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Tuberculostatic Action of Chloromycetin *In vitro* and *In vivo*.*

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Ehrlich, Bartz, Smith, Joslyn and Burkholder[1] reported the isolation of a *Streptomyces sp.* which produced a new antibiotic which they named Chloromycetin. This antibiotic was highly bacteriostatic *in vitro* for a variety of bacteria, but only moderately bacteriostatic for the virulent H37Rv strain of *M. tuberculosis*. Chloromycetin showed considerable activity against *Rickettsia prowazeki* in tests using chick embryos. Smadel and Jackson[2] have also reported that chloromycetin was an effective chemotherapeutic agent experimentally against a variety of Rickettsiae. In a recent, more detailed report Smith, Joslyn, Gruhzit, McLean, Penner and Ehrlich[3] have further described the properties of chloromycetin, including data on toxicity and chemotherapeutic activity. The *in vitro* tests of the bacteriostatic activity of chloromycetin for *M. tuberculosis var. hominis* (H37Rv) reported in the above publications were done in this laboratory. This work has now been extended to include *in vitro* tests on other strains of virulent human-type tubercle bacilli and the effect of chloromycetin administered subcutaneously and orally on experimental murine tuberculosis.

Methods. The *in vitro* bacteriostatic tests with chloromycetin† were performed by the technic previously reported[4,5,6] and the results recorded in terms of the least amount of chloromycetin which would completely inhibit the subsurface growth of 0.01 mg of tubercle bacilli per ml of synthetic medium. Duplicate tests were conducted in the same medium to which had been added enough sterile beef serum to make a final concentration of 10.0%. Nineteen strains of virulent human-type tubercle bacilli were employed, including 7 which were resistant to more than 1000.0 μg of streptomycin per ml. One bovine strain was also used. Similar *in vitro* tests were conducted in which chloromycetin was employed in combination with streptomycin and para-aminosalicylic acid (PAS).

The chemotherapeutic action of chloromycetin *in vivo* was determined by using mice infected intravenously with 0.1 mg of the H37Rv strain of *M. tuberculosis*. The technic employed in these tests and the evaluation of the results of the tests were in every respect similar to those previously reported employing streptomycin and PAS,[6,7] with the exception that chloromycetin, when administered subcutaneously to mice, was dissolved in 20% propylene glycol water solution and the experiments were terminated at the end of 21 days instead of 28 days.

Results. The results of the *in vitro* bacteriostatic tests with and without serum using the 19 human type strains and the one bovine strain are shown in Table I. There was as much as an 8-fold variation in the sensitivity of these strains to chloromycetin when serum was omitted from the medium. Since twofold dilutions were used, the majority of the strains (12 out of 19) required

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1 Ehrlich, John, Bartz, Quentin R., Smith, Robert M., Joslyn, Dwight A., and Burkholder, Paul R., *Science*, 1947, **106**, 417.

2 Smadel, J. E., and Jackson, E. B., *Science*, 1947, **106**, 418.

3 Smith, Robert M., Joslyn, Dwight A., Gruhzit, Oswald M., McLean, Wm. I., Jr., Penner, Mildred A., and Ehrlich, John, *J. Bact.*, 1948, **55**, 425.

† Crystalline chloromycetin obtained from Parke, Davis Company, Detroit, Mich.

4 Youmans, Guy P., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 119.

5 Youmans, Guy P., and Doub, Leonard, *Am. Rev. Tuberc.*, 1946, **54**, 287.

6 Youmans, Guy P., Raleigh, Gordon W., and Youmans, Anne S., *J. Bact.*, 1947, **54**, 409.

7 Youmans, Guy P., and McCarter, John C., *Am. Rev. Tuberc.*, 1946, **52**, 432.

TABLE I.
Bacteriostatic Effect of Chloromycetin on 20 Strains of Tubercle Bacilli.

Strain No.	Type	Concentration in μg per ml which completely inhibited growth: Without serum	With serum
H37Rv	Human	12.5	12.5
H37RvR*	"	12.5	12.5
1	"	12.5	12.5
1R	"	12.5	25.0
11	"	12.5	12.5
12	"	12.5	12.5
15	"	3.12	12.5
15R	"	3.12	12.5
18	"	1.56	12.5
21	"	6.25	12.5
24	"	12.5	12.5
24R	"	6.25	25.0
69	"	12.5	12.5
69R	"	12.5	12.5
97	"	6.25	12.5
97R	"	6.25	12.5
100	"	12.5	12.5
100R	"	12.5	12.5
111	"	12.5	12.5
48	Bovine	No growth	25.0

* R indicates a streptomycin-resistant strain.

between 6.25 and 12.5 μg per ml to completely inhibit growth. The bacteriostatic activity of chloromycetin for these same 12 strains in the presence of serum was not significantly reduced. The remaining 7 strains which, in the absence of serum were inhibited in their growth to a greater degree by chloromycetin, were, in the presence of serum, as resistant as the other 12. Whether the decrease in sensitivity to chloromycetin shown by the above 7 strains in the presence of serum was due to the inactivation of chloromycetin by serum or was merely a reflection of the stimulation of growth of tubercle bacilli which occurs in the presence of serum is not discernible from these data. While the figures given in Table I represent the least amount of chloromycetin which under the conditions of the test completely inhibited growth, partial retardation of growth was usually noted in one-half or one-fourth this concentration. In any event, chloromycetin *in vitro* is considerably less bacteriostatic for virulent human type tubercle bacilli than is either streptomycin or para-aminosalicylic acid since with these latter substances most strains of tubercle bacilli are completely inhibited in their growth by less than one or 2 μg per ml.[7,8]

TABLE II.
Bacteriostatic Action of Chloromycetin in Combination with PAS and Streptomycin.

	Conc. in μg per ml which completely inhibited growth
Chloromycetin	12.5
PAS	0.78
Streptomycin	0.78
Chloromycetin + PAS	1.56
" + Streptomycin	1.56
" + PAS + Streptomycin	1.56

Since the bacteriostatic activity of many chemotherapeutic agents such as the sulfonamides, sulfones and PAS is markedly affected by the number of organisms used in the test, the bacteriostatic action of chloromycetin was tested using larger numbers of tubercle bacilli. Since a 10-fold increase in the amount of inoculum used in the test resulted in only a 4-fold decrease in bacteriostatic activity, the action of the drug, in contrast to para-aminosalicylic acid[7] is apparently not appreciably influenced by the number of organisms present.

In spite of the relatively low tuberculostatic action of chloromycetin, it was thought advisable to determine *in vitro* the effect of combining this substance with para-aminosalicylic acid and streptomycin. Table II shows the results of these tests. It is apparent that chloromycetin contributed nothing to the bacteriostatic action of such combinations since in no case was the effect greater than that obtained with either para-aminosalicylic or streptomycin alone. There was in fact consistently a slight reduction in bacteriostatic action when chloromycetin was included in the medium. Since this difference is well within the experimental error of the method, no great significance at present can be attached to it.

The initial experiments with chloromycetin to determine its therapeutic effectiveness *in vivo* were conducted with intravenously tuberculized mice by treating subcutaneous-

[8] Raleigh, Gordon W., and Youmans, Guy P., *J. Inf. Dis.*, 1948, in press.

TABLE III.
Results of the Mice Infected with *M. tuberculosis* (H37Rv) and Treated with Chloromycetin in the Diet.

Compound	% of compound in diet	No. mice	% mortality	Avg wt loss or gain in g	Amt gross pulmonary tuberculosis	Type of lesion
Chloromycetin	0.5	20	0.0	—4.1	3.5+	P & NE
	0.25	20	30.0	—3.3	3.84+	P & NE
	0.125	20	65.0	—3.85	3.85+	NE
Para-amino-salicylic acid	1.0	20	0.0	+1.6	2.5+	P
Controls		20	75.0	—4.8	4.0+	NE

1+ — 0-10% involvement of lung with tuberculosis
2+ — 10-25% " " " " "
3+ — 25-50% " " " " "
4+ — 50-100% " " " " "
P — Proliferative lesions
NE — Necrotic-exudative lesions

ly with 5 mg of chloromycetin per day dissolved in 20% propylene glycol. This was divided in 2 daily doses of 2.5 mg each given approximately 8 hours apart. This total daily dosage was selected on the basis of the known toxicity of chloromycetin administered by this route[3] and approached closely the maximal tolerated dose. These experiments failed to demonstrate any suppressive effect on the tuberculous process. Furthermore, marked reaction developed at the site of injection with swelling, edema and eventually ulceration.

When it became apparent that chloromycetin was well absorbed from the gastro-intestinal tract, experiments with intravenously tubercularized mice were conducted in which the compound was administered with the diet. Table III details the results of a typical experiment. Included in this table in addition to the untreated controls is a group of animals treated with 1.0% para-amino-salicylic acid. From the data given in the table, it is readily apparent that there was a relationship between the per cent mortality of the treated mice and the concentration of chloromycetin administered. Those mice which received 0.5% chloromycetin in the diet survived the 21-day period of treatment. The group which received 0.25% showed 30% mortality and the group which received 0.125%, a 65% mortality. The last figure is very similar to the mortality figure for the untreated control group. There was no significant difference between the amount of weight lost by any of the chloromycetin treated animals and the untreated controls; in contrast, the para-aminosalicylic acid treated animals showed a slight increase in average weight. The data given for the amount of gross pulmonary tuberculosis demonstrable at the end of the experimental period in the various groups correlates with the weight response. On the basis of these findings one might question the significance of the mortality figures for the groups of animals receiving the 0.5% and the 0.25% chloromycetin. However, upon microscopic examination, whereas the total amount of involvement of lung substance was large, those animals treated with 0.5% and 0.25% chloromycetin showed a significantly higher proportion of proliferative type lesions as compared with the controls. These findings, as has previously been determined,[8] indicate that the treated animals had a slower evolution of the disease process. The histopathological findings in the mice which received the smallest amount of chloromycetin (0.125%) did not differ from the untreated controls. It can be stated with some confidence that chloromycetin administered orally to intravenously tubercularized mice in a concentration of 0.5%, and possibly 0.25%, exerted a slight suppressive effect upon the disease process. Before the animals which received 0.5% were sacrificed at the termination of the experiment they were bled by cardiac puncture approximately 2 hours after being fed and the small blood samples obtained were pooled. The serum thus obtained was tested for its content of chloromycetin‡ and was found to be 7.0 μg per ml.

‡ Performed by Dwight A. Joslyn, Parke, Davis & Company, Detroit, Mich.

If this one determination represented the average serum concentration of chloromycetin during the course of treatment one would expect the slight borderline therapeutic effect which was noted since this concentration of chloromycetin, while it does not completely inhibit the growth of H37Rv strain *in vitro,* will partially retard the multiplication of the bacilli.

Summary and Conclusions. The antibiotic chloromycetin has been found to be only moderately bacteriostatic for virulent human type tubercle bacilli *in vitro* as compared with streptomycin or para-aminosalicylic acid. The majority of 19 human type strains studied were completely inhibited in their growth by between 6.25 and 12.5 micrograms or more of the drug in the presence of serum. One bovine strain was equally sensitive. This degree of bacteriostatic activity was not markedly affected by the number of tubercle bacilli present nor was the bacteriostatic activity of para-aminosalicylic acid or streptomycin enhanced by the addition of chloromycetin.

When administered subcutaneously, chloromycetin has been shown to be ineffective, whereas, when admixed with the diet in concentrations of 0.5 and 0.25%, it was slightly effective for the suppression of experimental murine tuberculosis.

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Subtenolin. An Antibiotic from *Bacillus subtilis.* I. Bacteriologic Properties.

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In the course of a search for antibiotic agents in this laboratory, an organism was isolated in February, 1945, that produced a substance showing antibiotic activity for certain gram-positive and gram-negative bacteria. The organism was isolated from nutrient agar plates that had been seeded with *Escherichia coli* and *Staphylococcus aureus,* respectively, and inoculated with material from dusty surfaces in the laboratory. It was identified as *Bacillus subtilis.** Because of its microbic source and strong enolic properties[1] the name, subtenolin, has been given to the antagonist. The bacteriologic properties of the antibiotic are the subject of this report; its isolation from the harvest and its chemical properties are described in the succeeding report.[1]

Production of Subtenolin. Mediums containing peptones and other complex organic enrichments were found to be very poor for the production of subtenolin. A medium containing *dl*-alanine produced the highest yields and least destruction. With subtenolin, as with subtilin,[2] strong stimulatory action by manganese was observed. Production was slightly stimulated by copper.

The following medium was adopted for use in the production of subtenolin:

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1 Howell, S. F., and Tauber, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 432.

2 Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, **4**, 297.