

THE TUBERCULOSTATIC EFFECT OF SUBTILIN IN VITRO AND IN VIVO

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The antituberculosis activity of subtilin, an antibiotic agent introduced by Jansen and Hirschmann (1944), has been the subject of conflicting reports in the literature. Salle and Jann (1945) reported that subtilin in a dilution of 1:50,000 prevented surface growth of *Mycobacterium tuberculosis* on Long's synthetic medium, and that transfers into fresh medium made from tubes containing 1:10,000 subtilin produced no growth. They concluded that "subtilin is bacteriostatic in high dilution and germicidal in greater concentration."

Anderson and Wong (1946) and Wong, Hambly, and Anderson (1947) found that in the sorbitan monooleate albumin medium of Dubos and Middlebrook (1947), a 1:400,000 dilution of subtilin (2.5 μ g per ml) regularly inhibited the growth of *Mycobacterium tuberculosis* for 12 days. To study the bactericidal effect, they inoculated 1 ml of each dilution not showing visible growth into each of two guinea pigs. The animals receiving material from tubes containing 1:20,000 and 1:10,000 dilutions of subtilin did not develop tuberculosis. These workers also found that subtilin inhibited tubercle formation in the allantoic membrane of the chick embryo, but they could demonstrate no effect on tuberculous infection of rabbit corneas by topical application of the drug, or on tuberculous infection in hamsters by subcutaneous injections of subtilin for 6 weeks.

Knight and Tompsett (1948) observed that subtilin was effective in inhibiting the growth of tubercle bacilli only when the organisms were growing in a dispersed fashion, as in media containing wetting agents such as sorbitan monooleate. In media not containing wetting agents, where the bacilli were growing in aggregates, subtilin did not inhibit their growth. They reported that the course of tuberculous infection in mice was not affected by much larger daily doses of subtilin than those necessary for protection against pneumococcus or streptococcus infection.

Farber *et al.* (1948) reported the sensitivity to subtilin of 6 strains of tubercle bacilli recently isolated from patients. Three were sensitive to 1:400,000, two were sensitive to 1:200,000, and one to 1:100,000 dilution of the drug. Presumably these tests were done in sorbitan monooleate albumin medium.

This report describes experiments designed to clarify the status of subtilin as a tuberculostatic agent in the test tube and in guinea pigs.

In vitro results. The effect of subtilin¹ on the growth of the virulent, human type tubercle bacillus, H37Rv, was tested in four different liquid media: Pros-

¹ The subtilin was supplied through the courtesy of Dr. S. Binkley of the Bristol Laboratories, Syracuse, New York, lot number 529-8, with labeled potency of 55 units per mg.

kauer and Beck's (P and B) synthetic medium, the Youmans and Karlson modification (1947) of the P and B medium; P and B with 10 per cent beef serum, and the sorbitan monooleate albumin medium of Dubos and Middlebrook. They were dispensed in 5-ml amounts in 18-by-50-mm test tubes. The subtilin was dissolved in water and passed through a Seitz filter. The inoculum was a 7-day culture of H37Rv in sorbitan monooleate albumin medium. For the two en-

TABLE 1

Effect of subtilin on growth of the H37Rv tubercle bacillus in four different types of liquid media

SUBTILIN IN		GROWTH IN DAYS					SUBTILIN IN		GROWTH IN DAYS				
u/ml	μg/ml	4	11	15	21	27	u/ml	μg/ml	4	11	15	21	27
A. Sorbitan monooleate albumin liquid medium						B. Synthetic medium with 10 per cent beef serum							
Control		+	3+	4+	4+	4+	Control		+	3+	4+	4+	4+
"		+	3+	4+	4+	4+	"		+	3+	4+	4+	4+
0.055	1	±	3+	4+	4+	4+	0.055	1	+	3+	4+	4+	4+
0.55	10	0	±	2+	3+	4+	0.55	10	+	3+	4+	4+	4+
5.5	100	0	0	0	0	±	5.5	100	+	3+	4+	4+	4+
55	1,000	0	0	0	0	0	55	1,000	+	3+	4+	4+	4+
		(ppt)							(ppt)				
SUBTILIN IN		GROWTH IN DAYS					SUBTILIN IN		GROWTH IN DAYS				
u/ml	μg/ml	4	11	15	21	27	u/ml	μg/ml	4	11	15	21	27
C. Youmans and Karlson basic medium						D. Proskauer and Beck synthetic medium							
Control		+	3+	4+	4+	4+	Control		0	2+	3+	4+	4+
"		+	3+	4+	4+	4+	"		0	2+	3+	4+	4+
"		+	3+	4+	4+	4+	"		0	2+	3+	4+	4+
0.055	1	±	2+	3+	4+	4+	0.055	1	0	2+	3+	4+	4+
0.55	10	0	+	3+	4+	4+	0.55	10	0	2+	3+	4+	4-
5.5	100	0	0	2+	3+	4+	5.5	100	0	+	2+	3+	4+
55	1,000	ppt				pos.*	55	1,000	0				pos.*
									(ppt)				

* Turbid because of precipitation (ppt), but microscopic preparation reveals presence of many acid-fast bacilli.

riched media (sorbitan monooleate albumin and P and B with beef serum), 0.1 ml of the culture (about 0.03 mg, dry wt) was inoculated into each tube; twice this amount was used for the two synthetic media.

The results of these tests are presented in table 1. In the three media containing no sorbitan monooleate, in which the growth was granular, subtilin had very little inhibitory effect, even in concentrations of 100 or more μg per ml (5.5 units per ml). In the sorbitan monooleate albumin medium, in which the growth was dispersed, 10 μg per ml inhibited growth for about 12 days, but then progressive turbidity appeared. One hundred μg per ml prevented growth for 27 days.

TABLE 2
Effect of treatment with subtilin on guinea pig tuberculosis

G. FIG NO.	FATE	DAYS OF INFECTION	DAYS OF TREATMENT	AMOUNT OF TUBERCULOSIS* GROSSLY AT AUTOPSY
Controls				
1	Died	19	0	6+
2	"	63	0	9.5+
3	"	66	0	7.5+
4	"	67	0	14+
5	Killed	71	0	10+
6	"	71	0	9+
7	"	71	0	11+
8	"	71	0	8+
9	"	71	0	9+
10	"	71	0	7.5+
Average†.....				9.5+
Treated				
1	Died	19	13	6+
2	"	35	29	5+
3	"	35	29	6+
4	"	45	39	12.5+
5	"	47	41	14.5+
6	"	47	41	12+
7	"	50	44	10+
8	"	56	50	13+
9	"	67	61	13+
10	"	68	62	8.5+
11	"	70	64	6+
12	"	71	65	11.5+
13	Killed	70	64	9+
14	"	70	64	8+
15	"	70	64	8+
16	"	70	64	10.5+
17	"	70	64	11+
18	"	70	64	10.5+
19	"	71	65	11.5+
20	"	71	65	9+
21	"	71	65	9.5+
22	"	71	65	9.5+
23	"	71	65	11+
24	"	71	65	13+
Average†.....				10.0+

* Each organ (liver, spleen, lungs, lymph nodes) was assigned a value from 0 to 4+ according to the amount of tuberculous disease present. Figures in this column represent the total amount of disease, the maximum being 16+ for one animal.

† Average of those animals surviving at least 60 days after infection.

In vivo results. The effect of the same batch of subtilin on the course of tuberculosis in guinea pigs was also tested. Thirty-four guinea pigs, weighing be-

tween 400 and 600 g, were infected with the H37Rv microorganisms. Each animal received 0.05 mg (dry wt) of microorganisms subcutaneously in the inguinal region. Ten animals were kept as untreated controls, and 24 were started on subtilin treatment 6 days after infection.

The subtilin was made up in distilled water in concentration of 5 mg per ml. Each treated pig received 0.2 ml, or 1 mg, intramuscularly every 12 hours. After 37 days of treatment the dose was increased to 1.7 mg every 12 hours, and after 44 days of therapy the dose was increased again to 2.0 mg every 12 hours. Seventy days after infection the experiment was terminated by sacrificing all surviving animals.

At autopsy each pig was rated as to the gross tuberculous disease present: the spleen, liver, lungs, and lymph nodes were each given a value of from 0 to 4+, making a maximum total of 16+ for each animal. In table 2 are presented the results of this experiment. It may be seen from the table that there was no significant difference in the amount of tuberculosis between the control and the subtilin-treated groups.

DISCUSSION

The results herein described show that subtilin has only a very slight inhibitory effect on the growth of the H37Rv strain of the tubercle bacillus in fluid media without a dispersing agent. In the presence of sorbitan monooleate, which produces a dispersed growth, the drug is moderately effective. This confirms the observations of Knight and Tompsett (1948).

Anderson and Wong (1946), Wong *et al.* (1947), and Farber *et al.* (1948) define the minimal inhibitory concentration of subtilin for *Mycobacterium tuberculosis* as approximately 2.5 to 5.0 μg per ml in sorbitan monooleate albumin medium. The observation period was limited to 21 days because subtilin is not stable in neutral solutions, losing 68 per cent of its activity at 37 C at pH 7.3 in 6 days, and 57 per cent at pH 6.8 in 6 days (Dimick, Alderton, Lewis, Lightbody, and Fevold, 1947). In the tests reported here, growth in 10 μg of subtilin per ml was beginning to appear at about the twelfth day. Contributing to the greater inhibitory effect found by Anderson and his group is the fact that the medium they used contained 0.05 per cent sorbitan monooleate, 0.15 per cent albumin, and no glucose, whereas in this laboratory the formula contains less sorbitan monooleate (0.02 per cent), more albumin (0.5 per cent), and 0.2 per cent glucose. In addition, there is as yet no standard subtilin preparation, and it is therefore difficult to compare results obtained with different preparations.

Subtilin produced no significant effect on the tuberculosis of the guinea pigs included in this experiment. The dosage used was close to the maximal tolerated dose for prolonged therapy with this preparation. Progressive loss of weight was produced by 4.0 mg (220 units) per day, and it was deemed inadvisable to increase the dose further. The same negative results have been obtained with mice (Knight and Tompsett, 1948) and with hamsters (Anderson and Wong, 1946). The preparations of subtilin now available have a very low solubility in physiological saline and in serum. It has been found (Wilson, Lewis, and Humphreys,

1948) that the drug is precipitated at the site of subcutaneous or intramuscular injection and absorbed slowly. In rabbits, up to 100 mg per kg injected intramuscularly produced blood levels of no more than 2 parts per million (Wilson, Lewis, and Humphreys, 1948). This insolubility in physiological fluids undoubtedly contributes to the poor results obtained. Other infections, however, such as pneumococcus, streptococcus, and anthrax, have been reported to respond very well to parenteral treatment with subtilin in guinea pigs and mice (Salle and Jann, 1946*a,b,c*).

SUMMARY

Subtilin had a moderate inhibitory effect on the growth of the H37Rv strain of the tubercle bacillus in sorbitan monooleate albumin liquid medium, but very little effect in the other three media that did not contain sorbitan monooleate.

Subtilin did not influence the course of a tuberculous infection in guinea pigs produced by the H37Rv strain of the tubercle bacillus.

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