PARA-AMINOSALICYLIC ACID—PART III

SOME FURTHER STUDIES ON THE *IN VITRO* TUBERCULOSTATIC BEHAVIOUR OF *PARA*-AMINOSALICYLIC ACID AND RELATED COMPOUNDS

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IN our previous report[1], some preliminary results showed that simple substitution in the *p*-aminosalicylic acid molecule did not produce any compound possessing an *in vitro* tuberculostatic activity markedly higher when using *Mycobacterium tuberculosis* H37RV strain. This paper is concerned with the *in vitro* study of a wider range of 1-2-4-tri-substituted aromatic compounds, the majority of which have not been previously described; their chemistry will be reported upon elsewhere. The culture medium used throughout the work and the technique for the determination of activity was similar to that described in our earlier paper[1]. It may be of interest to note that we have confirmed the observations made by Youmans *et al.*[2] that the presence of tween 80 (polyoxyethylene sorbitan mono-oleate) in the medium markedly influences the tuberculostatic behaviour of *p*-aminosalicylic acid and other compounds; this

TABLE I

THE INHIBITORY CONCENTRATION OF *p*-AMINOSALICYLIC ACID AND RELATED COMPOUNDS. INOCULUM OF 0·001 MG/ML. OF THE H37RV STRAIN

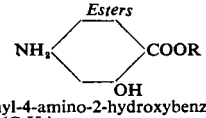
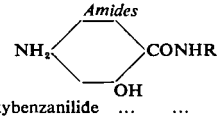
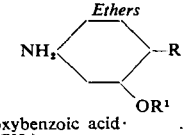
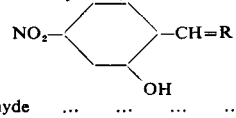
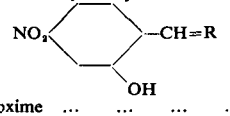
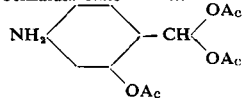
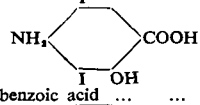
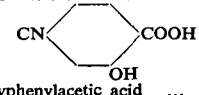
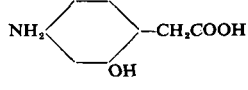
	Standards	*Inhibitory concentration mg. 100 ml. (after 14 days at 37° C.)*
GROUP A		
1	*p*-Aminosalicylic acid	0·0487—0·0243
2	Streptomycin	0·0121—0·006
GROUP B	*Esters* 	
3	β-Diethylaminoethyl-4-amino-2-hydroxybenzoate	0·1 — 0·01
	(R=-$CH_2.CH_2.N(C_2H_5)_2$	(0·195— 0·0975)
4	β-Hydroxyethyl-4-amino-2-hydroxybenzoate	100—10
	(R=-CH_2-CH_2-OH)	
GROUP C	*Amides* 	
5	4-Amino-2-hydroxybenzanilide	10—1
	(R=-C_6H_5)	
6	4-Amino-2-hydroxybenz-*p*-toluidide	10—1
	(R=-$C_6H_4.CH_3$)	
7	4-Amino-2-hydroxyhippuric acid...	100—10
	(R=-CH_2COOH)	
8	4-Amino-2-hydroxybenzoyl alanine	10—1
	(R=-CH(CH_3)-COOH)—	
9	4-Amino-2-hydroxybenzoyl-DL-aspartic acid	100—10
	(R=-CH(CH_2COOH)-COOH)	

TABLE I—*continued*

		Standards	*Inhibition concentration mg. per 100 ml. (after 14 days at 37°C.)*
GROUP C—cont.			
10		4-Amino-2-hydroxybenzoic acid hydrazide (R=-NH_2)	0·1—0·01 (0·0975—0·0487)
GROUP D		*Ethers* 	
11	*	4-Amino-2-methoxybenzoic acid· (R=-COOH.R′,-CH_3)	> 100
12		4-Amino-2-methoxytoluene hydrochloride (R=-CH_3,R′,-CH_3)	100—10
13		4-Amino-2-ethoxytoluene hydrochloride... (R=-CH_3,R′,-C_2H_5)	100—10
14		4-Amino-2-*n*-propoxytoluene hydrochloride (R=CH_3,R′,-CH_2-CH_2-CH_3)	100—10
15		4-Amino-2-*n*-butoxytoluene hydrochloride (R-CH_3,R′,-CH_2 $(CH_2)_2$ CH_3)	100—10
GROUP E		*4-Nitrosalicylaldehyde derivatives* 	
16		4-Nitrosalicylaldehyde (R=O)	1—0·1
17		4-Nitrosalicylaldoxime (R=N.OH)	10—1
18		4-Nitrosalicylaldehyde semicarbazone (R=N.NHCONH$_2$)	10—1
19		4-Nitrosalicylaldehyde thiosemicarbazone (R=N.NHCSNH$_2$)	1—0·1
GROUP F		*4-Aminosalicylaldehyde derivatives* 	
20		4-Aminosalicylaldoxime (R,N.OH)	10—1
21		4-Aminosalicylaldehyde thiosemicarbazone R=N.NHCS.NH_2)...	0·01 —0·001 (0·003 —0·0015)
22		4-Amino-2-acetoxybenzaldiacetate ... ... 	10—1
GROUP G		*Miscellaneous*	
23		3-5-diiodo-4-amino-2-hydroxybenzoic acid ... ... 	0·1—0·01 (0·0975—0·0487)
24		4-cyano-2-hydroxybenzoic acid ... ... 	100—10
25		4-Amino-2-hydroxyphenylacetic acid ... ... 	> 100

* Youmans, Raleigh, and Youmans' reported that this compound does not inhibit growth of 0·01mg/ml of *M. tuberculosis* H37RV at a concentration of 10 mg/100 ml.

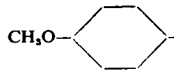
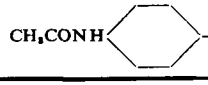
substance was, however, retained in our medium as its presence facilitates the turbidimetric standardisation of inoculum. The less soluble compounds were dissolved in propylene glycol, which was found to have no tuberculostatic effect when used in concentrations of 1 per cent. and below.

Table I shows the inhibitory concentrations of the compounds examined when using a standard inoculum of 0·001 mg./ml. (dry bacterial substance) of M. *tuberculosis* H37RV strain. The standards used were *p*-aminosalicyclic acid and streptomycin and compounds exhibiting activities of an order similar to the two standards were examined in closer dilutions.

The above results coupled with those obtained by others[3,4,5,6,7,8] suggest that there is no apparent relationship between chemical structure and *in vitro* tuberculostatic activity in this group of compounds, and from data obtained it would appear difficult to predict the effect of a simple variation in structure on *in vitro* activity. One can conclude that there is a high degree of specificity of the *p*-aminosalicylic acid molecule for tuberculostatic activity, and with the possible exception of the esters, alteration in molecular structure gives rise to markedly diminished activity. The anomalous results relating to the activity of the esters[1,4,5,6,7] may be due to their low solubility and tendency to hydrolyse under conditions of test. The effect of nuclear substitution with the exception of halogens has received no attention, and we propose to investigate this type of compound and report more fully.

We are of the opinion, however, that some of the compounds referred to in our previous paper, together with compounds No. 3,4,7 and 10, are worthy of a preliminary *in vivo* examination in view of the possibility that they may possess certain advantages over *p*-aminosalicylic acid by being less rapidly absorbed and excreted. The aldehyde derivatives (Groups E and F) have special interest for animal work in view of the reports by Domagk *et al.*[9] that certain benzaldehyde thiosemicarbazone derivatives have promising properties in the treatment of some forms of tuberculosis. Compound No. 21 (the thiosemicarbazone of *p*-aminosalicylaldehyde) is considered to possess sufficiently high *in vitro* activity to justify a trial in animals. For comparison purposes we report in Table II the *in vitro* activities of two of the compounds studied by

TABLE II

		Inhibition concentration mg./100 ml.
26	*p*-methoxybenzaldehyde thiosemicarbazone ... ... 	0·0487—0·0243
27	*p*-acetylaminobenzaldehyde thiosemicarbazone ... ... 	0·0975—0·0487

Domagk, namely, *p*-methoxybenzaldehyde thiosemicarbazone and *p*-acetylaminobenzaldehyde thiosemicarbazone.

The observation that the action of *p*-aminosalicylic acid is antagonised by *p*-aminobenzoic acid[1,7] has led us to study the possible effects of other members of the vitamin B group; at this stage it is possible to report that such antagonism is not displayed by pteroylglutamic acid. The significance of this cannot be realised until further results are obtained. It is interesting to note that the activity of sulphathiazole against *Staphylococcus aureus*[10] is similarly antagonised by *p*-aminobenzoic acid and not by folic acid.

SUMMARY

1. A series of tri-substituted aromatic compounds have been synthesised and their *in vitro* activities against the tubercle bacillus determined. The results of the study indicate no apparent relationship between structure and activity.

2. The thiosemicarbazone of *p*-aminosalicylaldehyde has been synthesised and its activity compared with the thiosemicarbazones of *p*-acetylaminobenzaldehyde and *p*-methoxybenzaldehyde.

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PARA-AMINOSALICYLIC ACID—PART IV

ATTEMPTS TO INDUCE RESISTANCE TO *PARA*-AMINOSALICYLIC ACID, IN STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

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THE unfortunate limitation of streptomycin in the treatment of tuberculosis, due to the development of drug resistance, suggested to us the necessity for an investigation as to whether similar phenomena might occur with *p*-aminosalicylic acid.

The investigation was concerned with attempts to induce drug-resistance *in vitro*, and with a study of strains of *M. tuberculosis* isolated from patients before and during *p*-aminosalicylic acid treatment. The H37RV strain was cultivated in Dubos medium containing decreasing amounts of the acid. After a suitable incubation period, the bacilli in the tubes containing the highest concentration of *p*-aminosalicylic acid which was still allowing growth were freed from it by washing, and used to inoculate a similar series of tubes. No increased resistance was demonstrated by this method, in fact, no growth at all occurred at the dilutions used after two or more passages. These results are confirmed by Hurni[1] who reported on a similar study while this work was in progress. In view of the unsatisfactory results obtained with the above method, the following procedure was adopted in an attempt to induce drug resistance. A large inoculum (0.5 mg./ml. of dry bacterial substance) of *M. tuberculosis* H37RV was introduced into Dubos medium containing *p*-aminosalicylic acid in a concentration of of 100 mg./ml. After 14 days' incubation, a similar concentration of *p*-aminosalicylic acid in Dubos medium was inoculated from this culture. Inhibition concentration tests were made at monthly intervals, and after 10 months (20 passages) the organisms showed a similar *p*-aminosalicylic acid sensitivity to that at the beginning of the experiment. A duplicate experiment was carried out using a medium containing no tween 80 with similar results.

These results suggest that under the above conditions the H37RV strain does not become resistant to the tuberculostatic action of *p*-aminosalicylic acid. In an attempt to obtain further and possibly more significant data we obtained cultures of *M. tuberculosis* isolated before and during treatment from patients suffering from pulmonary tuberculosis who received 20 g./day for 6/day week of sodium *p*-aminosalicylate given orally in divided doses. The strains, after cultivation in Dubos medium, were subjected to sensitivity tests by the method described in our previous paper[4] using a standard inoculum (0·001 mg./ml. of dry bacterial substance). The results of these experiments are shown in Table I and it will be seen that in only one instance (case 9) was there any indication of development of resistance to *p*-aminosalicylic acid. It is of interest to note, however, that there does exist a slight difference in sensitivity between different strains. These results together with those of

Lehmann[2], Hurni[1], and Seivers[3] suggest that " drug-fastness " is not a significant problem in the treatment of tuberculosis with this drug.

Whilst this paper was in course of preparation, Graessle and Pietrowski[5] reported that repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 120 days failed to produce an increase in the resistance of the strain.

TABLE I

Case No.	Before treatment	*p*-Aminosalicylic Acid treatment		
		2 months	3 months	4 months
1	0·0243—0·0121			0·0975—0·0487
2	0·0487—0·0243	0·0243—0·0121		
3	0·0243—0·0121			0·0487—0·0243
4	0·0243—0·0121			0·0487—0·0243
5	0·0243—0·0121			0·0487—0·0243
6	0·0243—0·0121			0·0243—0·0121
7	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
8	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
9	0·0243—0·0121			3·125 —1·56
10	0·0243—0·0121	0·0243—0·0121		0·0121—0·006
11	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
12	0·0487—0·0243	0·0243—0·0121		0·0487—0·0243
13	0·39 —0·195	0·39 —0·195		0·0975—0·0487
14	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
15	0·0243—0·0121	0·0243—0·0121		0·0243—0·0121
16	0·0243—0·0121			0·0243—0·0121
17	0·0487—0·0243		0·0487—0·0243	
18	0·0487—0·0243		0·0487—0·0243	
19	0·0243—0·0121	0·0243—0·0121		
20	0·0243—0·0121	0·0243—0·0121		
21	0·0487—0·0243			0·0487—0·0243
22	0·0243—0·0121			0·0243—0·0121
23	0·0243—0·0121	0·0243—0·0121		
24	0·0243—0·0121	0·0243—0·0121		
25	0·0487—0·0243		0·0487—0·0243	

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SUMMARY

1. After repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 10 months, no increase in resistance developed.

2. Out of a total of 25 strains of *M. tuberculosis* isolated from patients receiving *p*-aminosalicylic acid, only one developed any increase in resistance after four months' treatment.

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