STUDIES ON ANTIMICROBIAL SUBSTANCE B 44 P (STREPTOVARICIN) PRODUCED BY A STRAIN OF ACTINOMYCETES. IV

BIOCHEMICAL MECHANISM OF ACTION OF SUBSTANCE B 44 P

HISAJI YAMAZAKI, SATOSHI MIZUNO, KAZUO NITTA, RYOZO UTAHARA and HAMAO UMEZAWA

National Institute of Health, Tokyo

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The mechanism of action of the substance B44P, an antibacterial antibiotic, was studied with growing cells of *E. coli* B and *St. aureus* 209 P. This antibiotic was found to inhibit protein and RNA synthesis in these cells. With a cell-free system using S-30 fraction of *E. coli* B, this antibiotic was shown not to affect protein synthesis, in absence of RNA synthesis. Consequently, it was concluded that the substance B 44 P primarily inhibits RNA synthesis in bacterial cells. The difference in the mechanism of RNA synthesis inhibition by the substance B44P and actinomycin D was discussed.

The substance B44P, isolated by the authors[1] from the culture filtrate of a variant of *Streptomyces spectabilis*, is an antibiotic pigment identical with streptovaricin[2,3]. This antibiotic is active mainly against Gram positive bacteria and *Mycobacterium tuberculosis*, *in vitro*[4]. It exhibits a moderate curative effect against staphylococcal infection in mice[5].

The authors investigated the mechanism of action of the substance B 44 P using intact cells of *Escherichia coli* B and *Staphylococcus aureus* 209 P. Although the substance B 44 P inhibited both protein and ribonucleic acid (RNA) syntheses in these bacteria, it was ascertained by experiments with a cell-free system of *E. coli* B that the primary inhibitory action of the substance B 44 P was not against the protein synthesis but against the RNA synthesis.

Materials and Methods

Chemicals :

^{14}C-amino acid mixture employed (6.47 mc/mMC) was the radioactive chlorella hydrolysate prepared by Institute of Applied Microbiology, University of Tokyo. U-^{14}C-lysine (136 mc/mM), U-^{14}C-leucine (155 mc/mM), U-^{14}C-glycine (80 mc/mM), U-^{14}C-proline (120 mc/mM), 5,6-^{3}H-uridine (2.7 c/mM) and 6-^{3}H-thymidine (5.0 c/mM) were obtained from Radiochemical Centre, Amersham, England. U-^{14}C-Phenylalanine (118.8 mc/mM) was purchased from Daiichi Chemical Co., Tokyo. Adenosine triphosphate (ATP), creatine phosphate and creatine kinase [EC 2.7.3.2] (rabbit muscle) were obtained from Sigma Chemical Co. Guanosine triphosphate (GTP) was purchased from Nutritional Biochemicals Corp. Actinomycin D was obtained from Research Division, American Cyanamid Co.

Microorganisms :

E. coli B and *S. aureus* 209 P maintained in the authors' laboratory were used. The

Fig. 1. Effect of B 44 P on the growth of *E. coli* B.

E. coli B cultivated overnight at 37°C in nutrient broth was inoculated into fresh medium. After 2 hour-incubation, the substance B 44 P was added. The growth of the bacteria was measured by turbidimetry at 600 mμ.
a 6.25 mcg/ml, b 12.5 mcg/ml, c 25.0 mcg/ml, d 50.0 mcg/ml.

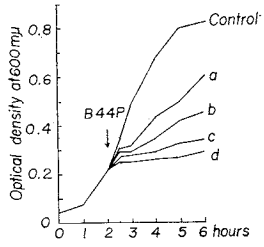


Fig. 2. Effect of B 44 P on the viability of *E. coli* B.

The conditions were the same as in Fig. 1. Colony-counting on nutrient agar was applied instead of turbidimetry.
a 6.25 mcg/ml, b 12.5 mcg/ml, c 25.0 mcg/ml, d 50.0 mcg/ml.

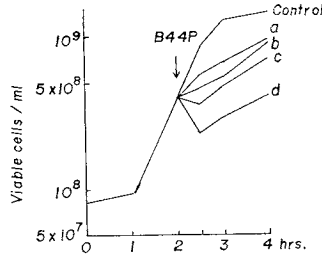
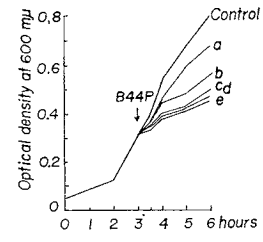


Fig. 3. Effect of B 44 P on the growth of *S. aureus* 209 P.

S. aureus cultivated overnight at 37°C in tryptose phosphate broth was inoculated into fresh medium. After 3-hour incubation, the substance B 44 P was added. The growth of the bacteria was measured by turbidimetry at 600 mμ.
a 0.5 mcg/ml, b 1.0 mcg/ml, c 2.0 mcg/ml, d 4.0 mcg/ml, e 8.0 mcg/ml.



growth of the *E. coli* B was suppressed by the substance B 44 P at a concentration of 25 mcg/ml when the cells were incubated with the substance for 20 hours in STEPHENSON-WHETHAM's synthetic medium. The responses of the *E. coli* B cultivated in nutrient broth to various concentrations of the substance B 44 P are illustrated in Figs. 1 and 2. The *S. aureus* 209 P was inhibited with the substance B 44 P at a concentration of 2~4 mcg/ml in tryptose-phosphate broth, if the substance was added at the beginning of the incubation. The pattern of the inhibition by adding the substance after 3-hour incubation is illustrated in Fig. 3.

E. coli S-30 fraction:

E. coli S-30 fraction was prepared by the method of MATTHAEI and NIRENBERG[6] with a small modification. The principle of the procedure was as follows: *E. coli* B cells harvested at the late logarithmic phase were washed and suspended in an equal volume of standard buffer composed of 0.02 M Tris-HCl (pH 7.8), 0.01 M magnesium acetate, 0.06 M KCl and 0.006 M β-mercaptoethanol. The suspended cells were disrupted in a French press. After decomposition of the contained deoxyribonucleic acid (DNA) by addition of deoxyribonuclease [EC 3.1.4.5] (manufactured by Worthington Co.), the whole preparation was centrifuged at 30,000×*g* for 20 minutes and the supernatant was centrifuged again under the same conditions. The supernatant was dialysed against the standard buffer described above for 18 hours at 4°C. The retentate was used as *E. coli* S-30 fraction. This preparation showed an optical density of 0.50 at 670 mμ and contained 17 mg protein/ml.

Measurement of protein and nucleic acids:

Protein content was determined by the method of LOWRY *et al.*[7] using bovine serum albumin as the standard. RNA and DNA were determined by orcinol and diphenylamine reactions, respectively.

Analyses:

For the determination of the incorporation of ^{14}C-amino acids, ^{3}H-uridine and ^{3}H-thymidine, the reaction was terminated by addition of trichloroacetic acid (TCA) at a final concentration of 5% in the cold. The precipitate was washed with cold 5% TCA three times by centrifugation. In case of the determination of amino acid incorporation,

the precipitate was heated at 90°C for 20 minutes in 5 % TCA followed by washing with cold 5 % TCA to give hot-TCA insoluble material. The hot-TCA insoluble or, for the determination of ^{3}H-uridine and ^{3}H-thymidine incorporation, the percipitate without heating, was washed with cold 95 % ethanol and then ethanol - ether (3 : 1) successively. The residual material was dissolved in 1 N NH_4OH and plated on planchets for drying and counting. Radioactivity was counted with a windowless gas flow counter without correction for self-absorption. Self-absorption by the sample did not affect the results of the experiments.

Results

Effect of the substance B 44 P on the synthesis of protein, RNA and DNA in growing bacteria

E. coli B cultivated overnight in nutrient broth at 37°C was washed twice with STEPHENSON-WHETHAM's medium by centrifugation. The washed cells were resuspended in STEPHENSON-WHETHAM's medium to make a suspension showing an optical density of 0.06 at 600 mμ. Twenty ml of the cell suspension were incubated with shaking at 37°C. After 3 hours, the substance B 44 P was added at a final concentration of 10 mcg/ml and the incubation with shaking was continued. Each hour, 6 ml were taken from the suspension, the reaction in the drawn samples was stopped by adding an equal volume of 10 % TCA and the samples were analyzed for protein, RNA and DNA contents. The results are shown in Fig. 4. The protein and RNA syntheses were inhibited by 10 mcg/ml of the substance B 44 P. If the percen tinhibition is expressed as $\frac{\Delta m_c - \Delta m_{tr}}{\Delta m_c} \times 100$ (where m_c is the increment of the amount of protein or RNA in time t in the control and Δm_{tr} is that in the B 44 P-treated; time zero is assigned to the time when the substance B 44 P is added), the inhibition of protein synthesis in 1, 2 and 3 hours was 33.3 %, 49.2 % and 26.0 %, respectively, and that of the RNA synthesis was 50.0%, 35.7 % and 38.5 %. DNA synthesis was not inhibited but rather accelerated.

Fig. 4. Effect of B 44 P on protein, RNA and DNA in *E. coli* B.

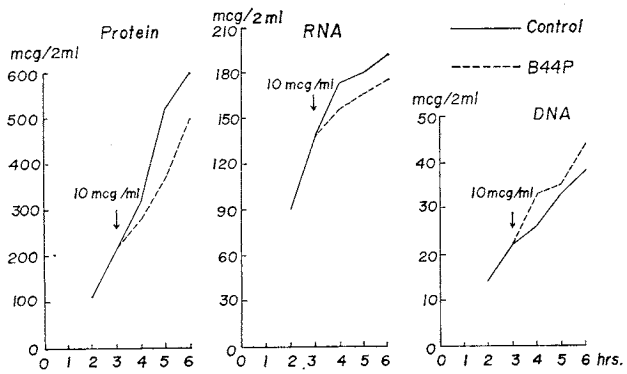
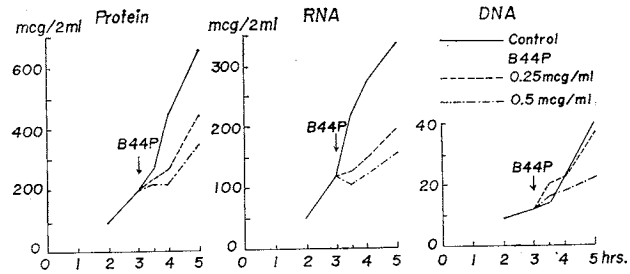


Fig. 5. Effect of B 44 P on protein, RNA and DNA syntheses in *S. aureus* 209 P.



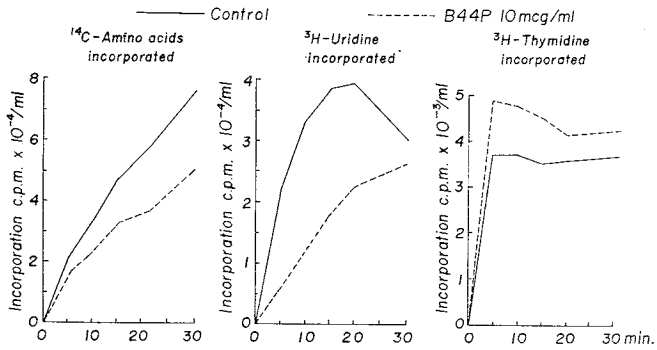
A similar experiment was carried out using *S. aureus* 209 P. In this case, however, tryptose-phosphate broth was used instead of STEPHENSON-WHETHAM's medium and nutrient broth. Two concentrations

of the substance B 44 P, 0.5 mcg/ml and 0.25 mcg/ml, were tested. Samples were taken for the determination of protein, RNA and DNA contents 0.5, 1 and 2 hours after the addition of the drug. As demonstrated in Fig. 5, the protein and RNA syntheses were markedly suppressed; the inhibition of RNA synthesis was particularly remarkable. The percent inhibition of the protein synthesis was 72.2 %, 91.7 % and 68.1 % in 0.5, 1 and 2 hours respectively at a concentration of 0.5 mcg/ml, and 54.9 %, 70.8 % and 47.3 % at a concentration of 0.25 mcg/ml. The inhibition of RNA synthesis was 113.5 %, 98.4 % and 82.8 % in 0.5, 1 and 2 hours respectively at 0.5 mcg/ml, and 92.5 %, 82.0 % and 64.4 % at 0.25 mcg/ml. The DNA synthesis was elevated at least in the first 30 minutes, and after 1 hour or later it was depressed at 0.5 mcg/ml.

Effect of the substance B 44 P on the incorporation of ^{14}C-amino acids, ^{3}H-uridine and ^{3}H-thymidine into protein, RNA and DNA in growing bacteria

The test bacteria and the incubating conditions were the same as in the experiments for measuring protein and nucleic acid content just described. After 3-hour incubation, the followings were added together with the substance B44P: for ^{14}C-amino acid incorporation, ^{14}C-amino acid mixture (^{14}C-chlorella hydrolysate) 0.25 μc/ml in case of the experiment with *E. coli* B or 0.1 μc/ml in case of the experiment with *S. aureus* 209 P, plus casamino acids 0.5 mcg/ml; for ^{3}H-uridine incorporation, ^{3}H-uridine 0.1 μc/ml plus uracil 0.5 mcg/ml; for ^{3}H-thymidine incorporation, ^{3}H-thymidine 0.1 μc/ml plus cold thymidine 0.025 mcg/ml. Sampling was done for counting radioactivty 5, 10, 15, 20 and 30 minutes after the addition, 2 ml each being taken every time. In *E. coli* B, as shown in Fig. 6, the incorporation of ^{14}C-amino acids and ^{3}H-uridine was inhibited by 10 mcg/ml of the substance B44P. Especially, ^{3}H-uridine incorporation was distinctly suppressed from the beginning. The percent inhibition of ^{14}C-amino acid incorporation was 26.8 %, 33.3 %, 31.6 %, 35.9 % and 34.2 % in 5, 10, 15, 20 and 30 minutes respectively. The low inhibition of ^{3}H-uridine incorporation in 20 and 30 miuntes is due to the decrease in the counts incorporated in the control at those times. The decrease in the counts in the control might have been caused by the exhaustion of available ^{3}H-uridine; the reduction of the RNA synthesis itself at thos etimes in the control was not likely from the experiment shown in Fig. 4. ^{3}H-Thymidine incorporation was promoted by the substance B 44 P.

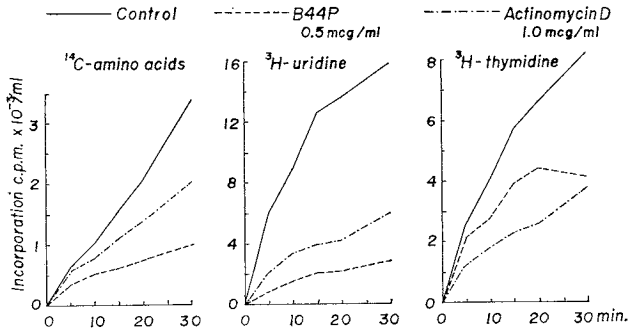
Fig. 6. Effect of B 44 P on the incorporation of ^{14}C-amino acids, ^{3}H-uridine and ^{3}H-thymidine into protein, RNA, and DNA in *E. coli* B.



In the experiments using *S. aureus* 209 P, actinomycin D (1 mcg/ml) was tested as a reference, besides the substance B 44 P (0.5 mcg/ml). As illustrated in Fig. 7, the

substance B 44 P at 0.5 mcg/ml strongly inhibited the incorporation of ^{14}C-amino acids and ^{3}H-uridine into protein and RNA in staphylococcus. The inhibitory activity of 0.5 mcg/ml of the substance B 44 P was much stronger than that of 1.0 mcg/ml of actinomycin D. The percent inhibition of ^{14}C-amino acid incorporation by 0.5 mcg/ml of the substance B 44 P was 44.4 %, 48.6 %, 60.6 %, 63.4 % and 70.4 % in 5, 10, 15, 20 and 30 minutes respectively, and that by 1.0 mcg/ml of actinomycin D was 4.8 %, 15.5 %, 28.1 %, 31.7 % and 39.4 % at the corresponding times. The inhibition of ^{3}H-uridine incorporation by 0.5 mcg/ml of the substance B 44 P was 86.7 %, 83.3 %, 84.1 %, 84.5 % and 82.3 % in 5, 10, 15, 20 and 30 minutes, and that by 1 mcg/ml of actinomycin D was 65.0 %, 63.3 %, 69.4 %, 69.1 % and 62.3 %. Contrary to the situation with *E. coli*, the substance B44P inhibited the ^{3}H-thymidine incorporation in staphylococcus, but the inhibition was weaker than that exhibited by 1.0 mcg/ml of actinomycin D.

Fig. 7. Effect of B 44 P on the incorporation of ^{14}C-amino acids, ^{3}H-uridine and ^{3}H-thymidine into protein, RNA and DNA in *S. aureus* 209 P.



Lack of action of the substance B 44 P on amino acids incorporation into protein by cell-free system of *E. coli* B

The question whether the substance B44P primarily inhibits protein synthesis in bacteria or not was examined in a cell-free system of *E. coli* B under conditions where RNA synthesis was prevented. The reaction mixture contained, in a total volume of 0.5 ml, Tris-HCl (pH 7.8) 50 μmoles, magnesium acetate 5 μmoles, KCl 30 μmoles, β-mercaptoethanol 3 μmoles, ATP 0.5 μmoles, creatine phosphate 2.5 μmoles, creatine kinase 25 mcg, GTP 0.05 μmoles, ^{14}C-amino acids (mixture of ^{14}C-lysine, ^{14}C-leucine, ^{14}C-glycine, ^{14}C-proline and ^{14}C-phenylalanine) 0.18 μc total, 15 other unlabeled amino acids 25 mμ moles each, and *E. coli* S-30 fraction 1.7 mg protein. The substance B 44 P, actinomycin D or puromycin was added prior to the S-30 fraction in doses as indicated in

Table 1. Lack of inhibitory activity of the substance B 44 P and actinomycin D against ^{14}C-amino acid incorporation into protein in cell-free system of *E. coli* B

		c.p.m./mg protein	% inhibition
Control		3,155	
+Substance B 44 P	10 mcg/ml	3,200	
	20 mcg/ml	3,430	
	50 mcg/ml	2,940	
+Actinomycin D	10 mcg/ml	3,070	
	20 mcg/ml	3,400	
	50 mcg/ml	3,480	
+Puromycin	5 mcg/ml	1,230	61.0

Reaction mixture : Tris-HCl (pH 7.8) 50 μmoles, magnesium acetate 5 mM, KCl 30 mM, β-mercaptoethanol 3 mM, ATP 0.5 mM, creatine phosphate 2.5 mM, creatine kinase 25 mcg, GTP 0.05 mM, ^{14}C-amino acids (mixture of ^{14}C-lysine, ^{14}C-leucine, ^{14}C-glycine, ^{14}C-proline and ^{14}C-phenylalanine) 0.18 μc, 15 other cold amino acids 25 mμmoles each, and *E. coli* S-30 fraction (DNAase-treated) 1.7 mg protein in a total volume of 0.5 ml.
Incubation : at 37°C for 30 minutes.
Analysis : see text (Materials and Methods).

Table 1. The mixture was incubated at 37°C without pre-incubation. After 30 minutes, the mixture was analyzed for ^{14}C-amino acid incorporation into the protein fraction.

As shown in Table 1, neither the substance B 44 P nor actinomycin D, even at 50 mcg/ml, inhibited amino acid incorporation into protein fraction in *E. coli* B, when RNA synthesis was prevented by decomposition of primer DNA, although puromycin tested as a reference showed a reasonable inhibition at 5 mcg/ml.

Discussion

In order to clarify the mechanism of the action of the substance B 44 P, an antibacterial antibiotic, the synthesis of protein, RNA and DNA in growing bacterial cells was quantitatively measured in the presence of the drug by two means : one was direct measurement of the total amount of the protein and the nucleic acids, and the other was counting the radioactivity of the labeled precursors incorporated into protein and nucleic acid fractions of those cells. The results in both experiments showed that the substance B 44 P inhibited the syntheses of protein and RNA in *E. coli* and *S. aureus*. Although the inhibition of the RNA synthesis was much stronger than that of protein synthesis, it cannot be concluded that B 44 P acts on the RNA synthesis. Protein synthesis inhibition occurred without any lag under the conditions employed, unlike the results seen with actinomycin D (Fig. 7). On the other hand, a cell-free experiment with *E. coli* S-30 fraction demonstrated that the substance B 44 P did not affect protein synthesis even at 50 mcg/ml. Considering this, one may conclude that the substance B 44 P inhibited primarily the RNA synthesis in bacteria.

The substance B44P exhibited stronger inhibitory activity against protein and RNA syntheses in staphylococcal cells than actinomycin D as shown in Fig. 7. On the other hand, the order of the activities of both antibiotics were reversed with respects to the inhibition of DNA synthesis. These facts suggest that the substance B 44 P inhibits RNA synthesis more specifically than actinomycin D and that the mode of action of the two differs.

The substance B 44 P has no antitumor activity against EHRLICH ascites carcinoma *in vivo*. In agreement with this, the substance B 44 P inhibited neither incorporation of ^{14}C-amino acids into protein nor incorporation of ^{3}H-nucleosides into nucleic acids in EHRLICH cells up to 100 mcg/ml (see Table 2). The difference in anti-tumor cell activity

Table 2. Effect of the substance B 44 P on the incorporation of ^{14}C-amino acids, ^{3}H-uridine and ^{3}H-thymidine into protein and nucleic acids in EHRLICH ascites carcinoma cells

		^{14}C-Amino acids c.p.m.$\times 10^{-3}$	^{3}H-Uridine c.p.m.$\times 10^{-3}$	^{3}H-Thymidine c.p.m.$\times 10^{-4}$
Control		2.50	3.03	13.9
Substance B 44 P	25 mcg/ml	2.52	3.43	13.9
	50 mcg/ml	2.66	2.96	13.6
	100 mcg/ml	2.34	2.94	12.2
	200 mcg/ml	2.52	2.24 (26.0)	10.5 (24.5)
Actinomycin D	0.5 mcg/ml	1.92	1.88 (38.0)	14.2
	1.0 mcg/ml	1.86	1.07 (64.6)	14.3
	2.0 mcg/ml	1.85	0.77 (74,6)	12.7 (8.6)

Reaction mixture contained Tris-HCl (pH 7.6) 0.02 mM, glucose 0.04 mM, NaCl 0.14 mM, radioactive precursor, EHRLICH cells 5×10^{6} in a total volume of 1 ml. The radioactive precursor added to the mixture was : ^{14}C-amino acids mixture (^{14}C-chlorella hydrolysate) 0.02 μc, ^{3}H-uridine 0.1 μc or ^{3}H-thymidine 0.05 μc. After 60-minute incubation at 30°C, the whole mixture was submitted to the analysis.

The figures in the parentheses represent percent inhibition.

between the substance B 44 P and actinomycin D might be due to the selective permeability of the cell membrane, or may suggest that the mechanism of the RNA synthesis inhibition of the substance B 44 P differs from that of actinomycin D.

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