STUDIES ON ANTITUBERCULOUS FACTORS IN THE LOW MOLECULAR FRACTIONS OF THE SERA OF VARIOUS ANIMAL SPECIES

REPORT II. PURIFICATION OF ANTITUBERCULOUS FACTORS AND THEIR CHEMICAL ANALYSIS

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Introduction

It has been demonstrated in the previous report[1] that the low molecular fraction in the serum of various animal species contains inhibitory substances on the growth of virulent tubercle bacilli *in vitro.*

As the substances were impure containing other substances in addition to the active factor, chemical purification was attempted. In this paper the results of purification and chemical analysis will be reported.

Materials and Methods

Preparation of materials

The outer fluid produced by dialysis of the sera of various animal species was used as starting materials. Bovine serum was used in vast majority of experiments.

The culture method using filter paper.

The technique was as follows.

10–20 mg of dried stating material were developed with water on a filter paper (Toyo-Roshi No. 50, 2×40 cm) at room temperature. After the development ended, the filter paper was cut in 1 cm long pieces. Each piece was sterilized by heating at 80°C for 3 hours. Each piece was placed on an Oka-Katakura's solid egg medium. 0.1 ml of bacillary suspension in benzine[3] was poured on the medium and the medium was incubated at 37°C.

The degree of growth of bacilli is designated as follows:

++ : Large colonies were found uniformly on the medium and on the paper.

+ : Small colonies were found on the medium but not on the paper.

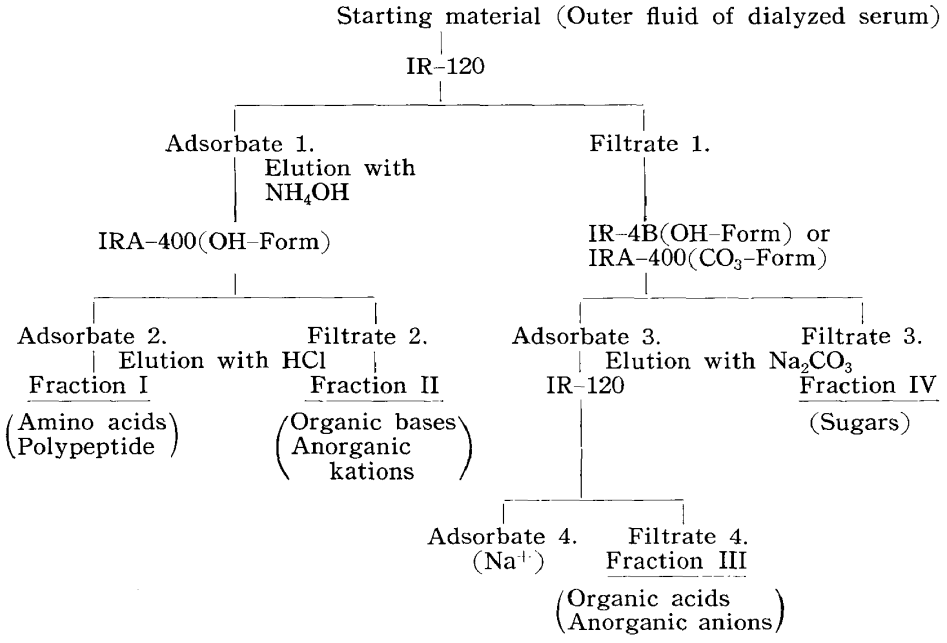
− : No bacillary growth.

In addition, pieces of the paper were treated by several chemical reagents in order to examine the relationship between their antibacterial activity and their chemical nature.

Procedure of systematic chemical fractionation using ion exchange resins.

As the technique was reported in the previous paper[4] in detail, only a diagram is presented in Table 1. The low molecular fraction of the serum was

Table 1. Procedures used in chemical fractionation of low molecular substances of serum.



separated into four fractions. Each fraction was concentrated under reduced pressure to dryness and was weighed.

Test for tuberculostatic activity

The technique was described in the previous report. As Fraction I and Fraction III were strongly acid, they were tested for tuberculostatic acitvity after being neutralized with NaOH.

Results

1. Experiment using the paper culture method.

The paper culture was carried out using the low molecular fraction of the sera of various animals.

The results are shown in Table 2. An apparent inhibition to the growth of H37Rv strain was found at about Rf 0.5 and weak inhibitions were noted also at

Table 2. The tuberculostatic zone of the low molecular fractions in the sera of various animals. (The paper culture method)

	Human		Bovine		Normal rabbit				Immunized rabbit	Dog		Cat		Goat		Guinea pig
	H37Rv	BCG	H37Rv	BCG	H37Rv	BCG	Avian Cho-Kyo	607	H37Rv	H37Rv	BCG	H37Rv	BCG	H37Rv	BCG	H37Rv
Rf																
1.0	+	—	—	—	+	+	++	++	++	++	+-	+-	++	—	—	++
0.9	—	—	+	+-	+	—	++	++	++	+	—	—	—	++	++	++
0.8	++	++	++	++	++	++	++	++	++	++	++	++	+-	—	++	++
0.7	+	—	++	++	++	++	++	++	++	+-	—	++	++	++	++	++
0.6	++	++	++	++	++	++	++	++	—	++	++	++	++	++	++	++
0.5	—	—	—	—	+	—	+	++	+	—	—	—	—	—	—	—
0.4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+
0.3	—	—	++	++	++	++	++	++	—	++	++	++	++	++	++	++
0.2	++	++	++	++	++	++	++	++	+-	++	++	++	++	++	++	—
0.1	+	—	—	—	+	—	++	++	++	+-	++	—	—	—	—	++

H37Rv and BCG was cultured by paper culture method for thirty days.
Avian (Cho-Kyo) strains of tubercle bacilli and Myco. 607 was cultured for seven days.
Immnized rabbit ; Rabbit was treated intramuscularly with 100 mg of heat killed H37Rv twice at two weeks intervals.

Rf 0.9, 0.3 and 0.1. The inhibitory spectrum of the growth of BCG strain was very similar to that of the H37Rv strain. To the avian type of tubercle bacilli and Myco. 607 no inhibition was noted.

Moreover, the paper culture method was performed using the low molecular fraction of rabbit's serum which was immunized with 100 mg of heat killed H37Rv strain twice at two weeks intervals. As shown in Table 2, the distinct inhibitory zone was found at about Rf 0.6 and 0.3. Although this result was a little different from that of normal rabbits, the difference may be due to the difference in the experimental conditions.

Chemical analysis of active substance.

Fig. 1 shows chemical reactions on the papers on which the low molecular

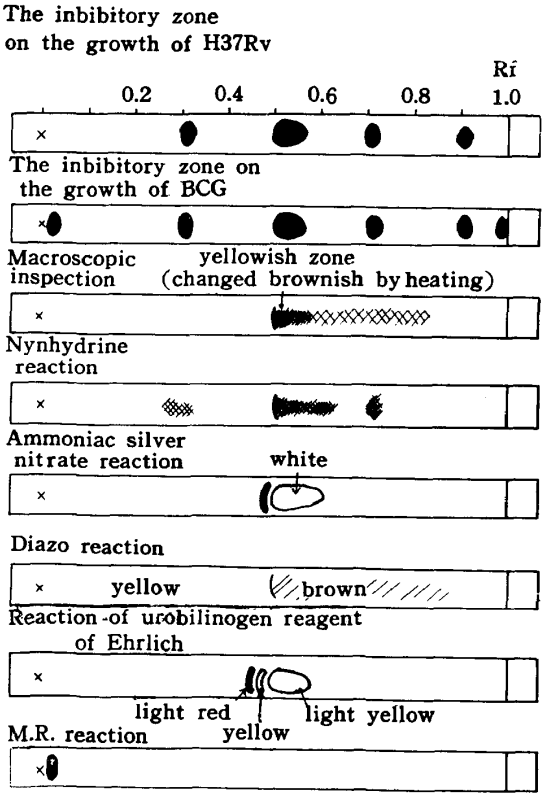


Fig. 1. Paperchromatogram of the low molecular fraction of human serum developed with water.

fraction of humans serum was developed. Strongest ninhydrine reaction was found at Rf 0.5 (the same site of the strongest inhibition). This fact suggests that the active substance may closely be related to amino acids or polypeptides.

2. The systematic fractionation using ion exchange resins.

The starting material was seperated into four fractions using ion exchange resins. Each fraction was tested for tuberculostatic activity.

a) The experiment on the normal serum.

Table 3. The tuberculostatic test of fractionated materials obtained from bovine serum and their minimum concentration for inhibiting the growth of H37Rv.

Material	pH (before neutralization)	Concentration of material						Minimum concentration for inhibiting the growth of H37Rv
		×32	×16	×8	×4	×2	×1	
Original material	8.6		—	—	—	+	++	33.7 mg/dl
Fraction I	2~3	—	—	+	++	++	+++	9.1
Fraction II	7.5~8.5	++	+++	+++	+++	+++	+++	—
Fraction III	1~2	—	—	+	++	++	+++	11.9
Fraction IV	7.0	+++	+++	+++	+++	+++	+++	—

Control : +++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

Bovine serum: The results are shown in Table 3. Both Fraction I and III inhibited the growth of H37Rv at the concentration of eight or sixteen times as high as that of the starting serum.

Fraction II did not inhibit even at 32 times concentration.

Fraction IV did not inhibit even at 128 times concentration.

The minimum amount of dried fractions for inhibiting the growth of H37Rv are also shown in Table 3.

The inhibitory activities on the growth of BCG strain indicated the similar results as those on the H37Rv strain.

Rabbit serum; The results are shown in Table 4. Both Fraction I and III could inhibit the growth of H37Rv at the concentration of four or eight times as high. as the starting serum The minimum amounts of dried fractions for inhibiting the growth of H37Rv are also shown in Table 4.

b) The experiment concerning immunized animals.

Rabbits were immunized by intramuscular inoculation with 100 mg heat-killed H37Rv strain twice at two weeks intervals. Using the serum of these rabbits the same experiments as described above were carried out. The results are shown in

Table 4. The tuberculostatic test of fractionated materials obtained from rabbit serum and their minimum concentration for inhibiting the growth of H37Rv.

Material	pH (before neutralization)	Concentration of material					Minimum concentration for inhibiting the growth of H37Rv
		×8	×4	×2	×1	×½	
Original material	8.6	—	—	—	+	++	22.0 mg/dl
Fraction I	2~3	—	-+	++	+++	+++	10.9
Fraction II	7.5~8.5	++	+++	+++	+++	+++	—
Fraction III	1~2	—	—	++	+++	+++	11.9
Fraction IV	7.0	+++	+++	+++	+++	+++	—

Control: +++
Slide culture using H37Rv strain in Kirchner's medium for ten days.

Table 5. The tuberculostatic test of fractionated materials obtained from serum of immunized rabbits.

Material	Treatment	pH (before neutralization)	Concentrtion of material				
			×8	×4	×2	×1	×½
Original material	Normal	8.6	—	—	—	+	++
	Immune	8.6	—	—	—	++	++
Fraction I	Normal	2~3	—	+	++	+++	+++
	Immune	2~3	—	+	++	+++	+++
Fraction II	Normal	7.5~8.5	++	+++	+++	+++	+++
	Immune	7.5~8.5	++	+++	+++	+++	+++
Fraction III	Normal	1~2	—	—	++	+++	+++
	Immune	1~2	—	—	+	++	+++
Fraction IV	Normal	7.0	+++	+++	+++	+++	+++
	Immune	7.0	+++	+++	+++	+++	+++

Control: +++
Slide culture using H37Rv strain in Kirchner's medium for ten days.
Immunized rabbit: Rabbit was inoculated intramuscularly with 100 mg of heat killed H37Rv strain twice at two weeks intervals.

Table 5. No significant difference between immunized and normal rabbits was noted.

3. Paperchromatographic analysis of the Fraction I and III.

Paperchromatographic analysis of the Fraction I and III was performed. The results are shown in Table 6. Ten or more amino acids and polypeptides seems to be contained in the Fraction I and there are five or more of organic acids in the Fraction III.

Table 6. Rf value of paperchromatogram of Fraction I and III.

Developer	Fraction I		Fraction III		
	Buthanol-acetic acid-water (4 : 1 : 2)	Phenol	Ether-acetic acid-water (13 : 3 : 1)	Phenol-formic acid-water (75 : 1 : 25)	Buthanol-formic acid-water (10 : 2 : 15)
Rf value	0.10～0.12	0.13	0.05	0.06	0.20
	0.23～0.25	0.20	0.14	0.13	0.28
	0.25～0.26	0.30	0.25	0.27	0.34
	0.40～0.45	0.35	0.72	0.67	0.73
	0.50～0.53	0.41	0.96	0.92	0.97
	0.64～0.65	0.48			
	0.69	0.60			
		0.69			
		0.82			
		0.88			

4. Stability of the Fraction I and III against heating and hydrolysis.

Fraction I and Fraction III of bovine serum were treated as described in the Report I[1].

a) Stability against heating.

The results are shown in Table 7. Active substances in the Fraction I and the Fraction IV were considerably heat stable.

Table 7. The stability against heating of fractionated materials and their inhibitory activities on the growth of H37Rv.

Treatment of material	Fraction I						Fraction III					
	×32	×16	×8	×4	×2	×1	×32	×16	×8	×4	×2	×1
Heating at 100°C for 30 min.	—	—	++	++	+++	+++	—	—	+	++	+++	+++
Heating at 100°C for 60 min.	—	—	++	++	+++	+++	—	—	+	++	+++	+++
Heating at 120°C for 30 min.	—	—	++	++	+++	+++	—	—	+	++	+++	+++
Heating at 120°C for 60 min.	—	—	++	++	+++	+++	—	—	+	++	+++	+++
No heating	—	—	++	++	+++	+++	—	—	+	++	+++	+++

Control : +++

Slide culture using H37Rv strain in Kirchner's medium for ten days.

b) Stability against hydrolysis by 6N HCl at 100°C for 15 hours.

The results were shown in Table 8. Although slight decrease of the inhibitory power of the Fraction I was noted after hydrolysis, the activity remained was sufficient to inhibit the growth of virulent tubercle bacilli *in vitro*. The active substance was thought to be considerably resistant to hydrolysis. This fact means

Table 8. The stability againct hydrolysis of fractionated matesials and their inhibitory activities on the growth of H37Rv.

Material	Treatment	Strain	Concentration of material					
			×32	×16	×8	×4	×2	×1
Fraction I	Before hydrolysis	H37Rv	—	—	±	++	+++	+++
		BCG	—	—	±	++	+++	+++
	After hydrolysis	H37Rv	—	—	++	++	+++	+++
		BCG	—	++	+++	+++	+++	+++
Fraction III	Before hydrolysis	H37Rv	—	—	+	++	+++	+++
		BCG	—	—	±	++	+++	+++
	After hydrolysis	H37Rv	—	—	±	++	+++	+++
		BCG	—	—	+	++	+++	+++

Control : +++
Slide culture in Kirchner's medium for ten days.

that the active substances are consisted of mainly amino acids or of not so high molecular peptides.

The inhibitory activity of the Fraction III did not decrease even after hydrolysis. Therefore, it seems to be low molecular organic acids.

Discussion

It has been demonstrated in the previous report[1] that the low molecular fractions, the outer fluid produced by dialysis of the serum of various animal species, could inhibit the growth of virulent tubercle bacilli.

As these substances were stable against heating and hydrolysis, it was presumed that these substances were very low molecular substances. In the present report purification of active materials was performed. First, the crude material was investigated by the paper culture method, which was an application of chromatography and bio-assay. The active substances were noted in three or more spots, (Rf 0.5, front and original spot). The spot of Rf 0.5 was always positive in ninhydrine reaction. No significant difference of the properties of active substances between immunized and normal rabbits was noted.

Using systematic fractionation by ion exchange resins, the low molecular substances were separated into four fractions. Fraction I (amino acid polypeptide fraction) and Fraction III (organic acid fraction) had activity and there were no activity in Fraction II and Fraction IV. As Fraction I was considerably stable against heating and hydrolysis, the active substances were presumed to be amino acids or low molecular peptides. And the active substances in Fraction III were

presumed to be stable organic acids.

Marschak[5], Dubos[6,7] and Lewis[8] reported that a certain kinds of amino acids or polypeptides in some organs had the antituberculous activity, but no one reported concerning the antituberculous amino acids or polypeptides in the serum.

Dubos[9], Bergstrom[10], McJunkin[11], Boissevain[12], Patnode[13] and Platonov[14] reported the antituberculous organic acids. These acids were thought to be fatty acids, but the antituberculous organic acids of Fraction III were insoluble in acetone and therefore, may not be fatty acids.

Oshima[2] and Nakashima[15] in our laboratory reported the similar antituberculous factors in human urine and in several organ extracts of rabbits. Therefore, it is inferred that the antituberculous substances are produced in living tissues and then transported by blood circulation and excreted into the urine.

It has been reported by several investigators that antituberculous activity in the serum increases by immunization[16–23]. In the present investigation, no significant difference in tuberculostatic activity of fractions separated by the ion exchange resins between immunized and normal serum was found. It seems that the increase of activity found in the immune serum is due to high molecular substances, perhaps protein[4,24].

Summary

1) The low molecular fractions of the serum of human, bovine, rabbit goat, dog, cat, and guinea pig were investigated by the paper culture method. The inhibiting zone to the growth of H37Rv and BCG existed at the nynhydrin positive part of Rf 0.5. No inhibition was noted by using avian type of tubercle bacilli and Mycobacterium 607.

2) The low molecular fractions of the sera of bovine and rabbits were investigated by systematic fractionation using ion exchange resins. The active factors were divided into two fractions—the amino acid-polypeptide fraction and the organic acid fraction. The fraction of the organic bases and the fraction of sugars had no activity.

3) The activity of both fractions was not changed after immunization.

4) The activity of both fractions were stable against heating. Slight decrease of the inhibitory power of the amino acid-polypeptide fraction was noted after hydrolysis, but the activity of the organic acid fraction were stable against hydrolysis.

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