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113. Kiichiro Kakemi, Hitoshi Sezaki, and Shikifumi Kitazawa: Studies on the Pharmaceutical Potentiation of Drugs. III.*1 Antagonistic Effects of PABA on Antitubercular Activities of PAS Derivatives.

(*Faculty of Pharmaceutical Sciences, Kyoto University**2)

Antagonistic effects of PABA on the tuberculostatic activities of synthesized ω-substituted alkyl esters of PAS were examined. It was found that these derivatives were competitively antagonized and their inhibition indices decreased to 0.1 or 0.01 from one of the parent compound, and the correlation between partition coefficients and inhibition indices of these derivatives seems to exist.

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That the antitubercular activity of *p*-aminosalicylic acid (PAS) is inhibited at the locus of action by the presence of structurally related *p*-aminobenzoic acid (PABA) has attracted attentions since the earliest days of the chemotherapy.[1] Further investigations made it clear that antagonistic effect of the latter on the former was competitive[2] and the inhibition index[3] was equal to unity.[4,5] In view of these facts the mechanism of tuberculostatic activity of PAS has been speculated as the inhibition of the formation of folic acid in *Myc. tuberculosis*.[6]

Apart from the mechanism, these antagonistic phenomena are regarded as one of the reasons why large dosage is necessary for clinical use of this drug in spite of its considerable activity *in vitro*.

A number of ω-substituted alkyl esters of PAS have been synthesized in the previous papers,[7,8] and demonstrated that, although their physico-chemical characteristics and intrinsic activities were notably affected by both of the chemical constitution on the end of the alkyl chain and the length of alkyl chain, sufficient amount of these derivatives could easily be transported to the locus of action.

The present investigation was undertaken to clarify the antagonistic effects of PABA on tuberculostatic activities of these synthesized derivatives by determining their inhibition indices using *Myc. tuberculosis* BCG strains, and to discuss the relationship between these inhibition indices and their physico-chemical properties of the derivatives.

Experimental

BCG Strain——The BCG strain of *Myc. tuberculosis* which was utilized in this study had been obtained from Takeda Chemical Industries, Ltd. and subsequently maintained on Ogawa's medium through serial transfer at intervals of three weeks. For use in the experiments, the inoculum were made by the treatment described by Goodacre.[9]

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*1 Part II. This Bulletin, in press.

*2 Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto (掛見喜一郎, 瀬崎 仁, 北沢式文).

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Kirchner Medium——Kirchner medium containing 10% of horse serum was used in this study. The component of the medium was as follows : Sodium phosphate, dibasic, 3 g., potassium phosphate, monobasic, 4 g., aspartic acid, 5 g., sodium citrate, 2.5 g., magnesium sulfate, 0.6 g., glycerol, 20 g., horse serum, 100 ml. and water q.s. to 1000 ml.

Compounds Examined——Compounds which were used in the investigation were synthesized derivatives of PAS, reported in the previous papers. With the intention of investigating the influences of both alkyl chain length and chemical constitution of ω-substituent on the antagonistic effect, compounds that have ethyl and decyl and appropriate length which coincide with the peak tuberculostatic activity of such ω-substituted series as alkyl, hydroxy, chloro, amino, alkylene bis, diethylamino, diphenylamino and phenylethyl amino were used in the study.

Experimental Procedures——To four ml. of Kirchner medium containing 10% of horse serum in each culture tube, were added 0.5 ml. of the solution which had the appropriate concentrations of PABA to the final concentrations of $10^{-3}\sim10^{-10}$ M/L. and 0.5 ml. of the compounds to final concentrations of 100 times, 10 times, MIC itself and 0.1 times of their minimum inhibitory concentration (MIC), and then sterilized. Each tube was inoculated with 0.1 ml. of inoculum which contains 1 mg. of organism per 1 ml. of Kirchner medium. After incubation at 37° for fourteen days the growth of the organism was observed macroscopically from the outside of the culture tubes. Inhibition index of the PAS ester and PABA system was determined for varied concentrations of the compounds following the methods described by Shive and Macow.[3]

Results

Antagonistic effects of PABA on the bacteriostatic activity of PAS on BCG strain of *Myc. tuberculosis* is shown in Table I. This is almost similar result as Hedgecock and Nishimura obtained respectively, using *Myc. tuberculosis* H37Rv strain in Kirchner medium. The inhibition index is $\frac{\text{PAS}}{\text{PABA}}=\frac{10^{-6}}{10^{-6}}=\frac{10^{-5}}{10^{-5}}=\frac{10^{-4}}{10^{-4}}=1$. From the facts that the inhibition index is constantly 1 over a wide range of the concentration and the boundary of + and − is linear in Table I, the type of the antagonism between PAS and PABA is regarded as competitive.

Table I. Antagonistic Effect of PABA on the Tuberculostatic Activity of PAS in Kirchner Medium

Conc. of PABA (M/L.) / Conc. of PAS (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-4}	+	+	−	−	−	−	−	−	−
10^{-5}	+	+	+	−	−	−	−	−	−
10^{-6}	+	+	+	+	−	−	−	−	−
10^{-7}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

Antagonistic effects of PABA on the activity of synthesized ethyl ester of PAS which has MIC of $10^{-5}M$/L. was investigated as an example of the derivatives with short alkyl chain. Bacteriostatic activity of this derivative at the concentration of $10^{-5}M$/L. was inhibited in the presence of $10^{-4}M$/L. concentration of PABA, so that the inhibition index was calculated as $\frac{\text{ethyl PAS}}{\text{PABA}}=\frac{10^{-5}}{10^{-4}}=0.1$, and the boundary of + and − is linear in Table II, the type of the antagonism between ethyl PAS and PABA is regarded as competitive. While in the case of decyl ester of PAS which has MIC of $10^{-6}M$/L. an example of the derivatives with long alkyl chain, although the type of the antagonism was similar to that of short one, inhibition index was calculated as $\frac{\text{decyl PAS}}{\text{PABA}}=\frac{10^{-6}}{10^{-4}}=\frac{10^{-5}}{10^{-3}}=0.01$. In the case of nonyl ester of PAS

9) C. L. Goodacre : Quart. J. Pharm. Pharmacol., **21**, 301 (1948).

which has the maximum tuberculostatic activity of MIC, $10^{-7}M$/L., in this alkyl series, the inhibition index at the concentration of $10^{-7}M$/L. was 0.01 and at $10^{-6}M$/L. was also the same, the type of the antagonism between nonyl PAS and PABA was also regarded as competitive, as shown in Table Ⅳ.

Table Ⅱ. Antagonistic Effect of PABA on the Tuberculostatic Activity of Ethyl PAS in Kirchner Medium

Conc. of PABA (M/L.) / Conc. of Deriv. (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-3}	−	−	−	−	−	−	−	−	−
10^{-4}	+	−	−	−	−	−	−	−	−
10^{-5}	+	+	−	−	−	−	−	−	−
10^{-6}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

Table Ⅲ. Antagonistic Effect of PABA on the Tuberculostatic Activity of Decyl PAS in Kirchner Medium

Conc. of PABA (M/L.) / Conc. of Deriv. (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-4}	−	−	−	−	−	−	−	−	−
10^{-5}	+	−	−	−	−	−	−	−	−
10^{-6}	+	+	−	−	−	−	−	−	−
10^{-7}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

Table Ⅳ. Antagonistic Effect of PABA on the Tuberculostatic Activity of Nonyl PAS in Kirchner Medium

Conc. of PABA (M/L.) / Conc. of Deriv. (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-4}	−	−	−	−	−	−	−	−	−
10^{-5}	+	−	−	−	−	−	−	−	−
10^{-6}	+	+	−	−	−	−	−	−	−
10^{-7}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

Schematic presentation of antagonistic effects of PABA on the bacteriostatic activity of ω-diethylaminoethyl p-aminosalicylate which has MIC of $10^{-6}M$/L. is illustrated in Table Ⅴ as an example of derivatives which have substituents on the end of the short alkyl chain. Since bacteriostatic activity of this compound at the concentration of $10^{-6}M$/L. was inhibited in the presence of $10^{-5}M$/L. concentraion of PABA, the inhibition index was calculated as $\frac{\text{PAS ester}}{\text{PABA}}=\frac{10^{-6}}{10^{-5}}=0.1$. It is apparent from the table that the type of this antagonism is also competitive.

Bacteriostatic activity of ω-diethylaminodecyl p-aminosalicylate at the concentrations of $10^{-7}M$/L., $10^{-6}M$/L. and $10^{-5}M$/L. were inhibited in the presence of $10^{-5}M$/L., $10^{-4}M$/L. and $10^{-3}M$/L. concentrations of PABA respectively. From the results obtained here,

Table V. Antagonistic Effect of PABA on the Tuberculostatic Activity of ω-Diethylaminoethyl PAS in Kirchner Medium

Conc. of Deriv. (M/L.) \ Conc. of PABA (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-4}	−	−	−	−	−	−	−	−	−
10^{-5}	+	+	−	−	−	−	−	−	−
10^{-6}	+	+	+	−	−	−	−	−	−
10^{-7}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

the inhibition index was calculated as $\frac{\text{PAS ester}}{\text{PABA}}=\frac{10^{-7}}{10^{-5}}=\frac{10^{-6}}{10^{-4}}=\frac{10^{-5}}{10^{-3}}=0.01$, and the type of the antagonism between this derivative which has substituent on the end of the alkyl chain and PABA seemed to be competitive. Schematic representation of this antagonism is illustrated in Table Ⅵ.

Table Ⅵ. Antagonistic Effect of PABA on the Tuberculostatic Activity of ω-Diethylaminodecyl PAS in Kirchner Medium

Conc. of Deriv. (M/L.) \ Conc. of PABA (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-4}	−	−	−	−	−	−	−	−	−
10^{-5}	+	−	−	−	−	−	−	−	−
10^{-6}	+	+	−	−	−	−	−	−	−
10^{-7}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

As shown in Table Ⅶ, bacteriostatic activity of ω-diethylaminoheptyl *p*-aminosalicylate at the concentrations of $10^{-6}M$/L. and $10^{-5}M$/L. were inhibited in the presence of $10^{-4}M$/L. and $10^{-3}M$/L., concentrations of PABA correspondingly. These results revealed that the inhibition index was $\frac{\text{PAS ester}}{\text{PABA}}=\frac{10^{-6}}{10^{-4}}=\frac{10^{-5}}{10^{-3}}=0.01$ and competitive antagonism was taken place between PABA and this derivative which have highest bacteriostatic activity of this series of the derivatives.

Table Ⅶ. Antagonistic Effect of PABA on the Tuberculostatic Activity of ω-Diethylaminoheptyl PAS in Kirchner Medium

Conc. of Deriv. (M/L.) \ Conc. of PABA (M/L.)	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	None
10^{-5}	+	−	−	−	−	−	−	−	−
10^{-6}	+	+	−	−	−	−	−	−	−
10^{-7}	+	+	+	−	−	−	−	−	−
10^{-8}	+	+	+	+	+	+	+	+	+
None	+	+	+	+	+	+	+	+	+

To investigate the influences of the ω-substituents and the length of the alkyl chain on the antagonistic effect of PABA, other synthesized derivatives with different ω-substituents and different length were surveyed. The inhibition indices of these

substances are summarised in Table Ⅷ. Type of their antagonism was all similarly competitive.

Table Ⅷ. Inhibition Indices of the Derivatives of PAS

ω-Substituent	C-chain	Inhibition index	ω-Substituent	C-chain	Inhibition index
Alkyl	ethyl	0.1	Alkylene bis	ethyl	0.01
	nonyl	0.01		heptyl	0.01
	decyl	0.01		decyl	0.01
Hydroxyl	ethyl	0.1	Diethyl amino	ethyl	0.1
	heptyl	0.01		heptyl	0.01
	decyl	0.01		decyl	0.01
Chloro	ethyl	0.1	Diphenyl amino	ethyl	0.1
	hexyl	0.01		amyl	0.01
	decyl	0.01		decyl	0.01
Amino	ethyl	0.1	Phenylethyl amino	ethyl	0.1
	octyl	0.1		heptyl	0.01
	decyl	0.01		decyl	0.01

Discussion

There have been a lot of studies in this field related to PAS itself but little is known about the derivatives, except *o*-hydroxyphenyl *p*-aminosalicylate and a complex of hydroxyprocainepenicilline G. Hasegawa[10] and Aoyagi[11] reported that these compounds decreased the inhibition indices but did not make any reference to the type of these antagonisms.

Antagonistic effects of PABA on the tuberculostatic activities of ω-substituted alkyl esters of PAS are revealed in this paper. The tuberculostatic activities of these ω-substituted alkyl esters of PAS were more or less antagonized in the existence of PABA and, furthermore, the type of these antagonisms were all competitive as that of the parent compound. This suggests that the modes of action of these derivatives are smilar to that of the parent compound. In admitting the speculation proposed by Winkler,[6] Shive[12] and Brown,[13] the mode of action of these derivatives might be estimated as the specific inhibitions of synthesis of *p*-aminobenzoylglutamic acid in the formation of folic acid.

That all of the inhibition indices are decreased to 0.1 or 0.01, that is, one tenth or one hundredth mole of these derivatives are equivalent to one mole of PABA in these antagonisms while one mole of PAS is equivalent to the same mole of PABA, reveals that these derivatives are not so widely antagonized as the parent compound at the locus of action, and even with the small amount of these derivatives are effective in clinical usage. In combining the results obtained here with those in the previous papers, it may be concluded that as the results of these chemical modifications, potentiations of PAS have been achieved successively by the extensive improvement of the defects of the substance in increasing the adaptability of transportations or distributions in biophase and decreasing the antagonistic effects by PABA at the locus of action as well.

10) A. Hasegawa : Keio Igaku, **36**, 711 (1959).
11) A. Aoyagi : *Ibid.*, **34**, 483 (1957).
12) W. Shive : Ann. N. Y. Acad. Sci., **52**, 1212 (1950).
13) G. M. Brown : J. Biol. Chem., **236**, 2534 (1961); *Ibid.*, **237**, 536 (1962).

From the results illustrated in Table Ⅶ, no specific relationships can be found between chemical constitutions of the substituents on the end of the alkyl chain and inhibition indices of these derivatives, nor can be detected the influence of the differences of intrinsic antitubercular activities of derivatives on inhibiton indices.

The inhibition indices of substances in each homologous series of the derivatives, relatively the derivatives with short alkyl chain, are 0.1 and indices decrease by increasing of the length of alkyl chain, thus the derivatives with decyl of the alkyl chain have 0.01 of the inhibition indices. These evidences support the speculation that the inhibition indices are seemed to be affected by the length of the alkyl chain rather than the chemical constitution of the ω-substituents and the difference of intrinsic antitubercular activity of the derivatives examined.

Some exceptions arise in the cases of ω-aminooctyl p-aminosalicylate and ethylene bis(p-aminosalicylate). The indices of the former is 0.1, in spite of its long alkyl chain and the latter is 0.01, in spite of its short alkyl chain length. From the point of view of lipid solubility, the former is less lipid soluble and the latter is more soluble. Since these considerations made us to relate the inhibition indices of these derivatives to partition coefficients between heptane and phosphate buffered system, which had been obtained in the previous papers, arrangements of these derivatives in order of their partition coefficients were made. The results thus obtained are listed in Table Ⅸ. The partition coefficient of PABA was 0.085 and was closely similar to that of PAS, and the inhibition index of PAS was 1. The inhibition indices of the more lipid soluble derivatives whose partition coefficients were between 0.1 and 10 were changed to 0.1 and in the cases of further more lipid soluble derivatives whose coefficients were more than 10 were changed to 0.01.

Table Ⅸ. Relationship between Partition Coefficients and Inhibition Indices of the Derivatives of PAS

Compounds	Partition coeff.	I. index	Compounds	Partition coeff.	I. index
P.A.S.	0.09	1.0	Hydroxy decyl	17.3	0.01
Hydroxy ethyl	0.15	0.1	Amino decyl	17.5	0.01
Amino ethyl	0.62	0.1	Diphenylamino ethyl	17.6	0.01
Diethylamino ethyl	1.3	0.1	Phenylethylamino heptyl	20.8	0.01
Chloro ethyl	3.1	0.1	Diethylamino decyl	28.2	0.01
Amino octyl	5.6	0.1	Alkylene bis	31.1	0.01
Phenylethylamino ethyl	6.3	0.1	Diphenylamino amyl	36.2	0.01
Hydroxy heptyl	7.31	0.01	Phenylethylamino decyl	42.3	0.01
Alkyl ethyl	9.24	0.1	Chloro decyl	48.1	0.01
Diethylamino heptyl	11.9	0.01	Alkyl nonyl	56.9	0.01
Chloro hexyl	14.8	0.01	Diphenyl decyl	70.1	0.01
			Alkyl decyl	121	0.01
P.A.B.A.	0.085				

In view of the above facts, the most reasonable conclusion to be drawn is that there may be some close correlation between the inhibition indices and partition coefficients of these derivatives. The larger the latter, the less the former and the inhibition index is changed from one to 0.1 at about 0.1 of the partition coefficient and from 0.1 to 0.01 at about 10 of the partition coefficients.

In considerations of Brodie's hypothesis[14] that permeabilities of substances through biological membranes that have characteristics of lipoid are mostly depended upon

14) B.B. Brodie, *et al.*: J. Pharm. Exptl. Therap., **123**, 81 (1958).

their partition coefficients and Freedlander's mention[15] that *Myc. tuberculosis* have thick lipid cell walls, it may be considered that the difference of these inhibition indices among 1, 0.1 and 0.01 occurred from the difference of permeabilities, that are, from the difference of their partition coefficients of the derivatives, through the cell wall of *Myc. tuberculosis*.

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15) B. L. Freedlander : Am. Rev. Tuberc., **56**, 376 (1947).