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

MICROBIOLOGICAL AND PHARMACOLOGICAL STUDIES

HISAJI YAMAZAKI

National Institute of Health, Tokyo

(Received for publication October 31, 1967)

An antibiotic substance B 44 P identical with streptovaricin showed a strong inhibitory activity *in vitro*, mainly against Gram positive bacteria including *Mycobact. tuberculosis*. It also inhibited the growth of *Sh. sonnei*, *Sh. boydii* and *Salm. enteritidis* at small doses, but exhibited no activity against fungi. The antibacterial activity was not virtually influenced by the inoculum number of test cells, the medium pH within the range of 5.0~8.5, or human blood serum added to the medium. The mode of activity was considered to be mainly bacteriostatic, while partly bactericidal. Gram negative bacteria such as *Sh. boydii* and *Salm. enteritidis* could readily be made resistant to the substance B 44 P by being transferred through medium containing this antibiotic. On the other hand, *Staph. aureus* developed resistance slowly step by step. The frequency of naturally resistant mutants were nil in *S. enteritidis* and *Sh. boydii* and $1.0 \sim 4.6 \times 10^{-6}$ in *Staph. aureus*. No cross resistance was found between the substance B 44 P and other antibiotics. The substance B 44 P exhibited low toxicity in mice by single or long-term administration. A dog showed practically no pathologic signs by oral administration of 200 mg/kg/day of the drug for 33 successive days. Another dog, however, which received 500 mg/kg/day, developed some symptoms. Liver, kidney and bone marrow were not considered to be damaged by oral administration of this antibiotic. When orally given, the substance B 44 P was absorbed and appeared in the blood and the urine quite rapidly. Excretion continued for about a day. Relatively dense distributions of the antibiotic were observed in the livers and stomachs of mice after oral administration.

As reported in a previous paper[1], the substance B 44 P, an antibiotic pigment, was isolated in the authors' laboratory and identified as streptovaricin[2,3,4]. Some chemotherapeutic experiments have been performed against tuberculosis[5,6,7] and murine leprosy[8] *in vivo*, but no investigators have yet reported treatment of staphylococcal infection *in vivo* with streptovaricin. The authors have studied the substance B 44 P in microbiological and pharmacological respects, and examined it for chemotherapeutic effects against staphylococcal infection in animals. Furthermore, they have investigated its mode of action. These studies will be described in this series of papers.

In this paper, the antimicrobial spectrum of the substance B 44 P, development of resistance in bacteria *in vitro*, toxicity, and distribution in tissues and excretion is presented.

Materials and Methods

The substance B 44 P:

The substance B 44 P was prepared by Toyo Jozo Co. Ltd., Ohito, Japan. The extraction and purification of the substance has been already reported[1]. The purified preparation used in the experiments contained three major principles, A, B and C, in a ratio of 65 : 26 : 1 and a few negligible minor components. Its purity was 80 % or higher.

Other antibiotics:

Penicillin G, dihydrostreptomycin, kanamycin, tetracycline, erythromycin, oleandomycin, novobiocin, spiramycin and capreomycin used conformed to the standards.

Microorganisms:

Clostridia were obtained from Dr. S. KAMEYAMA, Department of Bacteriology II, National Institute of Health of Japan, Tokyo. *Mycobacterium tuberculosis* $H_{37}Rv$, *Mycobact. tuberculosis* BCG, *Mycobact. kirchberg* and *Mycobact. smegmatis* were donated by Dr. H. TAKAHASHI, Department of Tuberculosis of the same institute. The other strains of microorganisms used were those maintained in the authors' laboratory.

Animals:

Animals used were 5 week old, male, highly inbred *dd* mice weighing 18~20 g, 5~7 week old, male Wistar rats weighing 80~130 g, female hybrid rabbits weighing 2.5 kg, and male hybrid dogs weighing 7.0 and 9.5 kg. Mice and rats were fed with solid chow manufactured by Oriental Yeast Industry Co. and water *ad libitum*. Rabbits were fed in the same way except solid chow made for guinea-pigs and rabbits by Funabashi Farm was used instead of Oriental's.

Antimicrobial spectrum:

Technique adopted were: for Gram negative and positive bacteria, except mycobacteria and clostridia, agar dilution method using Difco heart infusion agar (abbreviated as HIA); for *Mycobact. tuberculosis* $H_{37}Rv$, BCG, and *Mycobact. kirchberg* the liquid dilution method using KIRCHNER's broth; for *Mycobact.* 607, *Mycobact. smegmatis* and *Mycobact. phlei* agar dilution with HIA containing 2 % glycerol; and for clostridia and fungi, liquid dilution with thioglycolate medium and agar dilution with SABOURAUD's glucose agar containing 2 % glucose, respectively.

Effect of the substance B 44 P on growth curve of *Staphylococcus aureus*, strain SMITH:

St. aureus SMITH cultivated for 20 hours at 37°C in Difco brain heart infusion broth (abbreviated as BHI) was inoculated into the same medium at 1.4×10^5 cells/ml and cultivated at 37°C. The substance B 44 P dissolved in BHI was added at the same time or after 6-hour incubation at 37°C. Aliquots of the culture were taken at appropriate times during the incubation for the measurement of bacterial growth. The measurement of growth was done in two ways: turbidimetrically at 600 mμ with an electrophotometer (Spectronic 20, Shimadzu) or by counting living cells. In the latter, the culture samples to be tested were diluted with BHI and aliquots mixed with melted and cooled, but still liquid, BHI-agar to make plates in 90 mm Petri dishes. When solidified, the plates were incubated at 37°C. After 18~24 hours, colonies in the plates were counted. The actual number of living cells in test material was calculated by multiplying the number of colonies by the degree of dilution.

Development of resistance *in vitro* in bacteria against the substance B 44 P:

Test bacteria were cultivated at 37°C in Difco heart infusion broth (abbreviated as HIB) with the substance B 44 P at the maximum concentration where they could grow. When grown, these were transferred to be subcultured into HIB containing higher concentrations of the antibiotic. The development of drug-resistance in the bacteria was accomplished by repetition of this procedure.

Frequency of spontaneous appearance of mutants resistant to the substance B 44 P in sensitive bacteria:

The frequency of spontaneous resistance to the substance B 44 P in sensitive bacteria was examined by the modified method of NEWCOMBE[9]. Ten ml of BHI-agar containing 50 or 100 mcg/ml of the substance B 44 P were poured into 90 mm Petri dishes. When solidified, the plates were overlayed with 10 ml of BHI-agar containing 1×10^6 cells of sensitive bacteria and left standing at room temperature until the agar was solidified. After a 20-hour incubation at 37°C, colonies in the plates were counted.

Toxicity test:

For the acute toxicity test in mice and rats, the substance B 44 P was administered only once intraperitoneally, subcutaneously or orally. Following administration, the animals were observed for 15 days. For subacute or chronic toxicity tests, mice received the antibiotic once a day orally for 10 successive days, or intraperitoneally for 30 consecutive days. The number of dead, changes in body weight, and other pathologic signs were recorded for 30 days. Autoptic examination was done on the dead.

Toxicity of the antibiotic was also examined in dogs. A dog weighing 7.0 kg was orally medicated once a day for 33 days in succession with the substance B 44 P at a dose of 200 mg/kg/day. Another dog weighing 9.5 kg received the drug orally once a day for 30 days with an interruption of 4 days in doses of 500 mg/kg/day for the first 23 days and 1,000 mg/kg/day for the last 7 days. During the experimental course, general pathologic signs and changes in body weight were recorded. The number of blood cells, homoglobin content, hemogram, liver function (serum transaminase) and kidney function (serum urea-N) were examined every 5~7 days. One or two weeks after the termination of administration, the dogs were sacrificed for autopsy.

Measurement of the drug concentration in blood and urine:

After one oral administration of 200 mg/kg of the substance B 44 P in rabbits, the blood was taken at intervals and centrifuged to separate serum. At the same time, urine was drained by catheterization. Concentrations of the drug in specimens of blood serum and urine were determined by paper disc-plate assay using *Sarcina lutea* PCI 219 as test organism.

Distribution of the substance B 44 P in organs after oral administration:

Twenty mice were given 400 mg/kg of the substance B 44 P orally. After 1, 3, 5 and 7 hours, 5 mice randomly chosen were sacrificed by decapitation after blood and urine had been taken. Lungs, livers, spleens, kidneys, hearts, stomachs, testes and skeletal muscle were removed, washed with physiological saline, blotted with filter-paper and homogenized separately in M/15 phosphate buffer, pH 6.8, with Waring blendors. Each homogenate was centrifuged at 1,000 rpm for 3 minutes. Concentrations of the antibiotic in the supernatants of these organ homogenates were measured by paper disc-plate assay in the same way as described above. The actual concentration in the organs was calculated and expressed in mcg B 44 P potency/g wet weight of organ. In the case of blood serum or urine, it was expressed in mcg B 44 P potency/ml.

Results

Antimicrobial Activity

The minimum growth-inhibitory concentrations (MIC) of the substance B 44 P *in vitro* were determined by observation over 24 hours for Gram negative and positive bacteria except mycobacteria, over 10 days for *Mycobact. tuberculosis* $H_{37}Rv$, BCG and *Mycobact. kirchberg*, over 3 days for the other mycobacteria, and over 2 days for fungi. The antimicrobial spectrum is tabulated in Table 1. In general, Gram positive bacteria tested were sensitive to this antibiotic; particularly, *Microc. flavus* M-16, *Sar. lutea* PCI 1001 and *Coryn. xerosis* 53-K 1 were sensitive enough to be inhibited at 0.05, 0.1 and 0.2 mcg/ml, respectively. Although *Sh. sonnei*, *Sh. boydii*

Table 1. Antimicrobial spectrum of the substance B 44 P

Organisms	Medium*	M.I.C. (mcg/ml)	Organisms	Medium*	M.I.C. (mcg/ml)
Staphylococcus aureus FDA 209 P	A	6.25	*Shigella dysenteriae* EW 21684	A	50
			Shigella flexneri EW 8 1a-1701	A	>100
Staphylococcus aureus TERAJIMA	A	0.78	*Shigella flexneri* SR-1 2a-107-57	A	50
Staphylococcus aureus SMITH	A	3.125	*Shigella flexneri* SR-1 2b-5-57	A	50
Micrococcus flavus M-16	A	0.05	*Shigella boydii* 1-65	A	12.5
Sarcina lutea PCI 1001	A	0.1	*Sihgella sonnei* 11, 37148	A	1.56
Corynebacterium xerosis 53-K 1	A	0.2	*Pseudomonas aeruginosa* A 3	A	>100
Bacillus anthracis	A	12.5	*Proteus vulgaris* OX-19	A	>100
Bacillus subtilis PCI 219	A	12.5	*Mycobacterium tuberculosis* $H_{37}Rv$	B	1.56
Bacillus subtilis NRRL B-558	A	6.25	*Mycobacterium tuberculosis* BCG	B	1.56
Bacillus megaterium	A	0.78	*Mycobacterium kirchberg*	B	>100
Bacillus cereus ATCC 10702	A	6.25	*Mycobacterium* 607	C	100
Escherichia coli NIHJ	A	>100	*Mycobacterium smegmatis*	C	100
Escherichia coli PO 1495	A	>100	*Mycobacterium phlei*	C	6.25
Klebsiella pneumoniae 602	A	50	*Clostridium welchii* PB-6K	D	3.125
Salmonella typhi B-26	A	>100	*Clostridium bifermentans* 315	D	25
Salmonella typhimurium 1406	A	>100	*Clostridium sporogenus* 20	D	12.5
Salmonella paratyphi A 1015	A	>100	*Aspergillus niger* Tieghen	E	>100
Salmonella paratyphi B 8006	A	>100	*Candida albicans*	E	>100
Salmonella paratyphi C Hirschfeld	A	>100	*Trichophylon mentagrophytes*	E	>100
Salmonella cholerae suis 1348	A	>100	*Sacchharomyces cerevisiae*	E	>100
Salmonella enteritidis	A	12.5	*Torula utilis*	E	>100

* A=Heart infusion agar. B=KIRCHNER's broth (MIC after 10 days). C=Heart infusion agar containing 2 % glycerin. D=Thioglycolate medium. E=SABOURAUD agar containing 2 % glucose.

and *Salm. enteritidis* were inhibited at low concentrations, Gram negative bacteria were generally insensitive. The substance B 44 P exhibited a relatively strong inhibitory activity against *Mycobact. tuberculosis* $H_{37}Rv$, BCG and *Mycobact. phlei*, but not against the other mycobacteria tested or fungi.

Influence of inoculum size, pH and serum on the activity of the substance B 44 P

The influence on the antibacterial activity of this agent by the number of inoculum test cells, and by the pHs of medium and human serum added was examined. The results are given in Tables 2, 3 and 4. Table 2 shows the minimum growth-inhibitory concentration (MIC) of this antibiotic against *Staph. aureus* SMITH for various inoculum sizes in BHI (pH 7.0) after 20-hour incubation at 37°C. From this data, it was concluded that the MIC was hardly influenced by the inoculum size of the test organism. The effect of medium pH on the activity of this antibiotic was examined. *Staph. aureus* SMITH was inoculated at a level of 5×10^4 cells/ml into BHI containing the substance B 44 P, the pH of the medium ranging from 5.0 to 8.5. The MIC was determined after a 20-hour cultivation

Table 2. Influence of inoculum size of test bacteria on antibacterial activity of the substance B 44 P compared to streptomycin

Number of the bacterial cells inoculated	MIC* mcg/ml	
	Substance B 44 P	Streptomycin
5×10^6	1.25	5.0
5×10^5	0.625	2.5
5×10^4	0.625	2.5
5×10^3	0.625	2.5
5×10^2	0.625	2.5
5×10^1	0.625	1.25

* Minimum inhibitory concentration.
Staph. aureus SMITH strain.

Table 3. Influence of pH in medium on antibacterial activity of the substance B 44 P compared to streptomycin

pHs in medium	MIC* mcg/ml	
	Substance B 44 P	Streptomycin
5.0	0.625	40.0
5.5	0.625	20.0
6.0	0.625	10.0
6.5	0.625	5.0
7.0	0.625	2.5
7.5	0.625	2.5
8.0	0.625	1.25
8.5	0.625	1.25

* Minimum inhibitory concentration.
Staph. aureus SMITH strain.

Table 4. Influence of the human blood serum added to the medium on antibacterial activity of the substance B 44 P compared to streptomycin

Concentration of the serum in medium (%)	MIC* mcg/ml	
	Substance B 44 P	Streptomycin
50	1.25	2.5
25	0.625	2.5
10	0.625	2.5
5	0.625	2.5
2	0.625	2.5
0	0.625	2.5

* Minimum inhibitory concentration.
Staph. aureus SMITH strain.

at 37°C. Results shown in Table 3 demonstrated the independence of the activity of the substance B 44 P and the pH of the medium within the range tested. Some antibiotics are known to be reduced in the antimicrobial activity by the presence of serum. The substance B 44 P was found to be unaffected in activity by human serum at a final concentration of 25 % or below when tested against *Staph. aureus* SMITH in BHI (inoculum: 5×10^4 cells/ml) (Table 4).

Effect of the substance B 44 P on growth of *Staphylococcus aureus*, strain SMITH

The growth of *Staph. aureus* SMITH in BHI with and without the substance B 44 P was measured turbidimetrically against BHI as reference at 600 mμ. As shown in Fig. 1, when the antibiotic was added at the beginning, the lag phase was markedly prolonged, particularly, at a dose of 0.313 mcg/ml or higher. After 15~21 hours, however, logarithmic growth started. As seen in Fig. 2, when the antibiotic was added after 6-hour cultivation, that is, at the early stage of logarithmic growth phase, the growth of the bacteria was depressed for 12 hours at a concentration of 2.5 mcg/ml or for 18 hours at 5 mcg/ml or more, respectively. A similar experiment was carried out by counting the living cells in the culture instead of by turbidimetry. The result is illustrated in Fig. 3. The addition of the drug to final concentrations of 2.5~10.0 mcg/ml at the early stage of the logarithmic growth phase (6-hour culture) decreased the number of living cells to the order of the initial inoculum cell number

Fig. 1. Effect of the substance B 44 P on the growth of *Staphylococcus aureus* SMITH (Turbidimetry)

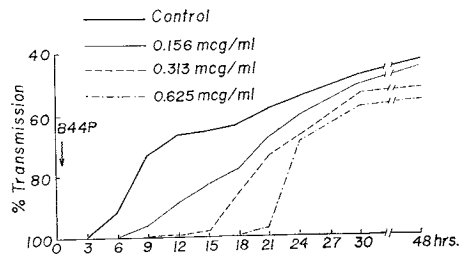


Fig. 2. Effect of the substance B 44 P on the growth of *Staphylococcus aureus* SMITH (Turbidimetry)

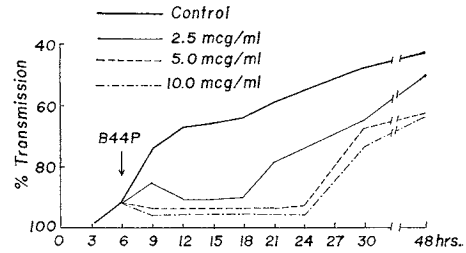


Fig. 3. Effect of the substance B 44 P on the growth of *Staphylococcus aureus* SMITH (Living cell count)

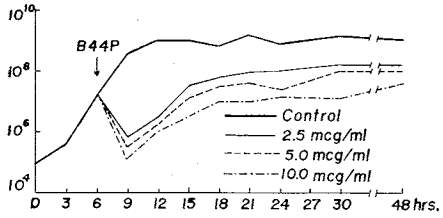
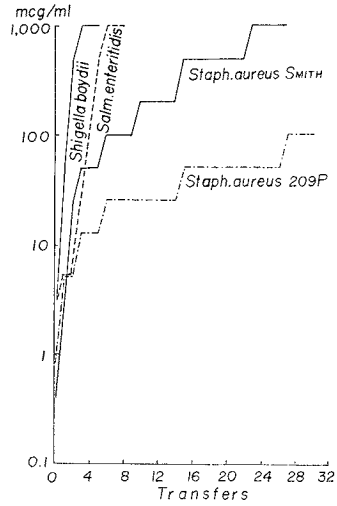


Fig. 4. Development of resistance *in vitro* of bacteria against the substance B 44 P



within 3 hours. Nevertheless, the remaining cells began to increase again over the next 3 hours to bring themselves into logarithmic growth. The maximum number of cells in stationary phase, however, was far less than that of the control.

Development of resistance in bacteria

Staph. aureus 209 P, *Staph. aureus* SMITH, *Salm. enteritidis* and *Sh. boydii* were tested for their development of resistance against the substance B 44 P. Results are shown in Fig. 4. Gram negative bacteria showed steep increments in resistance with a so-called one-step development; a high resistance to the substance B 44 P (1,000 mcg/ml) was observed after only 3 transfers of *Sh. boydii* and 6 of *S. enteritidis*. On the contrary, *Staph. aureus* demonstrated step by step development with an initial short but rapid jump; strain 209 P showed resistance to 100 mcg/ml after 30 transfers and strain SMITH displayed resistance to 1,000 mcg/ml after 23 transfers.

Frequency of spontaneous appearance of mutants resistant to the substance B 44 P

Frequency of spontaneous resistance was investigated on *Staph. aureus* 209 P, *Staph. aureus* SMITH, *Salm. enteritidis* and *Sh. boydii*. Results are tabulated in Table 5. As seen in the table, the frequency of mutants resistant at 25 mcg/ml spontaneously arising was 1.0×10^{-6} and 4.6×10^{-6} in *Staph. aureus* 209 P and *Staph. aureus* SMITH, respectively. In the case of strain SMITH, 2.2×10^{-6} cells were found to be resistant against the antibiotic at 50 mcg/ml. On the other hand, in

Table 5. Frequency of natural mutants resistant to the substance B 44 P

Organisms	Resistant to 25 mcg/ml	Resistant to 50 mcg/ml
Staphylococcus aureus 209 P	1.0×10^{-6}	0×10^{-6}
Staphylococcus aureus SMITH	4.6×10^{-6}	2.2×10^{-6}
Salmonella enteritidis	0×10^{-6}	0×10^{-6}
Shigella boydii	0×10^{-6}	0×10^{-6}

Table 6. Sensitivity to some antibiotics of *Staphylococcus aureus* SMITH which acquired resistance against the substance B 44 P *in vitro*

Antibiotics	Minimum inhibitory concentration (mcg/ml)	
	Original strain	Resistant strain
Substance B 44 P	0.313	>100
Penicillin	0.039	0.078
Dihydrostreptomycin	2.5	2.5
Kanamycin	0.313	0.313
Tetracycline	0.156	0.313
Erythromycin	0.313	0.313
Oleandomycin	0.313	0.313
Novobiocin	0.156	0.156
Spiramycin	2.5	5.0
Capreomycin	12.5	12.5

Salm. enteritidis and *Sh. boydii*, no cells resistant to 25 mcg/ml of the antibiotic were detected.

Cross resistance

The *Staph. aureus* SMITH which had acquired high resistance *in vitro* to the substance B 44 P was examined for its sensitivity to penicillin G, dihydrostreptomycin, kanamycin, tetracycline, erythromycin, oleandomycin, novobiocin, spiramycin and capreomycin. The test organism was inoculated at 5×10^3 cells/ml into BHI containing the antibiotics and cultivated for 20 hours at 37°C. The minimum growth inhibitory concentrations of these antibiotics were determined. As shown in Table 6, the test organism was resistant only to the substance B 44 P and sensitive to the other antibiotics tested, in other words, no cross resistance was observed.

Acute toxicity in mice and rats

Acute toxicity of the substance B 44 P was tested through one administration in mice and rats. The results are described in Tables 7 and 8. By an intraperitoneal injection of 500 mg/kg all the mice were killed, but 250 mg/kg did not cause death in any mice. In both oral and subcutaneous administrations all the mice tolerated 1,000

Table 7. Acute toxicity of the substance B 44 P in mice

Route	Dosis (mg/kg)	Average body weight before administration (g)	Number of the dead / Number of the tested		Average body weight after administration (g)	LD_{50} (mg/kg)
			1 day	15 days		
Intraperitoneal	500	18.0	5/5	5/5	—	375
	250	19.0	0/5	0/5	19.8	
	125	19.6	0/5	0/5	23.0	
	62.5	18.8	0/5	0/5	20.0	
Oral	2,000	19.6	2/5	2/5	20.5	>2.000
	1,000	18.5	0/5	0/5	23.7	
	500	19.0	0/5	0/5	22.1	
	250	18.8	0/5	0/5	21.9	
	125	19.0	0/5	0/5	23.9	
Subcutaneous	1,000	19.4	0/5	0/5	23.8	>1,000
	500	19.0	0/5	0/5	21.0	
	250	18.2	0/5	0/5	21.5	
	125	17.8	0/5	0/5	23.8	

Table 8. Acute toxicity of the substance B 44 P in rats

Route	Dosis (mg/kg)	Average body weight before administration (g)	Number of the dead / Number of the tested		Average body weight after administration (g)	LD_{50} (mg/kg)
			1 day	15 days		
Intraperitoneal	200	83	3/3	3/3	—	150
	100	90	0/3	0/3	133	
	50	98	0/3	0/3	138	
	25	90	0/3	0/3	148	
	12.5	105	0/3	0/3	158	
Oral	3,200	128	3/3	3/3	—	1,900
	1,600	137	1/3	1/3	168	
	800	132	0/3	0/3	153	
	400	123	0/3	0/3	152	
	200	107	0/3	0/3	150	

mg/kg. In rats, 200 mg/kg by the intraperitoneal route was lethal while 100 mg/kg was safe. The minimum lethal and the maximum tolerable doses by oral administration in rats were 3,200 mg/kg and 800 mg/kg, respectively. In the animals killed, no marked pathologic changes were observed upon autopsy.

Subacute and chronic toxicity in mice

Mice were given the substance B 44 P orally in various doses for 10 successive days. In each group, 8 mice were used. As shown in Fig. 5, among the 8 given 250 mg/kg/day, 2 mice died at 5 and 6 days respectively after the onset. No deaths were observed at 125 mg/kg/day or below. In any case, even among the controls, mice lost body weight during the period of medication, but once this was ceased, they rapidly gained weight. In the 2 mice which died, no particular pathologic change was found upon autopsy.

Fig. 5. Subacute toxicity of the substance B 44 P in mice by oral administration

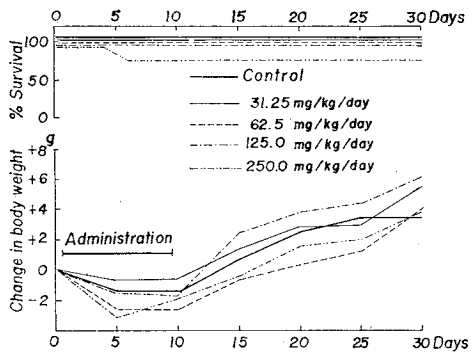


Fig. 6. Chronic toxicity of the substance B 44 P in mice by intraperitoneal administration

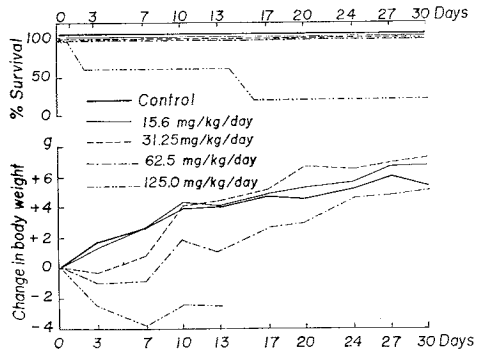


Fig. 6 illustrates the results of chronic toxicity studies on the substance B 44 P in mice which received the drug intraperitoneally for 30 consecutive days. Each group consisted of 10 mice. The mice given 125 mg/kg/day of the drug gradually lost weight and showed an unhealthy physical condition; 80 % of them died within 16 days. The livers and lungs of the dead were slightly atrophic with dull surfaces. Those mice which received 62.5 mg/kg/day of the drug showed a transitory losss in weight but soon after gained weight without death.

Toxicity in dogs

Two dogs were medicated with the substance B 44 P orally once a day for 30~33 days. One dog (Dog No. 1) received 200 mg/kg/day for 30 consecutive days, the other (Dog No. 2) received 500 mg/kg/day for 23 days followed by an interruption of 4 days and then continuation of the drug at 1,000 mg/kg/day for 7 days in succession. The data on clinical observations are tabulated in Tables 9 and 10. As seen in these, dog No. 1 showed a slight decrease in body weight, fluctuation in the percentage of lymphocytes, more or less high values in serum transaminase and transitory high values in serum urea-N, but his physical condition was good throughout the experimental period. Dog No. 2 displayed loss of appetite, vomiting, diarrhea, slight transitory ataxia and slight convulsions; his physical condition, in general, was not

Table 9. Clinical examination data for a dog during a course of long-term oral administration of the substance B 44 P (dog No. 1)

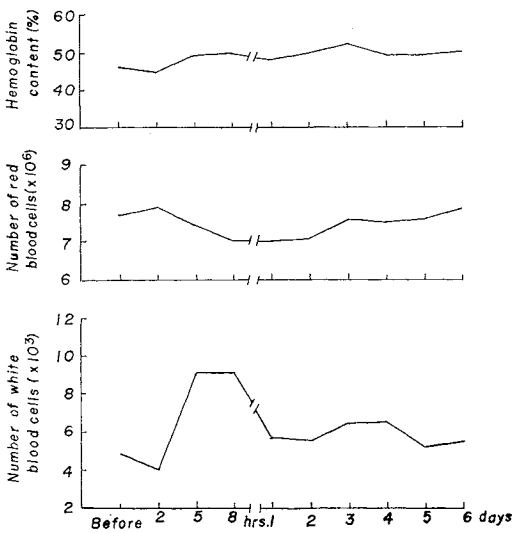
Number of the times		0	1～6	7～13	14～19	20～25	26～33	Two weeks after the termination
Dosis in one time (mg/kg)		0	200	200	200	200	200	
Dosis in total (g/kg)		0	1.2	2.6	3.8	5.0	6.6	
Number of red blood cells ($\times 10^6$/cmm)		7.39	6.25	7.33	5.81	5.42	5.80	6.69
Number of white blood cells ($\times 10^3$/cmm)		15.9	11.7	10.9	9.9	10.4	8.9	9.0
Neutrophile (%)		72	71	70	84	67	70	41
Eosinophile (%)		3	2	7	2	2	3	2
Monocyte (%)		4	10	13	10	10	16	14
Lymphocyte (%)		21	17	11	4	21	11	43
Liver and kidney function	GPT (u)	39	63	65	53	46	52	63
	GOT (u)	28	43	35	45	42	50	40
	Urea N (mg/dl)	17.5	19.0	15.0	35.0	20.0	24.7	11.5
Na (meq/1,000 ml)		142	145	—	170	170	—	156
K (meq/1,000 ml)		4.8	5.7	—	6.5	5.5	—	5.0
Cl (meq/1,000 ml)		99	104	—	130	112	—	100
Body weight (kg)		7.0	6.3	6.5	6.1	6.3	6.3	7.5

Table 10. Clinical examination data for a dog during a course of long-term oral administration of the substance B 44 P (dog No. 2)

Number of the times		0	1～4	5～9	10～11	12～17	18～23	24～30	A week after the termination
Dosis in one time (mg/kg)		0	500	500	500	500	560	1,000	
Dosis in total (g/kg)		0	2	4.5	5.5	8.5	11.5	18.5	
Number of red blood cells ($\times 10^6$/cmm)		6.20	5.23	6.19		6.68	6.02	5.12	7.15
Number of white blood cells ($\times 10^3$/cmm)		9.2	12.0	8.2		5.0	5.0	9.0	9.3
Hemoglobin content (%)		55	58	48		42	50	53	51
Neutrophile (%)		74	74	76		41	71	74	70
Eosinophile (%)		2	0	0		0	0	1	1
Monocyte (%)		7	9	8		9	8	2	7
Lymphocyte (%)		17	17	16		49	21	18	23
Liver and kidney function	GPT (u)	29	43	23		22	12	8	6
	GOT (u)	30	61	21		28	20	13	24
	Urea N (mg/dl)	13.5	12.1	9.1		8.0	13.0	18.5	7.5
Na (meq/1,000 ml)		—	147	138		—	145	145	140
K (meq/1,000 ml)		—	4.6	4.3		—	4.3	4.5	4.1
Cl (meq/1,000 ml)		—	108	—		—	114	112	92
Body weight (kg)		9.5	9.5	9.0		8.7	9.0	9.0	9.3
Remarks		Transitory slight ataxia, little appetite, vomiting			Interruption for 4 days, cheerless, little appetite, diarrhea, vomiting	Slight convulsions, little appetite, diarrhea			

good. The serum transaminase showed transitory high values only at the beginning but the values in serum urea-N were in the normal range. Both the dogs were sacrificed for autopsy, 2 weeks for No. 1 and 1 week for No. 2, after the termination of the medication. The main pathologic change observed was hemorrhage in the intestines. In dog No. 1, slight hyperemia and hemorrhage were observed in the mucous membranes of the duodenum and from the end of the ileum to the large intestines. In dog No. 2, many hemorrhage maculae were present in the mucous membranes through the whole intestinal tract, particularly intensive in the mucosa of the duodenum. In both animals, the surface of the livers looked dull. A few hemorrhage maculae were also observed in the left lobes of the livers in both animals and in the periphery of the spleen in dog No. 1. In other organs, that is, heart, lung, kidney, adrenal, pancreas, mesenterium, testis, and so on, no pathologic changes were detected.

Fig. 7. Hemoglobin content and number of red and white blood cells in the peripheral blood of a rabbit after an oral administration of 200 mg/kg of the substance B 44 P



Finding on the blood of a rabbit after oral administration of the substance B 44 P

Before and after oral administration of 200 mg/kg of the substance B 44 P, the hemoglobin content of the blood, the number of red and white blood cells and hemogram were determined for a rabbit. The results are shown in Table 11 and Fig. 7. A transitory leucocytosis was observed at 5 hours and 8 hours later, accompanying and related lymphopenia, but neither the number of the red cells nor the hemoglobin content was affected.

Table 11. Finding in the blood of a rabbit after an oral administration of 200 mg/kg of the substance B 44 P

	Hemoglobin content %	Number of red blood cells 10^6/cmm	Number of white blood cells 10^3/cmm	Neutrophile %	Eosinophile %	Monocyte %	Lymphocyte %
Before	45～48	7.50～7.92	3.7～5.1	35～43	1～2	5～9	50～70
2 hrs.	45	7.95	4.0	40	1	6	43
5	49	7.41	9.1	68	1	5	26.5
8	50	6.98	9.1	52	2	5	42
24	48	7.00	5.6	34.5	1	10	55
2 days	50	7.02	5.5	40	1	8	51
3	52	7.55	6.4	47	2	6	46
4	49	7.42	6.5	54	1	8	37
6	49	7.59	4.6	43	1	5	51
7	50	7.84	4.7	41	1	6	52

The concentration of the substance B 44 P in the blood and urine of rabbits

Two rabbits were given 200 mg/kg of the substance B 44 P orally, and then the blood and urine were taken at appropriate times for measurement of the drug concentration in them. The average concentrations in serum of the 2 rabbits are shown in Fig. 8. The drug appeared in the blood 30 minutes after administration and its concentration reached a maximum after 2.5 hours, then gradually decreased. After 9 hours, the drug was still detectable. Table 12 shows the amount of antibiotic excreted into the urine. The drug was found in the urine after only 30 minutes. The excretion of the drug reached a maximum after 3 hours, remained at a relatively high level for a few hours, and then went down very slowly. Even after 22~24 hours, the drug was still being excreted in small amounts.

Fig. 8. The concentration of the substance B 44 P in the blood of rabbits after oral administration of 200 mg/kg

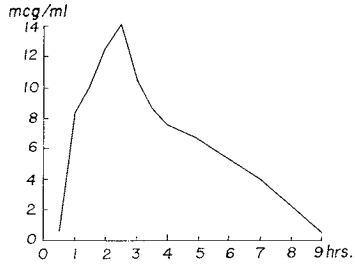


Table 12. Excretion of the substance B 44 P in rabbits after an oral administration of 200 mg/kg

Hours after the administration	Concentration in urine (mcg/ml)	Volume of urine (ml)	Amount excreted in urine (mcg)	pH of urine
10 min.	0	0.5	0	8.0
30	18	1.0	18	7.8
60	147	1.5	220	7.2
1.5 hrs.	340	1.0	340	7.0
2	850	2.0	1,700	6.8
2.5	1,440	2.5	3.600	6.4
3	1,600	3.0	4,800	6.0
3.5	1,030	3.5	3,600	6.0
4	850	4.0	3,400	5.6
5	750	4.0	3,000	5.2
7	534	6.0	3.200	5.6
9	560	5.0	2,800	6.4
22	19	50.0	950	7.6
24	2.5	10.0	25	7.6
Total (mcg)			27,653	
(%)			13.8	

Distribution of the substance B 44 P in organs after oral administration

One, three, five and seven hours after an oral administration of 400 mg/kg of the antibiotic, groups of 5 mice were sacrificed for the determination of drug distribution in the organs. The results are described in Table 13. The drug concentration in serum, urine or any organ was highest 1 hour after medication and then diminished at a rate depending on the nature of the organ. In serum, the 3-hour value was one-tenth of that of 1

Table 13. Distribution of the substance B 44 P in organs, serum and urine in mice after oral administration of 400 mg/kg

	Concentration mcg/g*			
	1 hr.	3 hrs.	5 hrs.	7 hrs.
Serum	13.5	1.3	0.95	0
Urine	460.0	260.0	170.0	12.0
Lung	38.0	12.0	4.0	4.5
Liver	42.0	30.0	25.0	14.0
Spleen	27.0	8.2	3.2	0.85
Kidney	20.0	8.0	5.0	5.8
Heart	38.0	11.4	7.0	4.2
Stomach	300.0	220.0	180.0	130.0
Testis	25.0	12.8	8.0	4.5
Skeletal muscle	17.0	7.5	4.2	—

* In the cases of the serum and urine, the concentration is expressed in mcg/ml.

hour, this relation was different from that in rabbits. The stomach contained relatively large amount of the antibiotic 1 hour after administration and maintained a comparatively high concentration. As one of the reasons for this high concentration, the adsorption of the drug on the mucosa of the stomach may be considered, although the organ was washed with phosphate buffer before homogenization. The concentration after 1 hour in liver, lungs and heart was higher than that in spleen, kidney, testis or skeletal muscle. The decrease in the liver with time was slow.

Discussion

An antibiotic pigment named substance B 44 P was isolated in the authors' laboratory and, later, was identified as streptovaricin[1]. Due to its low toxicity, the authors became interested in further detailed investigations.

The substance B 44 P was inhibitory mainly against Gram positive bacteria. Particularly, *Sar. lutea* and *Coryn. xerosis* were strongly inhibited by the antibiotic at a concentration of 0.1~0.2 mcg/ml. *Mycobact. tuberculosis* $H_{37}Rv$ and BCG were also inhibited at 1.56 mcg/ml. The substance B 44 P was active against clostridia and some Gram negative bacteria but the activity against the latter was not as strong except against *Sh. sonnei* and *Cl. welchii*. The substance was ineffective against fungi.

Staph. aureus SMITH was sensitive to the substance B 44 P. Organisms brought into contact with this antibiotic at 0.625 mcg/ml from the beginning or at 5~10 mcg/ml during the logarithmic growth phase were depressed in growth for 18~21 hours but thereafter started growing again. Thus, the effect of this antibiotic at these concentrations was thought to be bacteriostatic. The facts, however, that the living cells were diminished in number by the addition of this antibiotic and that the maximum growth in the culture with the drug was significantly depressed compared with that in the control culture suggested that the substance B 44 P might have some partial bactericidal action or might produce irreversible damage in the bacterial cells, even though it was not lethal.

S. enteritidis and *Sh. boydii* in which no natural resistant mutants were detected acquired resistance very rapidly *in vitro*, whereas *Staph. aureus* in which the frequency of natural mutant resistant to 25 mcg/ml of the substance B 44 P was $1.0 \sim 4.6 \times 10^{-6}$ gained resistance slowly *in vitro*. It has not been verified whether the bacteria resistant to the substance B 44 P have an R-factor or not. *Staph. aureus* SMITH resistant to the substance B 44 P did not exhibit any cross resistance against penicillin, dihydrostreptomycin, kanamycin, tetracycline, erythromycin, oleandomycin, novobiocin, spiramycin or capreomycin.

The toxicity of the substance B 44 P was relatively low. Uni- and multi-administration through various routes in mice demonstrated this. Rats seemed to be a little more sensitive than mice. A dog given the substance at 200 mg/kg/day orally for 33 consecutive days showed no striking toxic signs, but another dog administered orally with 500 mg/kg/day for 30 days developed toxic symptoms. Hepato- or nephrotoxicity might be disregarded, if present. Inasmuch the findings in hemoglobin content and blood cells in circulating blood were in the normal range except for a transitory leucocytosis after administration, the substance B 44 P was not considered to cause any severe damage to the bone marrow.

The substance B 44 P was readily absorbed from the digestive canals and rapidly transferred into the blood. The antibiotic administered to rabbits orally appeared in circulating blood after 30 minutes, showed a maximum concentration after 2.5 hours and then decreased gradually. The antibiotic was still detectable in the blood even 9 hours after administration. On the contrary, the antibiotic, when administered orally to mice, gave a maximum concentration in the blood much earlier and disappeared more quickly than in rabbits. The absorbed substance B 44 P was excreted into the urine, its first appearance there

occurring within 30 minutes in rabbits. The excretion in rabbits reached a maximum after 3 hours and continued, decreasing slowly, for 24 hours. The excretion in mice showed a steeper pattern.

In conclusion, the substance B 44 P had a strong antibacterial activity *in vitro*, mainly against Gram positive bacteria including *Mycobact. tuberculosis* and relatively low toxicity in mice, rats, rabbits, and dogs. Its chemotherapeutic effects should be as expected.

Acknowledgement

The author wishes to express his deep thanks to Dr. H. UMEZAWA and Dr. K. NITTA for guidances of this study, to Toyo Jozo Co. for the preparation of the substance B 44 P. The author also is indebted to Dr. S. KAMEYAMA and Dr. H. TAKAHASHI for their kind supply of clostridia and mycobacteria.

References

1) YAMAZAKI, H.: Studies on antimicrobial substance B 44 P (streptovaricin) produced by a strain of actinomycetes. I. Production, extraction and characteristics of substance B 44 P and the identity of the substance with streptovaricin. J. Antibiotics, 21 : 204~208, 1968.
2) SIMINOFF, P.; R. M. SMITH, W. T. SOKOLSKI & G. M. SAVAGE: Streptovaricin. I. Discovery and biologic activity. Am. Rev. Tuberc. Pulm. Dis. 75 : 576~583, 1957.
3) WHITFIELD, G. B., Jr.; E. C. OLSON, R. R. HERR, J. A. FOX, M. E. BERGY & G. A. BOYACK: Streptovaricin. II. Isolation and properties. Am. Rev. Tuberc. Pulm. Dis. 75 : 584~587, 1957.
4) DIETZ, A.; C. DE BOER, R. M. SMITH, P. SIMINOFF, G. A. BOYACK & G. B. WHITFIELD, Jr.: Antibiotic streptovaricin and process for its production. U. S. Patent 3,116,202 Dec. 31, 1963.
5) RHULAND, L. E.; K. F. STERN & H. R. REAMES: Streptovaricin. III. *In vivo* studies in the tuberculous mouse. Am. Rev. Tuberc. Pulm. Dis. 75 : 588~593, 1957.
6) STERN, K. F.; J. E. GRAY & L. E. RHULAND: Studies with streptovaricin in the tuberculous guinea pig. Am. Rev. Tuberc. Pulm. Dis. 77 : 976~982, 1958.
7) MCCUNE, R. M.; K. DEUSCHLE, C. JORDAHL, R. D. PREZ, C. MUSCHENHEIM & W. MCDERMOTT: The influence of streptovaricin used alone and with isoniazid in an experimental tuberculous infection in animals, and some clinical observations. Am. Rev. Tuberc. Pulm. Dis. 75 : 659~666, 1957.
8) CHANG, Y. T.: Effects of kanamycin, streptovaricin, paromomycin, novobiocin, and ristocetin on murine leprosy. Am. Rev. Tuberc. Pulm. Dis. 79 : 673~676, 1959.
9) NEWCOMBE, H. B.: Origin of bacteria variants. Nature 164 : 150~151, 1949.