THE ESSENTIAL PARTICIPATION OF AN ENZYME IN THE INHIBITION OF GROWTH OF TUBERCLE BACILLI BY SPERMINE

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(Received for publication, September 25, 1952)

Spermine, a naturally occurring organic base, inhibits the growth under certain conditions *in vitro* of several strains of human and bovine tubercle bacilli, exerting on them an apparently bactericidal action (1). This substance has little or no effect on the multiplication of saprophytic mycobacteria or of a variety of non-acid-fast microorganisms. The closely related compound spermidine possesses antimycobacterial properties resembling those of spermine, but several other biological and synthetic amines do not show similar activity (2).

Recent investigations directed towards a better understanding of some of the mechanisms involved in the action of spermine and spermidine on acid-fast bacteria have revealed that these compounds inhibit the growth of tubercle bacilli only if another tissue substance is present in the culture medium. This activating substance occurs as a chemical contaminant of the albumin in bovine plasma fraction V ordinarily added to the medium; it is also found in whole bovine and sheep serum and in aqueous extracts of the guinea pig kidney. The serum constituent responsible for the activation has the characteristics of a protein of the alpha globulin classification, and apparently exerts its effect by altering spermine enzymatically.

Evidence that a Substance Present in Bovine Plasma Fraction V Influences the Effect of Spermine on the Growth of Tubercle Bacilli

In the previous report (1) it was noted that spermine no longer inhibited the growth of tubercle bacilli when certain whole sera were substituted for the bovine plasma fraction V ordinarily added to the medium. Inhibitory activity was restored, however, by adding bovine albumin to the media containing whole serum. Experiments were therefore designed to determine the influence of bovine serum albumin preparations on the antimycobacterial action of spermine.

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The tests were performed in a liquid medium of the following composition: asparagine, 0.2 per cent; KH_2PO_4, 0.1 per cent; $Na_2HPO_4 \cdot 12\ H_2O$, 0.63 per cent; $MgSO_4 \cdot 7H_2O$, 0.001 per cent; $CaCl_2$, 0.000005 per cent; $CuSO_4$, 0.00001 per cent; $ZnSO_4$, 0.00001 per cent. No tween was included. All ingredients were dissolved in distilled water, and the medium was adjusted to pH 6.7. Aluminum-capped tubes 25 mm. in diameter containing 4 cc. of this medium were sterilized by autoclaving for 15 minutes at 15 pounds pressure.

Spermine tetrahydrochloride which had been prepared by chemical synthesis (Hoffmann-La Roche, Nutley, New Jersey) was dissolved in distilled water and adjusted approximately to neutrality by the addition of 0.1 N NaOH. 0.5 cc. of dilutions of this stock solution of spermine was added to the medium.

TABLE I

The Influence of the Concentration and Purity of the Bovine Albumin in the Medium on the Antimycobacterial Activity of Spermine

Bovine albumin preparation added to the medium	Final concentration	Inhibition of growth of tubercle bacilli				
		Molar concentration of spermine				
		7×10^{-5}	3.5×10^{-5}	1.75×10^{-5}	8.5×10^{-6}	None
	per cent					
Plasma fraction V	0.3	++++*	++++*	+*	0*	0*
	0.1	++++	++++	++++	0	0
	0.03	++++	++++	++++	0	0
	0.01	++++	++++	++++	+	0
	0.003	++++	+	0	0	0
	None	0	0	0	0	0
Crystalline albumin	0.3	0	0	0	0	0
	0.1	0	0	0	0	0
	0.03	0	0	0	0	0
	0.01	0	0	0	0	0
	0.003	0	0	0	0	0
	None	0	0	0	0	0

* ++++ = complete inhibition of growth. 0 = no inhibition of growth.

Stock cultures of an attenuated tubercle bacillus (BCG-Phipps) were maintained in Dubos tween-albumin medium. A 10 day old stock culture was diluted 1:1000 into sterile solutions containing 5 per cent glucose and varying concentrations of bovine plasma fraction V or crystalline bovine albumin (Armour and Company, Chicago). 0.5 cc. of the dilute suspension of the organisms was then added to each tube aseptically, producing a final concentration of 0.5 per cent glucose and 10^{-4} of the stock culture of tubercle bacilli. With this small bacterial inoculum, the amount of bovine plasma fraction V transferred from the stock culture was insignificant. The tubes were incubated at 38°C. When growth developed, the bacilli formed large clumps which could be partially dispersed by shaking. After incubation for 14 days, readings of the degree of inhibition of growth were made by visual examination and were graded from 0 (no inhibition of growth) to ++++ (complete inhibition of growth).

As is evident in the upper section of Table I, the concentration of bovine plasma fraction V in the medium had a remarkable effect on the antimyco-

bacterial activity of spermine. The presence of an excess of this preparation led to a slight decrease in the inhibition of growth, a result which is not surprising in view of the known protective action of albumin attributed to its binding properties (3). However, a progressive decrease in the concentration of bovine plasma fraction V also resulted in a diminution and finally a disappearance of the antimycobacterial effect of spermine. This unexpected finding suggested that the bovine albumin itself or some substance present as a contaminant in the relatively crude fraction V was instrumental in rendering spermine inhibitory for the growth of tubercle bacilli.

The lower section of Table I shows the results of an identical experiment in which crystalline bovine albumin was used in place of fraction V. Under these conditions spermine had no antimycobacterial effect regardless of the concentration of albumin added to the medium, thus indicating that some constituent of plasma fraction V other than the albumin was responsible for the activation of spermine.

Results similar to those presented in Table I were obtained when spermidine was used in place of spermine.

Distribution in Various Animal Sera and Tissues of the Agent Responsible for the Activation of Spermine

Experiments were next designed to assay the spermine-activating capacity of serum from several animal species. In addition, the effect of aqueous organ extracts on the spermine–tubercle bacillus system was studied in preliminary fashion.

In view of the large numbers of tests and the need for special conditions, new techniques were used in the experiments to be reported below. Since the tests were designed to determine the distribution and characteristics of the substances responsible for the activation of spermine, all components of the medium known to possess such spermine-activating capacity had to be excluded. Crystalline bovine albumin was therefore used in place of bovine plasma fraction V as the growth protective factor in the medium.

The crystalline bovine albumin was dissolved in 0.85 per cent saline at a concentration of 1 per cent. This solution was adjusted to pH 6.7 and sterilized by filtration through porcelain. A final concentration of 0.1 per cent crystalline bovine albumin was included in all tests and in the stock cultures. The remaining constituents of the medium were the same as previously described (see above).

The tests to be reported in the following sections were carried out in 12 by 100 mm. pyrex test tubes with a final volume of 1 cc. After inoculation, the cotton plugs were cut flush with the rim of the tube, depressed slightly with the finger, and the surface flooded with melted paraffin (M.P. 55–60°C). The paraffin seal was necessary in order to avoid evaporation of the small initial volume on prolonged incubation at 38°C.

The data presented in Table II demonstrate that beef and sheep serum contained a substance which rendered spermine inhibitory for the growth of

tubercle bacilli. Very small amounts of this activating material were required since these sera brought about the antimycobacterial effect even when diluted 1:100,000. On the other hand, even large amounts of these sera, in the absence of spermine, had no discernible effect on the growth of acid-fast bacteria. Sera obtained from man, rabbit, guinea pig, horse, and pig were ineffectual in activating spermine, regardless of their concentration in the medium. These results represent examinations of individual or pooled serum specimens from at least

TABLE II

The Activation of Spermine by Serum from Various Animals

Whole serum added to the medium	Final concentration	Inhibition of growth of tubercle bacilli		Whole serum added to the medium	Final concentration	Inhibition of growth of tubercle bacilli	
		No spermine	5×10^{-5} M spermine			No spermine	5×10^{-5}M spermine
	per cent				*per cent*		
Beef	10	0*	++++*	Rabbit	10	0	0
	1	0	++++		0.1	0	0
	0.1	0	++++		0.001	0	0
	0.01	0	++++				
	0.001	0	++++	Guinea pig	10	0	0
	0.0001	0	0		0.1	0	0
					0.001	0	0
Sheep	10	0	++++	Horse	10	0	0
	1	0	++++		0.1	0	0
	0.1	0	++++		0.001	0	0
	0.01	0	++++				
	0.001	0	++++	Pig	10	0	0
	0.0001	0	0		0.1	0	0
					0.001	0	0
Man	10	0	0				
	0.1	0	0	No serum added	—	0	0
	0.001	0	0				

* Symbols same as in Table I.

3 animals of each species, except for the horse and pig sera which were single samples. In the case of those sera containing activator, the dilution at which activation was no longer evident was remarkably constant on testing samples obtained at different times or from different individual animals.

An attenuated strain of bovine tubercle bacillus was used in the experiments summarized in Table II. In order to determine whether the spermine-activating capacity of sera from various animals was in any way related to the type of tubercle bacillus employed in the tests, similar experiments were conducted with several virulent and attenuated strains of human and bovine mycobacteria. The results were essentially the same as those presented in Table II.

Attempts were next made to test for the presence of spermine activator in the organs of animals whose serum was devoid of this substance.

Pooled guinea pig kidneys and hearts and rabbit kidneys and hearts obtained from normal animals were frozen and kept at −10°C. for a period of approximately 6 months. The organs were allowed to thaw at room temperature and were then mixed with an equal volume of 0.85 per cent saline and homogenized in a Waring blendor. The pH was adjusted to 6.7, and the homogenates were allowed to stand at 4°C. for 48 hours. Attempts at clarification by filtration and centrifugation were unsuccessful at this stage. Heating the suspensions at 56°C. for 30 minutes produced a coagulum. After disruption of this coagulum, ready filtration was accomplished. The clear tan to red filtrates were sterilized by passage through porcelain and were tested for activator content using techniques described in the preceding section. The

TABLE III

The Spermine Activator Content of Aqueous Extracts of Guinea Pig and Rabbit Organs

Organ extract added to the medium*	Final concentration	Inhibition of growth of tubercle bacilli		Organ extract added to the medium*	Final concentration	Inhibition of growth of tubercle bacilli	
		No spermine	5×10^{-5} M spermine			No spermine	5×10^{-5} M spermine
	per cent				*per cent*		
Guinea pig kidney	10	0‡	++++	Rabbit kidney	10	0	0
	2	0	++++		2	0	0
	0.4	0	0		0.4	0	0
Guinea pig heart	10	0	++	Rabbit heart	10	0	0
	2	0	0		2	0	0
	0.4	0	0		0.4	0	0

* See text for description of the method of preparation of the extracts.

‡ Symbols same as in Table I.

use of these extracts in concentrations higher than 10 per cent resulted in the appearance of a precipitate in the medium.

The results presented in Table III show that a substance conferring antimycobacterial properties on spermine could be extracted from guinea pig kidney and perhaps from guinea pig heart. Similar extracts of rabbit organs contained no detectable activating substance. Since the techniques used in this preliminary study were crude, the negative results are of little significance. The demonstration of spermine activator in the guinea pig kidney, however, emphasizes the fact that the distribution of this substance in the serum of various animals (Table II) does not necessarily reflect the situation in the organs or tissues of these animals.

Stability of the Activator Present in Bovine and Sheep Serum

In order to determine the stability of the spermine activator in bovine and sheep serum, serial dilutions of sera which had been exposed to a variety of

conditions were tested for their capacity to activate spermine. The tests were performed using techniques described in a previous section.

As is seen in Table IV, bovine serum stored at 4°C. over a period of many months remained unaltered in its capacity to activate spermine. Prolonged dialysis of bovine serum against many changes of distilled water did not modify its effect on the spermine–tubercle bacillus system, thus indicating that the substance responsible for this effect was stable in the absence of salts, and that it probably had a high molecular weight. The spermine-activating capacity of bovine serum was unaffected by heating at 56°C. regardless of the period of incubation or the ionic strength of the solution, within the limits studied. At temperatures higher than 56°C. loss of activity occurred, the rate of loss increasing with the duration of incubation and the height of the temperature. All activity was destroyed within a period of 1 hour at 80°C.

The capacity of sheep serum to render spermine toxic for mycobacteria remained constant at room temperature or at 56°C. for short periods of time between pH 5.4 and 7.4. At reactions more decidedly acidic or alkaline, this capacity was partially or completely lost, the rate of loss varying with the pH and the temperature.

As is shown in the table, the activating capacity of bovine serum was completely destroyed following incubation with trypsin.

The failure to pass through cellophane membranes, the inactivation at temperatures higher than 56°C., and the apparent destruction following incubation with trypsin all suggested that the spermine-activating substance was a protein.

The Spermine-Activating Capacity of Various Fractions of Bovine and Sheep Serum

In order to acquire further information pertaining to the chemical characteristics of the substance conferring antimycobacterial properties on spermine, bovine and sheep serum were subjected to various fractionation procedures, and the activity of the resulting fractions was then assayed, using the techniques described in a previous section.

Bovine serum was precipitated fractionally at 4°C. and pH 7.0 by the gradual addition of a neutral saturated solution of ammonium sulfate. The precipitates obtained at 40 per cent, 55 per cent, and 90 per cent saturation were collected by centrifugation and were dissolved in 0.85 per cent saline. Each fraction was reprecipitated one time at the corresponding original saturation of ammonium sulfate and was again collected and dissolved in saline. These final solutions were sterilized by filtration through porcelain.

Fractions of bovine serum prepared according to Cohn's method 5 were obtained through the courtesy of Armour and Company, Chicago. The dry powders were dissolved at 1 per cent concentration by stirring with 0.85 per cent saline, and these solutions were then sterilized by filtration.

Electrophoresis of sheep serum on filter paper was performed using the techniques described

TABLE IV

Stability of the Spermine Activator under Various Conditions

Preparation	Conditions of exposure	Spermine-activating capacity
Whole bovine serum	10°C. for 6 mos.	Unchanged
Whole bovine serum	Dialyzed against 10 volumes of distilled water three times at 4°C.	Unchanged
Bovine serum	1 per cent solution in distilled water at 38°C. for 24 hrs.	Unchanged
	" " " " " " " " 56 " " 4 "	"
	" " " " " " " " " " " 24 "	Slightly diminished
	" " " " " " " " 63 " " 1 hr.	Markedly "
	" " " " " " " " " " " 24 hrs.	Destroyed completely
	" " " " " " " " 80 " " 1 hr.	" "
	1 per cent solution in 0.07 M phosphate buffer pH 6.8 at 38°C. for 24 hrs.	Unchanged
	" " " " " " " " " " " " 56 " " " "	"
	" " " " " " " " " " " " 63 " " 1 hr.	Slightly diminished
	" " " " " " " " " " " " " " " 4 hrs.	Markedly "
	" " " " " " " " " " " " 80 " " 1 hr.	Destroyed completely
Sheep serum	1 per cent solution in 0.02 M phosphate buffer pH 2.4 at 20°C. for 2 hrs.	Destroyed completely
	" " " " " " " " " " 5.4 " " " " " "	Unchanged
	" " " " " " " " " " 6.7 " " " " " "	"
	" " " " " " " " " " 7.4 " " " " " "	"
	" " " " " " " " " " 10.0 " " " " " "	Slightly diminished
	" " " " " " " " " " 10.8 " " " " " "	Markedly "
	1 per cent solution in 0.02 M phosphate buffer pH 2.4 at 56°C. for 2 hrs.	Destroyed completely
	" " " " " " " " " " 5.4 " " " " " "	Unchanged
	" " " " " " " " " " 6.7 " " " " " "	"
	" " " " " " " " " " 7.4 " " " " " "	"
	" " " " " " " " " " 10.0 " " " " " "	Destroyed completely
Bovine serum	5 per cent solution in 0.85 per cent saline incubated with 0.1 per cent crystalline trypsin pH 8.5 at 38°C. for 1 hr.	Destroyed completely

by Kunkel and Tiselius (4). Preliminary experiments established the fact that the activator was stable when a 1 per cent alcoholic solution of brom-phenol blue saturated with mercuric chloride was added in sufficient quantity to impart a distinct blue color to the serum (final concentration of 0.002 per cent brom-phenol blue). Another set of preliminary experiments indicated that the activator could be eluted from serum on filter paper by soaking the paper overnight at 4°C. in saline or in mixed phosphate buffer at pH 6.8.

The techniques used in one of the experiments which involved electrophoretic separation will be described in detail.

Four sheets of Whatman No. 3MM filter paper approximately 7 by 30 cm. in size were used. A flap was cut through all sheets to facilitate placing the serum specimen on the paper. The paper was soaked briefly in 0.05 M mixed phosphate buffer at pH 7.5 and then blotted until only faintly moist. 0.5 cc. of whole sheep serum containing 0.002 per cent brom-phenol blue was then applied to the flap at the lifted leading edge. After the paper was placed between

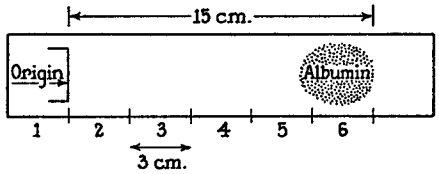

FIG. 1. Diagram to accompany Table V. At the conclusion of the electrophoretic fractionation of sheep serum described in the text, the filter paper was cut into sections as indicated in the schematic drawing above. The spermine-activating capacity of the eluates from each of these strips was determined. The stippled area labeled "albumin" corresponds to an oval area stained blue by brom-phenol blue dye bound to the albumin fraction of the serum. As is shown, the albumin fraction had migrated about 15 cm. from the original site of application of whole serum ("origin").

two glass plates measuring 8 by 20 cm., the lateral edges were sealed with silicone grease and the plates were clamped firmly together with "bulldog" paper clips. They were then placed between jars containing 0.05 M mixed phosphate buffer at pH 7.5 with the free edges of the paper dipping into the buffer solution. The entire apparatus was placed in a cold room at 4°C. and a current of 5 to 10 milliamperes was passed through the buffer solutions for a period of 24 hours. At this time the albumin had migrated 15 cm. from the origin as indicated by the position of the blue spot. Fig. 1 is a schematic drawing illustrating the appearance of the paper at the conclusion of the run and demonstrating the method of sectioning for analysis. The paper was removed and trimmed. The 15 cm. strip was divided into five 3 cm. strips, and each of these was soaked overnight at 4°C. in 5 cc. of 0.05 M mixed phosphate buffer at pH 6.8. The filter paper was then removed and discarded and the solutions were sterilized by filtration through porcelain. The activity of these specimens is recorded in Table V.

Table V presents the spermine-activating capacities of various fractions of bovine and sheep serum. It is seen that the fraction of bovine serum precipitated at 55 per cent saturation with ammonium sulfate was more active than the other fractions prepared by the salt technique. The activity of this fraction was, however, no greater than that of the original whole serum.

Of the various Cohn fractions of bovine serum, fraction IV was active in the highest dilution. A final concentration of 0.0005 per cent of dry fraction IV powder brought about inhibition of growth of tubercle bacilli when spermine was also present in the medium.

TABLE V

Spermine-Activating Capacity of Various Fractions of Bovine and Sheep Serum

Preparation added to the medium	Minimal concentration which rendered spermine inhibitory for the growth of tubercle bacilli
Ammonium sulfate fractions of whole bovine serum	
1. Fraction precipitated at 40 per cent saturation $(NH_4)_2SO_4$	0.05 per cent (as whole serum)
2. Supernatant from No. 1, precipitated at 55 per cent saturation $(NH_4)_2SO_4$	0.001" " " " "
3. Supernatant from No. 2, precipitated at 90 per cent saturation $(NH_4)_2SO_4$	5 " " " " "
Cohn fractions of bovine plasma	
I	No activation
II	" "
III	0.05 per cent (dry weight)
IV	0.0005 " " " "
V	0.01 " " " "
Fractions* separated by electrophoresis of sheep serum on filter paper	
1. Gamma globulin	No activation
2.	" "
3.	" "
4.	0.06 per cent (as whole serum)
5.	<0.005 per cent (as whole serum)
6. Albumin	0.08 per cent (as whole serum)
Whole bovine serum	0.001 per cent
" sheep "	0.001 " "

* See Fig. 1 and text for description of the fractions prepared by electrophoresis.

Because of the possibility that serum from other species might be inactive due to the presence of an antagonistic substance, and that the antagonistic substance might be removed in fractionation, ammonium sulfate fractions of rabbit serum and Cohn fractions of human serum were also tested. Like the corresponding whole sera, these fractions failed to activate spermine.

Fig. 1 and Table V illustrate the results of an experiment in which sheep serum was subjected to electrophoresis on filter paper. It is seen that the major portion of the spermine-activating capacity was recovered in the fraction mi-

grating adjacent to the albumin and corresponding in position to that usually occupied by alpha globulins. Several other electrophoresis experiments yielded similar results. In one instance nitrogen determinations were done on the eluate from a small strip which had been removed from the center of an area of high activity. After titration of this solution for its capacity to render spermine toxic for tubercle bacilli, calculations revealed that activation of spermine resulted from the inclusion of this solution in a concentration corresponding to the addition of 0.06 μg. of nitrogen to each cubic centimeter of medium.

Since many of the fractionation procedures did not result in a marked concentration of the activity as compared to the original whole serum, it seemed possible that two or more elements might be involved in the activation, and that partial loss of activity had resulted from the separation of these elements in the fractionation. To test this hypothesis whole sera were reconstituted from the fractions prepared by the various techniques. In no case was the activating capacity of the reconstituted serum greater than that of the most active fraction included therein.

The Mechanism of Action of the Spermine Activator

The experiments reported above indicated that the activator was a protein and that it was effective in very small quantities. Speculation as to the mode of action of this protein fell into three general categories:

1. The protein might act on the bacilli or on their surface to exert a toxic action in combination with spermine, or to render the bacterial cells susceptible to the toxic effects of spermine.
2. The protein might act on some constituent of the medium so as to alter conditions and render the bacilli sensitive to the action of spermine.
3. The protein might act on spermine, enzymatically or otherwise, and the product of this reaction might then be toxic for the tubercle bacillus.

Experiments were next designed to test these hypotheses. Cellophane membranes, impermeable to both the activator protein and the tubercle bacilli, were utilized to determine whether the activator would exert its effect when prevented from coming in contact with the bacteria.

Visking sausage casing was washed three times in hot distilled water in order to remove from it any substances toxic to tubercle bacilli. Preliminary experiments demonstrated that membranes treated in this fashion remained impermeable to the activator protein. Sheep serum or bovine serum was placed inside the washed cellophane casing and both ends were tied securely. The outer surface was rinsed repeatedly with distilled water to remove any contaminating serum which might have crept out during the tying. Sacs containing 10 cc. of serum were placed in sterile aluminum-capped tubes 25 mm. in diameter containing 10 cc. of water. Sterilization of the serum and cellophane was accomplished by heating these tubes overnight at 56°C.; such treatment was previously shown to have no destructive effect on the activator. The sacs were then removed and transferred aseptically to 500 cc. flasks containing 90 cc. of medium to which glucose, crystalline bovine albumin, and inoculum of tubercle

bacilli had been added in the same concentrations as those used in previous tests. Spermine was also present in the fluid outside the dialysis sac in a final concentration of 5×10^{-5} M. Appropriate controls contained water or rabbit serum inside the cellophane sac, and also omitted spermine from the outer fluid.

The results presented in Table VI demonstrate that the spermine activator exerted its effect when separated from the tubercle bacilli by a cellophane membrane. Under these conditions the activator could hardly have affected the surface of the bacterial cells or have exerted, in combination with spermine, a direct toxic action on the tubercle bacilli. The cellophane membrane was permeable to spermine, to various constituents of the medium, and to some of the metabolic products of the bacteria; it was thus probable that the serum protein acted on one of these substances. In view of the fact that even large

TABLE VI

The Spermine-Activating Capacity of Bovine and Sheep Serum When Separated from the Tubercle Bacilli in the Medium by a Cellophane Membrane

Cellophane sac containing:	Inhibition of growth of tubercle bacilli	
	Fluid outside cellophane sac containing medium, glucose, crystalline bovine albumin, inoculum of tubercle bacilli, and	
	No spermine	5×10^{-5}M spermine
Bovine serum	0*	++++
Sheep "	0	++++
Water	0	0
Rabbit serum	0	0

* Symbols same as in Table I.

amounts of bovine or sheep serum had no effect on the growth of tubercle bacilli in the absence of spermine, it seemed most likely that spermine was the dialyzable substance altered by the activator. This possibility was therefore studied.

Serum encased in cellophane sacs prepared as described above was added to flasks containing only spermine dissolved in 0.02 M mixed phosphate buffer at pH 6.7. Samples were removed from the fluid outside the sac after varying periods of incubation at 38°C. A concentrated solution of asparagine and metal salts was then added to these samples to complete the medium, which was inoculated with a dilute suspension of tubercle bacilli in glucose and crystalline bovine albumin in fashion similar to that described for previous experiments. All operations were carried out aseptically.

The results presented in Table VII show that the protein activator exerted its effect on a simple solution of spermine in phosphate buffer. Presumably the spermine moved inside the cellophane sac and was affected by the serum, and the altered spermine then dialyzed back into the outer fluid. The time needed

for the activation (approximately 1 day) under these conditions might be due to the slow rate at which the dialysis of spermine or the altered spermine proceeded, or the delay might result from an actual time factor involved in the reaction between spermine and the protein activator.

Evidence that the Protein Activator Is an Enzyme Which Alters Spermine

Preliminary experiments indicated that the antimycobacterial product formed on incubation of spermine and sheep serum retained its activity after being heated at 80°C. for 1 hour. This treatment completely destroyed the activating capacity of the serum (see previous section). These observations

TABLE VII

Evidence that the Protein Activator Affects Spermine

Inside of cellophane sac	Outside of cellophane sac	Inhibition of growth of tubercle bacilli by samples removed from fluid outside sac (and made up to complete medium) after incubation at 38°C. for:		
		1 hr.	1 day	5 days
Bovine serum	5×10^{-5} M spermine in phosphate buffer	0*	+++	++++
	Phosphate buffer	0	0	0
Rabbit serum	5×10^{-5} M spermine in phosphate buffer	0	0	0
	Phosphate buffer	0	0	0

* Symbols same as in Table I.

were utilized to design new techniques for investigating the nature of the reaction between spermine and the activator.

In testing for the formation of an antimycobacterial product, it was essential that the sheep serum be removed or inactivated before addition to the final system. In the experiments described in the preceding section, the dialysis membrane served to separate the serum from the culture medium. In order to avoid the cumbersome procedures and the limitations of the cellophane sac technique, the serum and spermine were mixed directly, and after appropriate periods of incubation, the activator was destroyed by heating at 80°C. for 1 hour. The solutions were then clarified by centrifugation if necessary, and serial dilutions were added aseptically to medium of the same final composition as that used in previous tests. Controls were always included to insure that the heating did not produce toxic substances apart from those due to the spermine-activator reaction.

The results of one such experiment are presented in Table VIII. In this experiment flasks containing 1 per cent sheep serum and 5×10^{-4} M spermine in 0.04 M mixed phosphate buffer at pH 6.8 were incubated at temperatures ranging from 4-65°C. After incubation for various periods of time, samples were removed. These samples were heated at 80°C. for 1 hour and were

then tested for antimycobacterial activity as described above. The dilutions were added so as to give final concentrations of 10, 20, and 40 per cent of the heated specimens. Since the concentration of spermine in the incubated flasks was 10 times that needed to inhibit the growth of tubercle bacilli in the presence of activator, inhibitory activity in those tubes containing 10, 20, and 40 per cent of the heated specimens would correspond to conversion of all, one-half, and one-fourth of the spermine respectively.

Control flasks handled in a manner identical to that outlined above contained sheep serum but no spermine. After heat inactivation these were tested directly and were also tested in the presence of 5×10^{-5} M spermine.

Table VIII summarizes the findings from the experiments inquiring into the influence of time and temperature on the activation of spermine. The more

TABLE VIII

The Development of Antimycobacterial Activity on Incubation of 5×10^{-4} M Spermine and 1 Per Cent Sheep Serum in 0.04 M Phosphate Buffer pH 6.8

Temperature of incubation	Antimycobacterial activity of specimens which were heat-inactivated after incubation for:			
	0 hrs.	2 hrs.	6 hrs.	24 hrs.
°C.				
4	0	0	0	++
24	0	0	+	++++
38	0	+	++	++++
50	0	+	++	++
65	0	0	0	0

0 = no antimycobacterial activity.

+ = antimycobacterial activity corresponding to activation of one-fourth of the spermine.

++ = antimycobacterial activity corresponding to activation of one-half of the spermine.

++++ = antimycobacterial activity corresponding to activation of all of the spermine.

rapid development of activity the higher the temperature, and the failure of the reaction to proceed at a temperature higher than 56°C. are findings compatible with an enzymatic reaction. The lack of activity in any of the specimens withdrawn at the start of the period of incubation attests to the fact that heating at 80°C. for 1 hour destroyed the activator. A set of control tubes, not recorded in the table, contained no spermine; after heat inactivation these specimens manifested no antimycobacterial activity when tested in the presence or the absence of added spermine.

Proof of the enzymatic nature of the reaction would, however, demand the demonstration of alteration in the proposed substrate, spermine. In order to fulfill this requirement, attempts were made to demonstrate chemically the disappearance of spermine on incubation with sheep or bovine serum. Testing

for spermine by the addition of picric acid with the formation of the insoluble spermine picrate was found to be suited to this purpose.

Spermine forms a picrate derivative which is insoluble in aqueous solutions at room temperature. Preliminary experiments revealed that a 10 per cent solution of sheep serum in 0.05 M phosphate buffer at pH 6.8 gave no precipitate when mixed with an equal volume of a saturated aqueous solution of sodium picrate at pH 6.5. When spermine was added to similar solutions of sheep serum in phosphate buffer the spermine could be detected and roughly estimated quantitatively by noting the precipitate which formed on mixing with a solution of sodium picrate. This technique readily detected spermine in a final concentration of 5×10^{-5} M. The tubes containing varying amounts of spermine and sheep serum in phosphate buffer were allowed to stand at room temperature for 1 hour after the addition of the sodium picrate solution, and the amount of precipitate formed was then graded from 0 (no precipitate) to ++++ (dense precipitate) by visual examination and comparison with controls. Attempts to adapt this method for more quantitative readings using a spectrophotometer were not successful.

TABLE IX

Demonstration of a Chemical Alteration in Spermine Following Incubation with Sheep Serum

3×10^{-4} M spermine — 1 per cent sheep serum in 0.05 M phosphate buffer pH 6.8	Spermine content as measured by precipitation of spermine picrate
Tested promptly after mixing	++++*
Incubated at 38°C. for 48 hrs.	0
Heated at 80°C. for 1 hr., then incubated at 38°C. for 48 hrs.	++++
Held at 4°C. for 48 hrs.	++

* Density of precipitate of spermine picrate graded by visual examination from 0 (no precipitate) to ++++ (dense precipitate).

The value of this method for following the disappearance of spermine was dependent on the fact that the alteration brought about by the enzyme yielded products which did not form insoluble picrates under the conditions of the test.

The data presented in Table IX indicate that spermine underwent alteration on incubation with sheep serum at 38°C. Similar changes took place more slowly at 4°C. Sheep serum which had been heated at 80°C. for 1 hour was no longer capable of attacking the spermine so as to render it soluble in the presence of a solution of sodium picrate.

In Table X further observations on the alteration of spermine under various conditions are recorded. There was a direct relationship between the concentration of sheep serum and the rate at which spermine was changed on incubation with this serum. This finding was also in keeping with an enzymatic mechanism.

The lower section of Table X shows the ability of various sera and serum fractions to attack spermine as measured by the picrate tests. Low concentra-

tions of bovine plasma fractions IV and V brought about a gradual decrease in the amount of unchanged spermine, but rabbit serum, guinea pig serum, and crystalline bovine albumin were completely inactive.

These observations were in agreement with the data presented in previous sections in which the content of spermine activator of various sera was measured in biological tests by noting the development of antimycobacterial activity. All these facts considered together make it safe to conclude that bovine and sheep sera contain an enzyme which acts on spermine. In an accompanying communication (8) it will be shown that this enzyme is an amine oxidase with properties differing from previously recognized enzymes of this classification. Presumably, some product of the reaction between this enzyme and spermine

TABLE X

Chemical Alteration in Spermine Following Incubation with Various Sera and Serum Fractions

3×10^{-4} M spermine in 0.05 M phosphate buffer pH 6.8 with a final concentration of:	Residual spermine as measured by precipitation of spermine picrate after the mixture had been incubated at 38°C. for:	
	4 hrs.	18 hrs.
0.01 per cent sheep serum	++++*	++
0.1 " " " "	+++	+
1 " " " "	+	0
10 " " " "	0	0
1 " " rabbit serum		++++
1 " " guinea pig serum		++++
0.1 " " bovine plasma fraction IV		+
0.1 " " " " " V		++
0.1 " " crystalline bovine albumin		++++

* Symbols same as in Table IX.

exerts a toxic action on mammalian tubercle bacilli. Present studies are concerned with the identification of the products of the reaction and the determination of their antibacterial properties.

DISCUSSION

Recent investigations in this laboratory have been directed toward a better understanding of the effect of local biochemical conditions in tissues on the host-parasite relationships in tuberculosis. The inhibition of growth of tubercle bacilli *in vitro* by organic acids (5), by the biological amines spermine and spermidine (1, 2), by the basic proteins lysozyme, histone, and protamine (6), and by a substance of unknown composition extracted from thymus (7) provides abundant evidence that tubercle bacilli can be suppressed in their growth or even killed by substances known to occur physiologically. This is in sharp

contrast to the widely held concept that mycobacteria possess unusual resistance to the toxic action of chemicals in general.

The task of determining whether or not spermine can act against tubercle bacilli *in vivo* has been made more difficult by findings reported in this communication—namely, that a heretofore unrecognized tissue enzyme plays an essential role in the antimycobacterial activity of spermine as observed in the test tube. Mere determinations of the spermine content of healthy and diseased tissues are of no conclusive value since it is now clear that this substance, by itself, has no effect on the growth or viability of acid-fast bacteria. It thus becomes necessary also to determine the tissue content of the enzyme which is responsible for the alteration of spermine and for the production of a substance toxic for the tubercle bacillus. Methods for measuring with reliability the amount of this enzyme present in various tissues are not yet available. Even if high concentrations of spermine and the enzyme were found in tissues, it would also be important to study the influence of local biochemical conditions in affecting the rate and eventual products of this enzymatic reaction before speculating about any role of this system in inhibiting the growth of tubercle bacilli *in vivo*.

In view of the numerous technical difficulties involved in an investigation of the factors outlined above, a more direct approach might be available *via* studies directed toward the isolation and characterization of the product of the enzymatic reaction which exerts the toxic effect on tubercle bacilli, and investigation of the occurrence and antimycobacterial activity of this product *in vivo*.

Although considerable quantities of spermine and small amounts of spermidine are known to be present in various animal and human tissues (9, 10), little is known of the origin or fate of these substances. The occurrence in animal tissues of an enzyme which has an apparently specific action on spermine and spermidine (8) is of considerable interest *per se*. Obviously, further study of this enzymatic reaction may shed light on the fate of these amines *in vivo*.

It is of interest to reconsider some of the data gathered in the previous report (1) in view of the new information relating to the mechanism of action of spermine on tubercle bacilli. The findings pertaining to the range of antibacterial action of spermine were opened to question since the bacteria resistant to the inhibitory action of the spermine system were organisms multiplying much more rapidly than the mammalian mycobacteria. Perhaps, then, cultures of these bacteria grew extensively before sufficient time elapsed for the conversion of spermine into the toxic substance. In order to investigate this possibility, tubes containing medium, glucose, spermine, and sheep serum were allowed to incubate at 38°C. for 5 days and were then inoculated with various mycobacteria and several types of non-acid–fast microorganisms. Under these conditions the results obtained were identical to those reported

previously; mammalian tubercle bacilli were the only organisms whose growth was prevented. It thus appears that bovine and human acid-fast bacteria are indeed peculiarly susceptible to the effects of some product formed during the enzymatic attack on spermine.

The new information concerning the mechanism of action also has possible bearing on the previous observations concerning the bactericidal effect of spermine on tubercle bacilli. Studies of this effect had revealed a "delayed" bactericidal action which appeared only after 2 to 3 days of incubation. It now seems likely that part if not all of this latent period may have been due to the fact that the alteration of spermine and consequent formation of a bactericidal substance took place only slowly.

SUMMARY

Spermine inhibits the multiplication of tubercle bacilli *in vitro* only if another tissue substance is present in the culture medium. This activating substance occurs as a chemical contaminant of the albumin in commercial bovine plasma fraction V ordinarily added to culture media; it is also found in whole bovine and sheep serum and in aqueous extracts of the guinea pig kidney. No detectable spermine activator is contained in whole human, guinea pig, or rabbit serum.

The serum constituent which renders spermine inhibitory for the growth of acid-fast bacteria has the properties of a protein of the alpha globulin classification. This protein is an enzyme which acts on spermine. Presumably, some product of the enzymatic reaction exerts a toxic effect on mammalian tubercle bacilli.

BIBLIOGRAPHY

1. Hirsch, J. G., and Dubos, R. J., *J. Exp. Med.*, 1952, **95,** 191.
2. Hirsch, J. G., *J. Exp. Med.*, 1953, **97,** 323.
3. Davis, B. D., and Dubos, R. J., *J. Exp. Med.*, 1947, **86,** 215.
4. Kunkel, H. G., and Tiselius, A., *J. Gen. Physiol.*, 1951, **35,** 89.
5. Dubos, R. J., *J. Exp. Med.*, 1950, **92,** 319.
6. Dubos, R. J., and Hirsch, J. G., unpublished observations.
7. Dubos, R. J., unpublished observations.
8. Hirsch, J. G., *J. Exp. Med.*, 1953, **97,** 345.
9. Dudley, H. W., and Rosenheim, O., *Biochem. J.*, 1925, **19,** 1034.
10. Hämäläinen, R., *Acta Soc. med. Duodecim*, 1947, series A, **23,** 97.