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Chemotherapy of Tuberculosis III. *In Vitro* and *in Vivo* Activities of Various Compounds.

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Continuing our studies of antituberculosis agents we have examined a heterogeneous group of compounds *in vitro,* and in addition, the sodium formaldehyde bisulfite addition product of 2-butoxy-5-aminopyridine has been evaluated *in vivo.* The results of these studies are set forth in the experimental part.

Experiments in vitro. The compounds, listed in Table I, were dissolved in Dubos' medium,[1] using the two-fold serial dilution method. The starting concentration was 64 mg%, or if the substance was not soluble to that extent, a saturated solution was used. Controls were included in every series, and each experiment was run in duplicate. The organisms were the pathogenic tubercle bacilli, H37RV and B1. The standard inoculum was 0.1 ml of a 14 day culture in Dubos' medium which had been diluted to an optical density of 0.2 as measured in the Lumetron colorimeter. Two weeks after inoculation tubes were examined for growth, and when readings were doubtful Ziehl-Neelsen stained smears were examined.

It is apparent from Table I, that none of the chemicals listed, with the exception of the p-aminosalicylic acid preparations, possess any great activity *in vitro* against tubercle bacilli. The differences in bacteriostatic activity between the different samples of p-aminosalicylic acid undoubtedly may be ascribed to differences in the purity of the samples. It should be noted that the findings with Compound 13 are completely different from those previously reported.[2] The differences in activities of Compound 13, and p-aminosalicylic acid from those previously reported[2,3,4] are almost certainly due to important differences in technique. Compounds numbered 6, 7, 8, and 14 are being further studied. Compound 19, a commercial product sold under the name "Mandelamine," has important antibacterial properties[5,6] apart from its antimycobacterial activity. Its effect on experimental tuberculosis is now under study.

Experiments in vivo. Mice of the highly susceptible[7] dba strain, average weight 20 g, were injected intravenously with 0.1 ml of a suspension containing 0.1 mg of *My. tuberculosis,* strain B1. One week after infection, the animals were divided into 3 groups. One group was given Compound 13 mixed into the ground diet in 2% concentration. A small group of mice was treated similarly, for comparative purposes, with p-aminosalicylic acid, the therapeutic effect of which is well known.[3,4,8] The third group of mice remained untreated. The food cups were weighed daily and the amount of drug which each mouse ingested was estimated. The mice were weighed twice weekly. Surviving animals were sacrificed 32 days after infection. At autopsy the disease present in the lungs, liver, spleen, and kidneys was graded according to its extent and character. The scores for all organs were averaged and the average score, graded from 0 to 4+, was calculated for each group. A 4+ rating indicates caseous progressive tuberculosis of more than 50% of each organ.

The results of these experiments are given in Table II. While p-aminosalicylic acid

1 Dubos, R. J., and Davis, B. D., *J. Exp. Med.,* 1946, **83**, 409.

2 Feinstone, W. H., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 153.

3 Lehmann, J., *Lancet,* 1946, **1**, 15.

4 Youmans, G. P., *Northwestern Univ. Bull. M. School,* 1946, **20**, 420.

5 Duca, C. J., and Scudi, J. V., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 123.

6 Scudi, J. V., and Duca, C. J., *J. Urol.,* in press.

7 Pierce, C., Dubos, R. J., and Middlebrook, G., *J. Exp. Med.,* 1947, **86**, 159.

8 Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Proc. Staff Meet. Mayo Clin.,* 1947, **22**, 473.

TABLE I.

Lowest Bacteriostatic Concentrations mg %* of the Various Compounds Which Inhibit the Growth of Strains of Pathogenic Mycobacteria. Dubos' medium, inoculum 0.1 ml, incubation time, 14 days.

Number	Compound	H37RV	B1
1	*p*-aminosalicylic acid HCl	0.125	0.125
2	" " " Lehmann†	1.0	1.0
3	" " " Youmans‡	1.0	1.0
4	*p*-phenetidin HCl	16.	32.
5	acetophenetidin	>32.	>32.
6	*p*-acetamidobutoxybenzene	8.	16.
7	diethylaminoethoxy-2,4,5-trichlorobenzene HCl	8.	—
8	" -2-naphthalene HCl	8.	16.
9	3-hydroxy-4-methylpyridine	>64.	>64.
10	" " -N-oxide	>64.	>64.
11	" -4-methyl,1-2',3'-dihydroxypropyl-pyridinium chloride	>64.	>64.
12	2-methoxy-5-aminopyridine dihydrochloride	32.	>64.
13	2-butoxy-5- " sodium formaldehyde bisulfite	>64.	>64.
14	2-butoxy-5-acetamidopyridine	8.	32.
15	2',6'-diamino-2-butoxy-5,5'-azopyridine	>4.	> 4.
16	2-amino-6-hydroxypyridyl-3,4'-azo-1'-hydroxybenzene	>4.	> 4.
17	2-amino-6-hydroxypyridyl-3,4'-azo-1'-hydroxybenzene	>4.	> 4.
18	p,p'-di(4-hydroxy-3-carboxy-phenylazo) diphenyl sulfone tetrasodium salt	—	32.
19	methenamine mandelate	16.	16.

* Numbers preceded by the symbol > indicate the concentrations of saturated solutions.

† Obtained through the kindness of Professor Jorgen Lehmann, Sahlgrenska Sjukhuset, Gothenburg, Sweden.

‡ Obtained through the kindness of Dr. Guy P. Youmans, Northwestern University Medical School, Chicago, Ill.

TABLE II.

Results of Treating Tuberculous dba Mice with *p*-Aminosalicylic Acid (No. 1), and with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13).

Compound No.	Drug conc. in diet	No. mice	Avg amt drug ingested g/kg/day	No. survivors	Amt tuberculosis
0	—	15	—	15	2+
13	2%	15	4.5	4	3+
1	2%	9	3.9	9	1+

exerts a favorable influence on tuberculous dba mice, Compound 13 appears to exert deleterious effects. Because the amount of Compound 13 ingested by the mice is more than twice its acute LD_{50},[9] it seems probable that the poor results may be in part attributed to toxic effects of the drug.

Guinea pigs were employed in the second *in vivo* test of Compound 13. Eighty-five tuberculin negative guinea pigs, average weight 300 g, were infected subcutaneously in the groin with 0.5 mg (moist weight) B1 per 500 g body weight. Eight days later, when all animals reacted to 1 mg O. T. (Mantoux), they were divided into 5 groups and treated as follows:

Group 1. 20 guinea pigs received Compound 13 in their food in 2% concentration.

Group 1A. 15 guinea pigs, receiving the same treatment as Group 1, were used for blood studies.

Group 2. 20 guinea pigs received Compound 13 subcutaneously, 0.17 g per kg 3 times daily at 9:00 A. M., 5:00 P. M., and 12:00 M.

Group 2A. 15 guinea pigs, receiving the same treatment as Group 2, were used for blood studies.

Group 3. 15 guinea pigs were used as

9 Feinstone, W. H., Friedman, H. L., Rothlauf, M. V., Kelly, A. M., and Williams, R. D., *J. Pharm. and Exp. Therap.*, 1947, **89**, 153.

untreated controls.

All animals were weighed and tuberculin tests (Mantoux) with 1 mg O. T. were performed at weekly intervals. Blood, obtained by cardiac puncture from the animals in the subgroups, was used for the estimation of hemoglobin and Compound 13. Hemoglobin was measured in the Lumetron colorimeter and Compound 13 was measured according to the Marshall method.[10] Blood for the latter test was drawn in the morning from the animals of Group 1A, and at specified intervals from animals of Group 2A. Each animal was autopsied as soon as possible after death. Surviving animals were sacrificed 90 days after infection. Upon autopsy the amount of tuberculosis involvement in the lungs, liver, spleen, and the lymphatic system was expressed in values proportional to the extent and severity of the disease. As in the preceding experiment, a rating of 4+ indicates that the organ exhibited widespread caseous lesions. The average ratings for the various organs represent a summary of the tuberculosis present in the animal. Representative blocks of tissue were sectioned and examined microscopically after staining with hematoxylin-eosin and with Ziehl-Neelsen stains.

Upon treating the tuberculous guinea pigs with the drug it was found that subcutaneous administration of the drug caused the appearance of broad shallow ulcers at the site of injection. These developed 2 to 3 days after treatment was started, were about 1 cm in diameter, and sometimes penetrated to the underlying muscle. Six or 7 such ulcers were present in most of the injected animals 2 weeks after beginning treatment. At autopsy, the muscle underlying these ulcers was found to be heavily congested over an area 3 to 4 times the size of the ulcer. At no time did any of the ulcers become visibly infected. After about a week the ulcers healed completely, leaving a scar covering the site of injection.

All the guinea pigs reacted to 0.1 mg O. T. (Mantoux) throughout the experiment. There were no significant differences between the weight curves of the different groups of animals. Comparison of the hemoglobin 20 and 70 days after treatment was begun disclosed a 5 to 10% decrease. This decrease in hemoglobin is usually found in tuberculous animals after the disease has become widespread and progressive. No differences between the guinea pigs treated subcutaneously or orally were noted. Average concentrations of Compound 13 present in the blood are summarized in Table III. In terms of the previously reported *in vitro* activity[2] adequate concentrations of the drug were present at all times in the circulating blood, but as will be seen, these levels are completely ineffective in the treatment of experimental tuberculosis

The amount of tuberculosis present at autopsy, and the percent of the animals which survived the full 90 days of the experiment, are listed in Table IV. Inspection of this table discloses no significant differences between the different groups either in the degree of tuberculosis or in the number of animals surviving. The same results were observed on histological examination of representative sections.

Discussion. The description by Dubos and Davis[1] of a liquid, synthetic medium in which pathogenic mycobacteria grow rapidly has provided investigators in the field with a most useful tool. In our hands, it has provided consistent and reproducible results. We, therefore, agree with the observation[11] that, "The use of Tween 80 medium has greatly facilitated the study of the effects of antibacterial agents upon mycobacteria." It was noted above that the findings with 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite are completely different from those previously reported,[2] and it was pointed out that the difference was due to important differences in technic. The results of the *in vivo* studies with this substance indicate that the drug is ineffective even in doses which produce serious toxic effects as evidenced by early death and severe local ulceration. These findings serve to emphasize the fact that the type of organism and the medium used will

[10] Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

[11] Middlebrook, G., and Yegian, D., *Am. Rev. Tub.*, 1946, **54**, 553.

TABLE III.
2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (No. 13) Blood Concentrations in Guinea Pigs.*

Group	No.	Blood taken	Compound No. 13 mg % avg	Range mg %
1A	13	9 A.M.	.65	.33-1.13
2A	12	1 hr after injection	3.2	1.4 -5.5
2A	9	3 " " "	1.0	.6 -3.2
2A	11	5 " " "	.66	.44-1.1
2A	9	8 " " "	.23	.14-3.1

* Group 1A received Compound No. 13 in 2% concentration in the diet, and ingested it *ad libitum*.

Group 2A animals received the Compound subcutaneously, 0.17 g per kg 3 times daily at 9:00 A.M., 5:00 P.M., and 12:00 M.

TABLE IV.
Average Amount of Tuberculosis in Guinea Pigs Treated with 2-Butoxy-5-aminopyridine Sodium Formaldehyde Bisulfite (Compound No. 13).*

Group	No. of guinea pigs	Lungs	Liver	Spleen	Lymph glands	Summary	% survivors
1	19	1.1	1.7	1.9	2.5	1.7	50
1A	14	1.7	1.7	2.1	2.6	2.3	34
2	18	1.8	1.5	1.9	2.9	1.9	30
2A	13	1.1	1.4	2.1	2.1	1.6	30
3	13	1.2	1.2	1.9	1.9	1.9	29

* Groups 1-1A received Compound No. 13 in 2% concentration in the diet.

Groups 2-2A received Compound No. 13 subcutaneously, 0.17 g/kg 3 times daily at 9 A.M., 5:00 P.M., and 12:00 P.M.

Group 3—controls.

The sub-groups (A) were bled by cardiac puncture for the blood studies listed in Table III.

profoundly influence the results. When 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite was tested *in vitro* using non-virulent mycobacteria growing in Proskauer-Beck medium,[2] the drug appeared to have a high order of activity. When tested against virulent tubercle bacilli growing in Dubos' medium the activity appeared greatly lessened. Since the compound is ineffective in the treatment of tuberculosis *in vivo*, it would appear that in this instance the latter method of testing gave a better indication of its *in vivo* activity.

The finding that Mandelamine (methenamine mandelate) possesses activity against virulent mycobacteria *in vitro* is of interest because this drug is known to be effective in the treatment of urinary tract infections caused by non-acid-fast bacteria.[12] Its effect upon urinary tract tuberculosis is under investigation.

Conclusions. 1. The synthetic medium described by Dubos and Davis provides an efficient method of studying antimycobacterial agents. 2. Of 19 compounds tested *in vitro*, 4 showed sufficient activity to warrant further investigation. 3. 2-butoxy-5-aminopyridine sodium formaldehyde bisulfite is ineffective in the treatment of experimental tuberculosis in mice and guinea pigs.

12 Carroll, G., and Allen, H. N., *J. Urol.*, 1946, **55**, 674.