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Biosynthesis of Streptothricin Antibiotics. II.[1] Catabolite Inhibition of Glucose on Racemomycin-A Production[2]

Yosuke Sawada, Hiroshi Sakamoto, Tetsuzo Kubo, and Hyozo Taniyama

Faculty of Pharmaceutical Sciences, Nagasaki University[3]

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The production of the antibiotic, racemomycin-A, was found to be inhibited in the presence of glucose in the medium, although glucose was the best carbon source for *Streptomyces lavendulae* ISP 5069. The repression by 1% glucose was gradually restorde by the addition of Pharmamedia or amino acid mixture. A severe repression by glucose was also observed at the rapid phase of antibiotic production. On the repression, racemomycin-A produced was incorporated into the mycelium. Xylose and mannitol were weakly repressed on racemomycin-A formation, and maltose caused a mild stimulation of the formation. The production of streptothricin antibiotics in all organisms tested was inhibited by glucose.

In the previous paper,[1] we reported that the biogenetic origin and the presumed pathway leading to the synthesis of racemomycin-A, one of the streptothricin antibiotics, by *Streptomyces lavendulae* ISP 5069 and that the gulosamine and β-lysine moieties of this antibiotic were generally derived from glucose and lysine, respectively. However, in the fermentation to obtain the antibiotic in a high yield, antibiotic production was found to be under severe catabolite repression by glucose. Therefore, we selected a fermentation medium containing minimum amount of glucose which gives a carbon source for racemomycin-A formation and an energy source for mycelial growth.

Catabolite repression of antibiotic production by glucose has been reported in a number of instances.[4-8] In all cases, glucose has been found to repress the formation of enzymes involved in their biosynthesis. We have examined the effect of glucose on racemomycin production in or er to elucidate the reason for the repression by glucose.

Materials and Methods

Chemicals——Various carbohydrates were purchased from the Nakarai Chemical Co., Kyoto, and Pharmamedia was kindly provided by the Traders Oil Mill Co., Texas. All other chemicals employed were purchased from commercial sources.

Cultivation of the Organism——To prepare a seed culture, spores of *Streptomyces lavendulae* ISP 5069 were transferred aseptically to a 500-ml flask containing 100 ml of basal medium (1% polypeptone, 0.5% yeast extract, and 0.3% NaCl in H_2O, pH 7.2) and this flask was incubated with resiprocal shaking for 3 days at 27°. Two ml sample of the seed culture was inoculated to a flask containing 100 ml of the same medium and then cultured under the same conditions.

Determination of Mycelial Dry Weight——Cultures were filtered on a paper filter (Toyo Roshi No. 2), washed 4 times with 50 ml of H_2O, and the filter papers with mycelia were dried for 3 days over P_2O_5 in a dessicator at room temperature *in vacuo*. The paper was reweighted and the weight of the mycelium was determined.

1) Part I: Y. Sawada, T. Kubo, and H. Taniyama, *Chem. Pharm. Bull.* (Tokyo), **24**, 2163 (1976).
2) A part of this work was read at the 94th Annual Meeting of the Pharmaceutical Society of Japan, Sendai, April 1974.
3) Location: *1-14, Bunkyo-machi, Nagasaki, 852, Japan.*
4) A. Kimura, *Agr. Biol. Chem.* (Tokyo), **31**, 837, 845 (1967).
5) R. Marshall, B. Redfield, E. Katz, and E. Weissbach, *Arch. Biochem. Biophys.*, **123**, 317 (1968).
6) A.L. Demain and E. Inamine, *Bacteriol. Rev.*, **34**, 1 (1970).
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8) L.H. Hurley and D. Bialek, *J. Antibiotics*, **27**, 49 (1974).

Determination of Racemomycin-A——Racemomycin-A concentration in the media was determined by a disk assay method employing a plate seeded with *E. coli* NIHJ (10^7 cells/ml). The plate was cultured for 18 hr at 37° after diffusing the antibiotic for 2 hr at 5°. Antibacterial activity was also examined by a shaking culture method: a mixture of brain-heart infusion (Difco) broth (4 ml), cultured broth (0.5 ml) of the *Streptomyces*, and diluted culture (10^7 cells/ml, 0.5 ml) of *E. coli* was incubated with gentle shaking at 37°. After 3.5 hr of cultivation, the optical density (OD) of the culture was measured at 540 nm.

Sterilization——Carbohydrates, amino acid mixture, and Pharmamedia were sterilized by the method of Gottlieb, *et al.*[9] Media were sterilized in an autoclave.

Incorporation of ^{14}C-labeled Racemomycin-A into Mycelium——After 30 hr of incubation, ^{14}C-racemomycin-A (1 mg, 1×10^5 dpm/μM)[1] was added into the broth (100 ml). Glucose at concentrations of 0.5, 1, 3, and 5% was also added into the broth. An experiment without glucose was simultaneously performed as a control. After 30 min of further cultivation, a part of the mycelium in the broth was collected on a filter paper by suction and rapidly washed 3 times with 10 ml each of cold H_2O containing 5% glucose or 0.9% NaCl. Then the mycelium was dried as described above, and ground into a powder in an agate mortar. The powder (10 mg) was suspended in 3 ml of 1 M-scintilamine-OH, MeOH solution and allowed to stand for 72 hr at 50° to be dissolved. The clear solution was neutralized with AcOH and then the radioactivity in 1 ml of the solution was measured with a scintillation counter (Aloka LSC-601) by use of H_2O-soluble scintillator (10 ml) consisting of 7 g PPO (2,5-diphenyloxazole), 0.3 g POPOP {1,4-bis[2-(4-methyl-5-phenyloxazoyl)]benzene}, and 100 g of naphthalene in 1 liter of dioxan.

Results

The influence of varing concentrations of glucose as sole carbohydrate source on racemomycin-A formation by *Streptomyces lavendulae* ISP 5069 is shown in Fig. 1. When the concentrations of glucose were more than 0.2%, racemomycin-A formation was found to be reduced markedly.[10] Especially, more than 2% concentrations of glucose suppressed the formation of racemomycin-A almost completely.

The addition of glucose resulted in an increase in the weight of mycelium with the increase of glucose concentration as shown in Fig. 2. This fact suggests that there is no direct relationship between the growth of the organism and the antibiotic formation.

Fig. 3 shows the effect of glucose on the production of racemomycin-A at the rapid production phase. A significant decrease in antibacterial activity in the broth was observed when

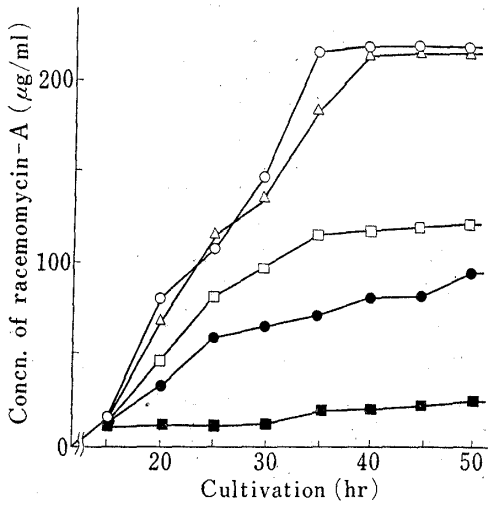

Fig. 1. Inhibition Effect of Glucose at Various Concentrations

Glucose additions were made at zero time.
glucose concentration; ○: none, △: 0.1%, □: 0.2%, ●: 1%, ■: 2,3,5%

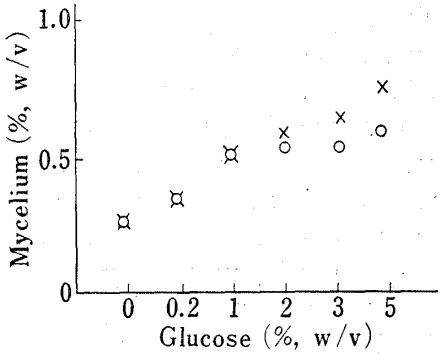

Fig. 2. Mycelial Weight under Various Concentrations of Glucose

The mycelium was harvested after 50 hr of cultivation.
○: Glucose was added at zero time.
×: Glucose was added at 30 hr of cultivation.

9) D. Gottlieb and E. Shirling, *Intern. J. Systematic Bacteriol.*, **16**, 313 (1966).
10) The inhibitory effect of glucose and its catabolites on the formation of some enzymes has been referred to as "Catabolite repression," by B. Magasanik, *Cold Spring Harbor Symp. Quant. Biol.*, **26**, 249 (1961).

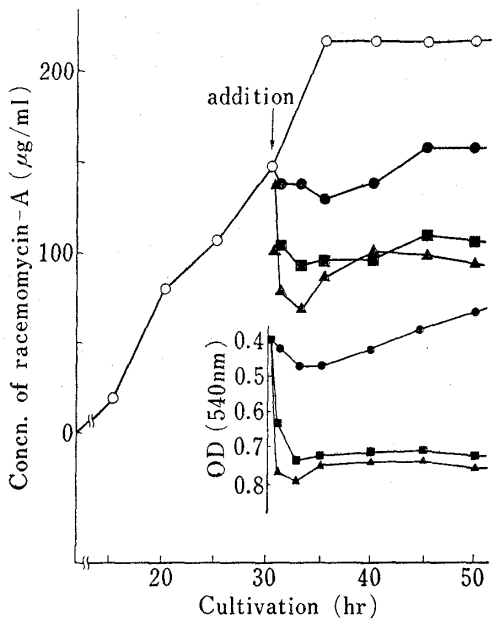

Fig. 3. Effect of Glucose Concentrations at Rapid Production Phase of Racemomycin-A

glucose concentration;
○: none, ●: 1%, ■: 3%, ▲: 5%

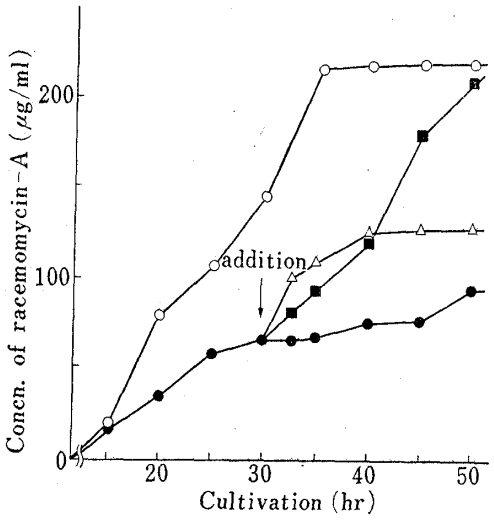

Fig. 4. Effects of Pharmamedia, and Amino Acid Mixture against Catabolite Repression of Racemomycin-A Formation

○: control, ●: 1% glucose (0 hr), ■: 1% glucose plus 1% Pharmamedia (30 hr), △: 1% glucose plus 1% amino acid mixture (30 hr)

glucose at concentrations of more than 1% was added. Antimicrobial activity of the broth was also measured by a shaking culture method. As shown in the Figure, the addition of glucose resulted in a decrease of racemomycin-A, that is, the increase of optical density in the case of glucose addition shows the growth of *Escherichia coli*. Although the recovery of the antibiotic formation on this repression was more moderate for further cultivation, the cell growth was observed during the subsequent 20 hour incubation as shown in Fig. 2. This fact also suggests that racemomycin-A formation is independent of mycelial growth.

By these results, glucose was found to repress racemomycin-A synthesis at any period of the culture and the repression was not resumed completely by further cultivation.

Fig. 4 shows the restorative effect of the catabolite repression by the addition of Pharmamedia, and amino acid mixture. The repression of the antibiotic formation by 1% glucose was gradually restored by the addition of 1% Pharmamedia. The addition of 1% amino acid mixture also resulted in the recovery of antibiotic formation to some extent. This reversible effect of glucose on the repression of the antibiotic formation signifies that glucose did not destroy the antibiotic-synthesizing machinery itself but suppressed the function of this machinery.

The effects of Pharmamedia, and amino acid mixture on the repression which was caused by the addition of glucose at the rapid production phase were also examined. The rapid repression by the addition of 1% glucose was gradually restored by the simultaneous addition of 1% Pharmamedia as shown in Fig. 5. Addition of amino acid mixture also caused a restorative effect on racemomycin-A formation to little extent. In contrast, the repression by the addition of 3% glucose was not restored by the addition of Pharmamedia or amino acid mixture.

The rapid decrease in antimicrobial activity by glucose at the rapid production phase of racemomycin-A seemed to be due to the absorption of synthesized racemomycin-A into mycelium, or to its inactivation by the formation of inducible enzyme(s). Possibility of the former was considered by a rapid decrease in antimicrobial activity after glucose addition. Moreover, the absorption of ^{14}C-labeled racemomycin-A into the mycelium in the presence of glucose supported this consideration. As shown in Fig. 6, the absorption of radioactivity was observed in the experiments of 3, and 5% of glucose additions, respectively. The mycelium was washed

with 0.9% NaCl, or 5% glucose to compare the differences of radioactivity in the cell by washing materials. Both relative radioactivities of the mycelium were almost the same. This result suggests that the absorption of racemomycin-A, once produced, into mycelium contributes to elucidate the mechanism of rapid decrease in antimicrobial activity.

The effect of various carbon sources on the biosynthesis of racemomycin-A was examined by the cultivation for 50 hours in a basal medium containing carbohydrates. Among the various carbon compounds evaluated, only glucose was found to repress racemomycin-A formation. Xylose and mannitol decreased it to little degree, whereas maltose caused a mild stimulation of the production. Galactose, fructose, mannose, rhamnose, sucrose, lactose, cellobiose, starch, cellulose, sorbitol, inositol, and dulcitol all had no appreciable effect as shown in Table I.

The effect of glucose on the production of streptothricin antibiotics by several organisms of the genus *Streptomyces* is shown in Table II. The production of antibiotics was suppressed by 1% glucose in all organisms tested which showed a various suppression-rate on the formation of antibiotics.

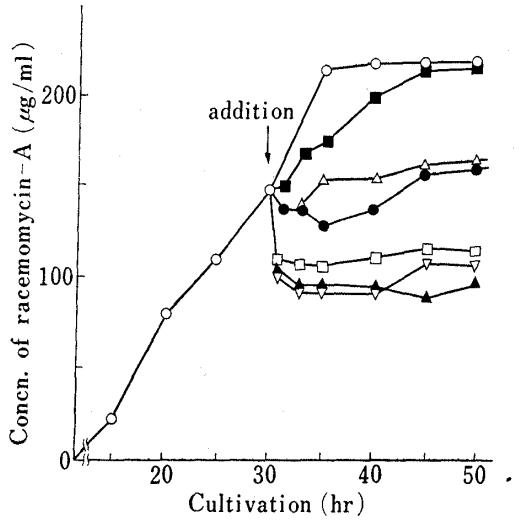

Fig. 5. Effects of Pharmamedia, and Amino Acid Mixture at Rapid Production Phase of Racemomycin-A Formation

○: control, ●: 1% glucose (30 hr), ■: 1% glucose plus 1% Pharmamedia, △: 1% glucose plus 1% amino acid mixture, ▽: 3% glucose (30 hr), □: 3% glucose plus 1% Pharmamedia, ▲: 3% glucose plus 1% amino acid mixture

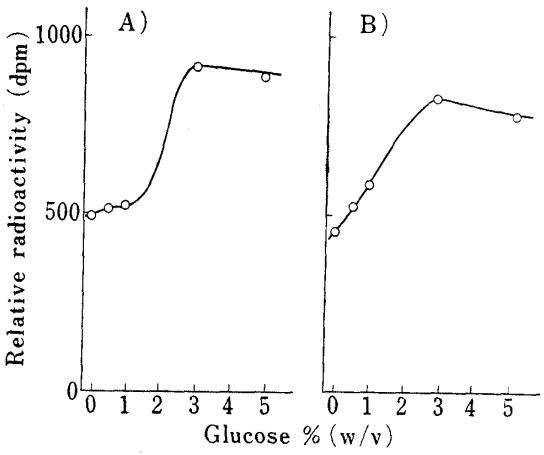

Fig. 6. Incorporation of ^{14}C-Racemomycin-A into Cells by the Addition of Various Concentrations of Glucose

A) Cell was washed with 0.9% NaCl.
B) Cell was washed with 5% glucose.

Table I. Effect of Carbohydrates on Racemomycin-A Production

Carbohydrate	Antimicrobial activity (μg/ml)	Carbohydrate	Antimicrobial activity (μg/ml)
Control	220	Lactose	270
Glucose	70	Cellobiose	270
Galactose	265	Starch	230
Fructose	225	Cellulose	245
Mannose	260	Sorbitol	220
Xylose	170	Inositol	270
Rhamnose	230	Mannitol	170
Sucrose	250	Dulcitol	270
Maltose	300		

Antimicrobial activity at 50 hr after addition of 1% carbohydrate was shown.

Table II. Suppressive Effect of Glucose on the Production of Streptothricin Antibiotics

Strain	Identification with racemomycins[a]	Antimicrobial activity[b] (μg/ml)		Inhibition (%)
		Control	Glucose addition[c]	
S. racemochromogenus 229-0	A, B, C	220	14	96
S. racemochromogenus 229-0-2	A, B, C	150	16	89
S. lavendulae KCC S-0055	C	22	8	64
S. lavendulae KCC S-0056	C	58	6	90
S. lavendulae ISP 5069	A	150	58	61
S. lavendulae IN 309 T	A	35	16	49
S. lavendulae R4	A, B, C	50	30	40
S. lavendulae R4GB	A, B, C	80	58	28
S. lavendulae IFO 12343	A	150	16	89
S. lavendulae IFO 3145	B	130	26	80
S. roseochromogenus KCC S-0075	B, C	110	16	85
S. roseochromogenus KCC S-0077	A	80	12	85
S. albidoflavus KCC S-0003	B, C	58	0	100

a) Identification was made by paper chromatography using Toyo Roshi No. 51 UH paper and solvent system of BuOH–pyridine–HOAc–H_2O-*t*-BuOH (15: 10: 3: 12: 4). Ninhydrin and *E. coli* were used as indicators.
b) Concentration indicates the antimicrobial activity as racemomycin-A.
c) Glucose concentration is 1% in final concentration.

Discussion

Glucose has been shown to repress the synthesis of enzymes that play a role in the primary methabolism of microorganism.[10–12] Recently, it has been demonstrated that the hexose inhibits the synthesis of secondary metabolites such as penicillin [13,14] and siomycin[4], and that of enzymes such as α-D-mannosidase[6,15] and phenoxazinone synthetase.[5]

Glucose was an excellent carbon source for the growth of *Streptomyces lavendulae* ISP 5069, but it was unsatisfactory for racemomycin-A synthesis. It was shown that the supply of glucose into the medium led to the repression of racemomycin-A formation (Fig. 1) to give a rapid decrease in antimicrobial activity (Fig. 3). These repressions were gradually resumed by the addition of Pharmamedia or amino acid mixture. Pharmamedia contains various nutrients such as amino acids, proteins, carbohydrates, fatty acids, vitamins and so on. The component of Pharmamedia which contributes to a better influence on racemomycin-A formation should be investigated.

The possibilities of physiological mechanisms on glucose effect have been considered by many workers. These include: a) repression of the synthesis of enzymes that play a role in antibiotic formation, b) inactivation of antibiotic by enzyme (s) produced when glucose was supplied, c) absorption of antibiotic into the mycelium. The inhibition of racemomycin-A formation by glucose or its catabolites (Fig. 1) may be related to the inhibition of synthesis of the enzyme that catalyzes the biosynthesis of the antibiotic. Pharmamedia may promote the enzyme synthesis.

11) K. Paigen and B. Williams, Edt. by A. Rose, "Advances in Microbial Physiology," Vol. 4, Academic Press Inc., New York, 1970, p. 251.
12) I. Pastan and R. Perlman, *Science*, **169**, 339 (1970).
13) A.L. Demain, *Lloydia* (Cincinnati), **31**, 395 (1968).
14) M.J. Johnson, *Bull World Health Organ*, **6**, 99 (1952).
15) E. Inamine, B.D. Lago, and A.L. Demain, Edt. by D. Perlman, "Fermentation Advances, Academic Press Inc., New York, 1969, p. 199.

A rapid decrease of racemomycin-A by glucose addition (Fig. 3) seemed to result from the absorption of the antibiotic into mycelium. Extraction of racemomycin-A was carried out on the disrupted mycelium which had been incubated with glucose. The repressed mycelium did not contain any racemomycin-A, but ^{14}C-racemomycin-A was incorporated into the cell (Fig. 6). Therefore, it seemed that racemomycin-A was converted to the inactive structure in the mycelium. These results proved that the repression contained the absorption and inactivation of racemomycin-A. The further investigations should be needed to measure the levels of enzymes involved in the inactivation of racemomycin-A.