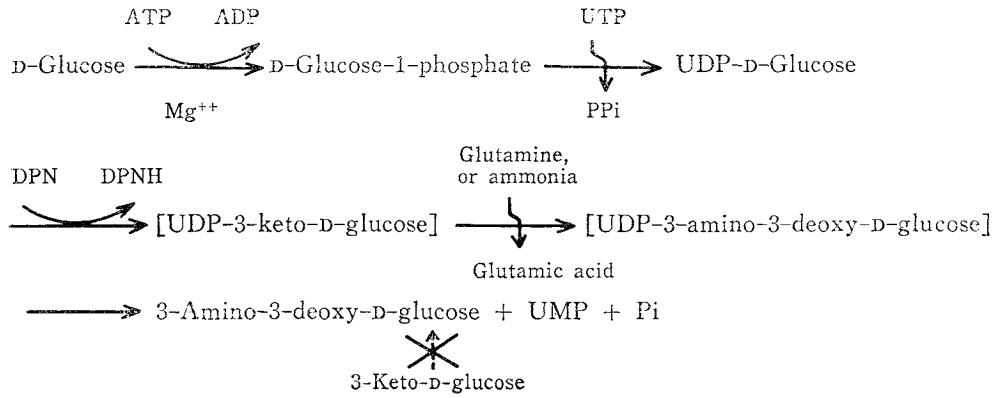
The effects of omission and addition of cofactors on the biosynthesis of the aminosugar were studied with the results shown in Table 1. For maximum synthesis, ATP and UTP are required. Replacement of UTP by GTP or CTP reduces the biosynthesis of the aminosugar. Mg^{++} ion and glutamine are also required. The synthesis of the aminosugar was not inhibited by azaserine or DON, and in another experiment it was shown that glutamine could not be replaced by glutamic acid, but glutamine could be replaced by ammonia. The requirement for UTP suggested that UDP-glucose may be an intermediate in the production of 3-amino-3-deoxy-D-glucose. Therefore, the enzymatic synthesis of the aminosugar from UDP-D-glucose in the cell-free system was studied. The labeled UDP-glucose was synthesized by the method of MOFFATT and KHORANA[2]. As shown in Fig. 3, a mixture of UDP-D-glucose, the enzyme solution, glutamine, and DPN in phosphate buffer incubated at 30°C for 30 minutes yielded 3-amino-3-deoxy-D-glucose, identified by high voltage electrophoresis, paper chromatography and thin-layer chromatography.

In this experiment, when DPN was omitted, no formation of 3-amino-3-deoxy-D-glucose was observed, indicating that DPN was required in this biosynthesis. A cell-free system containing all the enzymes necessary for synthesis of 3-amino-3-deoxy-D-glucose can also be prepared by treatment of cells with a sonic oscillator (10 Kc for 20 minutes). The suspension of the disrupted cells was centrifuged at $8,000 \times g$ for 30 minutes at −2.0°C, and dialyzed against distilled water. In the reaction mixture containing the enzyme solution 1.0 ml (5.1 mg as protein), D-glucose-U-^{14}C 1.0 μC, ATP 1.0 μmole, UTP 1 μmole, DPN 5 μmoles, glutamine 5 μmoles, $MgSO_4$ 20 μmoles and phosphate buffer ($\frac{1}{4}$M, pH 6.4) 1.0 ml, 3-amino-3-deoxy-D-glucose was synthesized incorporating 0.3 % of the radioactivity of the added D-glucose-U-^{14}C. With the same reaction mixture with glucose-U-^{14}C replaced by 2.0 μC of D-glucose-1-phosphate-U-^{14}C and no ATP, 0.31 % of the added D-glucose-1-phosphate-U-^{14}C was incorporated. When NaF was added at 1 mM, the incorporation was decreased to 0.21 %.

In the experiment shown in Table 1, 3-amino-3-deoxy-D-glucose was produced from glucose in the presence of ATP, even in the absence of UTP. ADP-D-glucose-U-^{14}C was synthesized by the method reported by KHORANA *et al.*[2] The synthesis of 3-amino-3-deoxy-D-glucose was examined in a reaction mixture containing ADP-D-glucose-U-^{14}C 2.3×10^6 cpm (2.2 μmoles), DPN 5.0 μmoles and glutamine 3.6 μmoles, the enzyme solution (5.1 mg as protein) prepared by the sonic disruption 1.0 ml and phosphate buffer ($\frac{1}{4}$M, pH 6.4) 1.0 ml at 30°C for 1 hour. No aminosugar was synthesized, although a control in which ADP-D-glucose-U-^{14}C was replaced by UDP-D-

glucose-U-^{14}C (2.5×10^6 cpm, 2.5 μmoles) yielded 3-amino-3-deoxy-D-glucose (total activity 5,500 cpm). It is certain that ADP-D-glucose is not used for synthesis of 3-amino-3-deoxy-D-glucose. The synthesis of 3-amino-3-deoxy-D-glucose in the experiment shown in Table 1 without addition of UTP is considered to be due to the contamination in the enzyme solution by uridine, UMP, UDP or UTP which might be converted to UTP in the presence of ATP.

The data described above indicate that 3-amino-3-deoxy-D-glucose is biosynthesized from glucose through UDP-D-glucose. The requirement for DPN obviously suggests that the UDP-D-glucose is oxidized to UDP-3-keto-D-glucose, although it has not been isolated. The next reaction is thought to be the amination of UDP-3-keto-D-glucose in the presence of glutamine or ammonia. Other pathways to be considered would involve 3-keto-D-glucose as an intermediate, which may be finally converted into 3-amino-3-deoxy-D-glucose. However, replacement of UDP-D-glucose with 3-keto-D-glucose in the cell-free system resulted in no distinct formation of the aminosugar. Moreover, an experiment in which D-glucose-U-^{14}C was diluted with 3-keto-D-glucose in the cell-free system showed no distinct supression of incorporation of ^{14}C. Thus, the pathway which involves 3-keto-D-glucose as an intermediate is unlikely, and the following pathway which involves UDP-D-glucose, UDP-3-keto-D-glucose and UDP-3-amino-3-deoxy-D-glucose is suggested.



Experimental

Materials:

D-Glucose-1-^{14}C (14.3 mC/mM), D-glucose-2-^{14}C (3.2 mC/mM), D-glucose-6-^{14}C (4.2 mC/mM), sodium acetate-1-^{14}C (10.0 mC/mM), sodium pyruvate-U-^{14}C (6.5 mC/mM) and D-glucose-U-^{14}C (5.0 mC/mM) were purchased from Daiichi Pure Chemicals Co., Ltd. Glycerol-1-^{14}C (4.7 mC/mM) was obtained from The Radiochemical Center, and D-glucose-1-phosphate-U-^{14}C (179 mC/mM) from New England Nuclear Corp.

Measurement of the radioactivity:

The radioactivity was counted by a liquid scintillation counter CPM-200 (LS-II) of Beckman Instruments, Inc. The scintillation medium for radioactivity determination consisted of 2,5-diphenyloxazole 6 g, and naphthalene 100 g in 1 liter dioxane. After the radioactive compounds were subjected to paper chromatography or high voltage electrophoresis, the chromatograms were scanned with a paper chromatogram scanner (PCS-5 with Ratemeter TRM-1 and Recorder ER-1-10-12) of Japan Radiation and Medical Electronics, Inc.

Incorporation of radioactive glucose, glycerol, pyruvate and acetate into 3-amino-3-deoxy-D-glucose produced by the shaking culture of *B. aminoglucosidicus*:

B. aminoglucosidicus was shake-cultured in 100 ml of a medium consisting of 1.5 % soybean meal, 1.0 % glucose and 0.3 % NaCl at 28°C. Five μC of D-glucose-U-^{14}C, glycerol-1-^{14}C, sodium pyruvate-U-^{14}C or sodium acetate-1-^{14}C was added at 16 hours of the shaking culture, and the shaking culture was continued for 20 hours thereafter. The cultured broth was filtered and the filtrate was passed through a column (20 ml, diameter of the column 13 mm) of Amberlite IRC-50 resin in the NH_4^+ salt form. The column was washed with 100 ml of distilled water and eluted with 20 ml of 1.0 % aqueous ammonia. The yields (weight of the aminosugar) and the specific activities of the aminosugar were as follows: 96 mg, 1.2×10^4 cpm/mg in the case of addition of D-glucose-U-^{14}C; 143 mg, 1.2×10^3 cpm/mg with glycerol-1-^{14}C; 225 mg, 5.3×10^2 cpm/mg with sodium acetate-1-^{14}C; 120 mg, 9.2×10^2 cpm/mg by addition of sodium pyruvate-U-^{14}C. Ninty mg of the aminosugar obtained by addition of D-glucose-U-^{14}C was N-acetylated in the following reaction mixture overnight at room temperature: methanol 5.0 ml, water 0.5 ml and acetic anhydride 1.0 ml.

The N-acetylated product was recrystallized from ethanol. Sixty-seven mg of 3-acetamido-3-deoxy-D-glucose with a specific activity of 1.0×10^4 cpm/mg was obtained. The N-acetyl aminosugar was subjected to paper chromatography followed by the radio scanning. The result is shown in Fig. 1. The N-acetyl derivative showed no melting point depression on admixture with an authentic sample of 3-acetamido-3-deoxy-D-glucose.

Incorporation of D-glucose-1-^{14}C, D-glucose-2-^{14}C and D-glucose-6-^{14}C into 3-amino-3-deoxy-D-glucose produced by the shaking culture of *B. aminoglucosidicus*:

Five μC of the labeled compound was added to 100 ml of the cultured broth at 16 hours of the shaking culture. The procedure and the medium composition were the same as in the previous experiments. After addition, the shaking culture was continued for 1, 3, 7, 9 and 24 hours thereafter. In each case, the aminosugar was isolated by the Amberlite IRC-50 resin process. The result is shown in Fig. 1. The aminosugar obtained from 100 ml of the 40-hour cultured broth was N-acetylated. The yield (weight) and the specific activity were as follows: 85 mg, 1.3×10^4 cpm/mg by addition of D-glucose-1-^{14}C; 174 mg, 0.9×10^4 cpm/mg by addition of D-glucose-2-^{14}C; 73 mg, 1.4×10^4 cpm/mg by addition of D-glucose-6-^{14}C. They were N-acetylated in the reaction mixture consisting of methanol 6.0 ml, water 0.8 ml and acetic anhydride 1.2 ml, and the yield and the specific radioactivity of the N-acetyl derivative were as follows: 40 mg, 7.3×10^3 cpm/mg by addition of D-glucose-1-^{14}C; 49 mg, 11.1×10^3 cpm/mg from D-glucose-2-^{14}C; 50 mg, 6.1×10^3 cpm/mg from D-glucose-6-^{14}C. The radioactivity from D-glucose-1-^{14}C or D-glucose-6-^{14}C was confirmed to exist mainly in C-1 or C-6 of the aminosugar respectively. Determination of distribution of the radioactivity in 3-amino-3-deoxy-D-glucose produced from D-glucose-6-^{14}C or -1-^{14}C: 3-Acetamido-3-deoxy-D-glucose-6-^{14}C obtained as described above (73 mg; total counts: 4.4×10^5 cpm) was diluted with 130 mg of non-radioactive 3-acetamido-3-deoxy-D-glucose and dissolved in 2.5 ml of 15 % aqueous acetic acid. To this solution, 0.4 ml of phenylhydrazine was added and the mixture heated at 100°C for 30 minutes to give 150.5 mg of N-acetyl-6-^{14}C phenylosazone. N-Acetyl-6-^{14}C phenylosazone (150.5 mg) in 50 % aqueous ethanol (30 ml) was oxidized overnight with sodium metaperiodate (2.3 equiv.). Then, the solution was extracted with 20 ml of chloroform and the water layer was neutralized with aqueous $Ba(OH)_2$ followed by separation of precipitate. The clear solution was adjusted to pH 4.5 with 1 N HCl, treated with dimedone (93 mg) and allowed to stand for three days at 0°C to give 50 mg of methylenebismethone. Specific activity: 960 cpm/mg. Theoretical yield: 266 mg.

3-Acetamido-3-deoxy-D-glucose-1-^{14}C (total counts; 2.84×10^5 cpm, 39 mg) obtained from D-glucose-1-^{14}C was diluted with 150 mg of non-radioactive 3-acetamido-3-deoxy-D-glucose and dissolved in 1.0 ml of water. To this solution, 182 mg (one equivalent) of

sodium metaperiodate was added and allowed to stand overnight at 0°C. Then, 2 ml of aqueous barium acetate solution (5 %) was added, and the resulting precipitate was separated by filtration. The filtrate was adjusted to pH 9.0 with 1 N NH_4OH and placed at room temperature for 30 minutes. The resulting solution was then distilled *in vacuo* at room temperature, and the distillate was cooled in dry ice-acetone. The distillate was then adjusted to pH 8.0 followed by evaporation into a small volume and readjusted to slightly acidic state followed by addition of 157 mg of S-benzylthiouronium chloride. Upon standing at 0°C overnight, S-benzylthiouronium salt of formic acid was obtained; 54 mg, specific activity: 1.15×10^3 cpm/mg. Theoretical yield was 179 mg.

The synthesis of 3-amino-3-deoxy-D-glucose in the cell-free system of *B. aminoglucosidicus* prepared by disruption using French pressure cell:

B. aminoglucosidicus was shake-cultured in a medium consisting of 1.5% soybean meal, 1.0 % glucose and 0.3 % NaCl. The cells of 100 ml of the cultured broth (3 g as wet weight) were collected by centrifugation at 3,000 rpm for 20 minutes, washed with cold physiological saline, suspended in phosphate buffer ($\frac{1}{2}$ M, pH 6.8, 4 ml) and disrupted in a French pressure cell (5,800 lb/in^2). The resulting suspension was centrifuged at $5,000 \times g$ for 30 minutes and thereafter the supernatant was dialyzed against 3,000 ml of distilled water for 16 hours at 5°C. The protein content of the enzyme solution measured by the method of LOWRY *et al.*[3] was 6.9 mg/ml. The reaction mixture contained the enzyme solution 1.0 ml, D-glucose-U-^{14}C 1.0 μC, ATP 1.0 μmole, UTP 1.0 μmole, glutamine 5.0 μmoles, $MgSO_4$ 20 μmoles, DPN 5.0 μmoles, and phosphate buffer ($\frac{1}{4}$ M, pH 6.8) 0.5 ml. The reaction was carried out at 28°C for 30 minutes and the reaction mixture was then heated at 100°C for 3 minutes. The reaction solution was passed through a column (4.0 ml, diameter 9.0 mm) of IRC-50 resin in ammonium form and after 100 ml of distilled water was passed, the aminosugar adsorbed was eluted with 20 ml of 1.0 % aqueous ammonia. The eluate was evaporated *in vacuo*. The resulting powder was subjected to the high voltage paper electrophoresis at 3,500 volts for 10 minutes, using formic acid - acetic acid - water (25 : 75 : 900, pH 1.8). The paperstrip was scanned with a radio paper chromatogram scanner and the total count of the aminosugar produced in the reaction mixture was calculated to be 2,800 cpm. The necessity of ATP, UTP, glutamine, and $MgSO_4$ for the synthesis of the aminosugar was confirmed by studying the total radioactivity of the aminosugar produced in cases when these substances were omitted. The result is shown in Table 1.

The synthesis of 3-amino-3-deoxy-D-glucose from UDP-D-glucose-U-^{14}C in the cell-free system:

The reaction mixture consisted of the enzyme solution (6.9 mg as protein) 1.0 ml, UDP-D-glucose-U-^{14}C 15 mg (21.9 μmoles, 1.5×10^6 cpm), glutamine 5 mg (35.7 μmoles), DPN 30 mg (30 μmoles), phosphate buffer (1/2 M, pH 6.8) 0.5 ml and distilled water 1.0 ml. The reaction was carried out at 30°C for 30 minutes. The reaction mixture was subjected to Amberlite IRC-50 resin chromatography and the aminosugar obtained by the elution with 1.0 % aqueous ammonia was subjected to the high voltage paper electrophoresis as described above. As shown in Fig. 2, the production of 3-amino-3-deoxy-D-glucose was confirmed. In another experiment, glutamine was replaced by ammonia and the synthesis of the aminosugar was confirmed.

Synthesis of 3-amino-3-deoxy-D-glucose in the cell free system of *B. amionoglucosidicus* prepared by sonic oscilator:

B. aminoglucosidicus was grown in a medium consisting of 1.5 % soybean meal, 1.0 % glucose and 0.3 % NaCl at 28°C for 18 hours. The cells collected from 100 ml of the cultured broth were washed with cold physiological saline by repetition of the centrifugation. Two grams of the wet cells was suspended in 5.0 ml of 1/20 M phosphate buffer of pH 6.4 and disrupted by sonic oscilator under 10 Kc for 20 minutes. It was centrifuged at $8,000 \times g$ for 30 minutes at −2°C. The supernatant was used as the crude enzyme solution. Then

in the reaction mixture consisting of the supernatant 1.0 ml, D-glucose-U-^{14}C 1.0 μC, ATP 1 μmole, UTP 1 μmole, DPN 5 μmoles, glutamine 5 μmoles, $MgSO_4$ 20 μmoles and phosphate buffer ($\frac{1}{4}$ M, pH 6.4) 1.0 ml at 30°C for 2 hours, 3-amino-3-deoxy-D-glucose (total count 21,600 cpm) was produced. The reaction mixture was heated at 100°C for 3 minutes, the aminosugar was isolated by the Amberlite IRC-50 resin process, and the eluate was subjected to the high voltage paper electrophoresis.

The supernatant (4.0 ml) of the disrupted cells which was obtained by the procedure described above was dialyzed against distilled water (3 liters) overnight at 5°C. Then, the total volume of the dialyzed enzyme solution was 4.8 ml and the protein content was 5.1 mg/ml. In the reaction mixture consisting of the same composition as above described except employing the dialyzed enzyme solution at 30°C for 1 hour, synthesis of 3-amino-3-deoxy-D-glucose was confirmed. The total radioactivity of the aminosugar synthesized was 6,700 cpm (that is, 0.3 % of the radioactivity of labeled glucose was incorporated). The reaction was carried out at 30°C for one hour with ADP-D-glucose-U-^{14}C 2.3×10^6 cpm (2.2 μmoles) or UDP-D-glucose-U-^{14}C 2.5×10^6 cpm (2.5 μmoles), DPN 5.0 μmoles, glutamine 3.6 μmoles, phosphate buffer (1/4 M, pH 6.4) 1.0 ml and distilled water 0.5 ml. No synthesis of the aminosugar from ADP-D-glucose-U-^{14}C was observed. When UDP-D-glucose was added, then 3-amino-3-deoxy-D-glucose was produced, and the total radioactivity was 5,500 cpm. Synthesis of 3-amino-3-deoxy-D-glucose in the reaction mixture containing D-glucose-1-phosphate, UTP, DPN, $MgSO_4$ and glutamine was also studied. The reaction mixture consisted of the dialyzed enzyme solution 1.0 ml, D-glucose-1-phosphate-U-^{14}C 2.0 μC, UTP 1.0 μmole, DPN 5.0 μmoles, glutamine 5.0 μmoles, $MgSO_4$ 20.0 μmoles and phosphate buffer (1/4 M, pH 6.4) 1.0 ml. The reaction was carried out at 30°C for 2 hours. The total count of 3-amino-3-deoxy-D-glucose which was obtained by the purification using the resin process followed by the high voltage electrophoresis was 13,800 cpm, that is, 0.31 % of the radioactivity of D-glucose-1-phosphate-U-^{14}C was incorporated. When sodium fluoride was added to the reaction mixture at 1 mM, the rate of the incorporation was 0.21 % and the inhibition of the synthesis of the aminosugar was observed.

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