MECHANISMS INVOLVED IN THE ANTIMYCOBACTERIAL ACTIVITY OF CERTAIN BASIC PEPTIDES

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A substance isolated from calf thymus has been found to inhibit the growth of tubercle bacilli under certain conditions *in vitro* (1). Microbiological and chemical studies on this substance indicated that a basic peptide or peptides were responsible for the antimycobacterial activity (2). The present report deals with investigations designed to determine some of the mechanisms involved in the action of this thymus peptide preparation on tubercle bacilli. These studies suggest that this material exerts its effect, at least in part, by binding or by otherwise rendering unavailable to the microorganisms sulfate ion, thus depriving them of a readily available source of sulfur which is essential for their growth. Additional experiments show that some other basic peptides derived from animal tissues possess antimycobacterial activity similar to that of the thymus peptide(s).

Experimental Methods

The medium employed for the cultivation of tubercle bacilli had the following composition: asparagine, 0.2 per cent; KH_2PO_4, 0.1 per cent; $Na_2HPO_4 \cdot 12H_2O$, 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 reaction was adjusted to pH 7.0.

The tests were carried out in screw cap culture tubes measuring 16 × 100 mm. The final volume in each tube was always 2 ml. In most instances the medium described above was prepared at double strength of all ingredients and 1 ml. of this solution was placed in each of the tubes. Thymus peptide (1) dissolved and diluted to various concentrations in distilled water was then added in 0.2 ml. volumes. Unless otherwise noted in the text, solutions of the substances to be tested for their neutralizing activity were added in similar fashion and the total volume was brought to 1.8 ml. per tube with distilled water. The tubes were then capped and autoclaved at 15 pounds' pressure for 15 minutes. Appropriate control tubes were included in each experiment.

Stock cultures of an attenuated strain of bovine tubercle bacillus (BCG-Phipps) were maintained by transfer in standard tween-albumin medium every 2 weeks. Fully grown cultures of this organism were diluted 1:100 into a sterile solution of 1 per cent bovine plasma fraction V and 5 per cent glucose in 0.85 per cent saline. Each tube to be tested was then inoculated aseptically with 0.2 ml. of this bacterial suspension, resulting in a final concentration of 0.1 per cent albumin, 0.5 per cent glucose, and 10^{-3} of the fully grown stock culture of BCG-Phipps.

The tubes were incubated at 38°C. in an upright position. When growth developed, the bacilli formed white flakes or clumps which could be partially dispersed by shaking. After incubation for 10 to 14 days, readings of growth were made by visual examination and were graded on an arbitrary scale from 0 (no growth) to ++++ (heavy growth).

In several instances, duplicate experiments were conducted in which the final volume in each tube was increased to 5 ml., and the tubes were incubated in a slanting position. Tween-80 was included in these media, and the diffuse growth which resulted was measured nephelometrically in a Coleman photoelectric colorimeter. In all instances this method gave results which were essentially the same as those obtained with the visual method of estimation mentioned above.

RESULTS

The Effect of Acidic and Basic Tissue Substances on the Antimycobacterial Activity of Thymus Peptide.—The antibacterial activity of some basic compounds is neutralized when acidic tissue substances such as nucleic acids are included in the culture medium (3). Presumably, these acidic substances bind the base and thus prevent its combination with components of the bacteria. It has also been demonstrated that the biological action of certain toxic basic compounds can be antagonized by the presence of other non-toxic bases (4).

TABLE I

The Effect of Acidic and Basic Tissue Substances on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps)					
	Acidic or basic tissue substance added to the medium at a final concentration of 100 μg. per ml.					
	Heparin	Thymus nucleic acid	Yeast nucleic acid	Cadaverine	Arginine	None
μg. per ml.						
100	0*	0*	0*	0*	0*	0*
30	+	0	+	0	0	0
10	+	+	+	+	+	+
3	+++	+	+	+	+	+
1	++++	++	++	+++	+++	++
0.3	++++	++++	++++	++++	++++	++++
None	++++	++++	++++	++++	++++	++++

* Amount of growth estimated by visual examination after incubation for 10 to 14 days and graded from 0 (no growth) to ++++ (heavy growth).

In this case it is supposed that the non-toxic base competes with the toxic one for the receptor positions on or within the cell, and in this manner affords a measure of protection to the cell. In order to determine whether such phenomena were connected with the antimycobacterial activity of the strongly basic thymus peptide, various acidic and basic tissue substances were added to

the medium and their effect on the thymus peptide–tubercle bacillus system was studied.

The techniques used were those described in the preceding section. Solutions of the acidic and basic tissue compounds in water were adjusted approximately to neutrality with 0.1 N NaOH or 0.1 N HCl before they were added to the medium.

As demonstrated in Table I, the addition of 100 μg. of heparin per ml. of medium produced a slight but definite reduction in the inhibitory power of thymus peptide. However, similar concentrations of other acidic compounds (thymus and yeast nucleic acids) and of basic compounds (cadaverine, arginine) had no effect on the activity of thymus peptide. As will be seen in a series of experiments to be presented later in this communication, the antagonistic activity of heparin may well be due to its sulfur content rather than to the acidic nature of the molecule. It thus appears unlikely that the capacity of thymus peptide to inhibit the multiplication of tubercle bacilli is influenced significantly by the presence of acidic or basic substances in the environment.

Neutralization of the Antimycobacterial Activity of Thymus Peptide by Beef Heart Infusion Broth.—It was noted in earlier experiments dealing with the action of thymus peptide on tubercle bacilli (1) that the addition of beef heart infusion broth to the medium brought about a marked reduction in the antimycobacterial activity of this substance. Further experiments were therefore performed in which various modified forms of beef heart infusion broth were added to the medium, and the effect on the quantitative relationships of the inhibition of growth of tubercle bacilli by thymus peptide was observed.

TABLE II

Neutralization of the Antimycobacterial Activity of Thymus Peptide by Beef Heart Infusion Broth

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps)				
	Preparation of beef heart infusion broth added to the medium to give a final concentration of 6.6 per cent				
	None	Untreated	Alkali precipitated	Acid precipitated	"Incinerated"
μg. per ml.					
100	0*	+*	+*	+*	+*
30	0	+++	+++	++	+
10	+	++++	++++	++++	++++
3	+	++++	++++	++++	++++
1	++	++++	++++	++++	++++
None	++++	++++	++++	++++	++++

* Symbols the same as in Table I.

The beef heart infusion broth was composed of a standard beef heart infusion base to which was added 1 per cent Pfanstiehl peptone and 0.5 per cent NaCl. The preparation of the various modified forms of this broth was as follows:—

Alkali precipitated: 5 ml. of beef heart infusion broth was brought to pH 12 by the addition

of 1 N NaOH. The precipitate which formed was removed by filtration through paper. The filtrate was then adjusted to pH 7.2 with 1 N HCl.

Acid precipitated: 5 ml. of beef heart infusion broth was adjusted to pH 2.5 with 1 N HCl. The resulting precipitate was removed by filtration through paper, and the filtrate was brought to pH 7.2 by the addition of 1 N NaOH.

"Incinerated:" 10 ml. of beef heart infusion broth was evaporated to dryness by boiling in a beaker over a Bunsen burner. The residue was then heated in the beaker over the flame for an additional 10 minutes. No record was made of the actual temperature attained. After cooling to room temperature, 10 ml. of water was added. The resulting solution was clarified by filtration and was adjusted to pH 7.2 with 1 N HCl.

Table II presents the results of the experiments in which modified forms of beef heart infusion broth were used. The antimycobacterial activity of thymus peptide was diminished approximately 30-fold in the presence of any one of these tissue extract preparations. The fact that acid treatment, alkali treatment, and heating to high temperatures did not destroy the capacity of beef heart infusion broth to antagonize the action of thymus peptide suggested that the substance or substances responsible for this antagonism were soluble at acidic or alkaline reactions, and that they were more likely to be inorganic than organic compounds.

The Effect of Various Inorganic Salts on the Antimycobacterial Activity of Thymus Peptide.—The above observation immediately brought to mind the possibility that thymus peptide exerted its effect on tubercle bacilli by binding a metal ion or ions. Cation chelation is a recognized property of some polybasic compounds, and the removal of a metal ion essential for growth of the bacteria could produce the observed effects. In order to test this hypothesis further, the inhibitory activity of thymus peptide on the multiplication of tubercle bacilli was tested in media in which all the ingredients had been dissolved in tap water, and was compared with the activity in standard medium made in distilled water. Thymus peptide was approximately 10 times less active in the tap water medium. This finding strongly supported the concept that an inorganic substance, perhaps a metal ion, was capable of antagonizing the antimycobacterial action of thymus peptide.

Accordingly, numerous metal salts were added to the medium and the effect on the thymus peptide–tubercle bacillus system was observed. Among the first metal ions studied was magnesium, since this substance is thought to be essential for optimal growth of acid-fast bacteria. Table III shows the effect on the antimycobacterial activity of thymus peptide of the inclusion of increasing quantities of magnesium sulfate in the medium. It is seen that the activity of thymus peptide was inversely proportional to the concentration of magnesium sulfate.

In the course of these studies, approximately 30 inorganic salts, including some containing rare earth metals, were investigated in empirical fashion. Only 3 of these were found to antagonize the inhibition of growth of tubercle

bacilli in the presence of thymus peptide. These 3 cations were, it so happened, the only ones in the entire group which had been added in the form of sulfate salts. The question therefore arose: was the antagonism due to the sulfate ion rather than to the particular cation?

TABLE III

The Influence of the Concentration of $MgSO_4$ in the Medium on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps) in a medium containing a final concentration of $MgSO_4 \cdot 7H_2O$ of			
	0.1 μg. per ml.	1 μg. per ml.	10 μg. per ml.	100 μg. per ml.
μg. per ml.				
100	0*	0*	0*	+*
30	0	0	+	+++
10	+	+	++	++++
3	+	+	++++	++++
1	+	+	++++	++++
0.3	+	++++	++++	++++
0.1	++	++++	++++	++++
0.03	++++	++++	++++	++++
None	++++	++++	++++	++++

* Symbols the same as in Table I.

TABLE IV

The Influence of Various Magnesium Salts in the Medium on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Effect on the growth of tubercle bacilli of the addition to the medium of 50 μg. per ml. of				
	$MgSO_4 \cdot 7H_2O$	$MgCl_2 \cdot 6H_2O$	$Mg(NO_3)_2 \cdot 6H_2O$	Mg citrate	None
μg. per ml.					
10	++++*	+*	+*	+*	+*
None	++++	++++	++++	++++	++++

* Symbols the same as in Table I.

In order to determine whether the observed neutralizing capacity of magnesium sulfate was due to the anionic or cationic portion of the molecule, the effect of various magnesium salts on the antimycobacterial activity of thymus peptide was studied. These studies are summarized in Table IV. Of the several magnesium salts, the sulfate was the only one which was antagonistic to the inhibition of growth of tubercle bacilli by thymus peptide.

In Table V it is shown that the addition of sodium sulfate to the medium resulted in antagonism of the action of thymus peptide on tubercle bacilli, thus indicating strongly that the sulfate ion was indeed to be incriminated in this

phenomenon. On comparison with Table III, it is apparent, however, that more sodium sulfate than magnesium sulfate was required to produce a given degree of neutralization of thymus peptide. The reasons for this quantitative difference are unknown. Ammonium sulfate and potassium sulfate manifested neutralizing capacities comparable to the capacity of sodium sulfate.

TABLE V

Antagonism of the Antimycobacterial Activity of Thymus Peptide by the Addition of Sodium Sulfate to the Medium

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps) in the presence of Na_2SO_4 added to the medium to give a final concentration (μg. per ml.) of			
	None	30	100	300
μg. per ml.				
50	0*	0*	0*	0*
25	0	+	+	+
12.5	+	+	+	+++
6.25	+	+	++	++++
3.125	+	++	++++	++++
1.56	++	+++	++++	++++
None	++++	++++	++++	++++

* Symbols the same as in Table I.

Examination of several types of organic compounds containing sulfur revealed that some of these also antagonized the inhibitory action of thymus peptide on the growth of tubercle bacilli. Among compounds showing such neutralizing capacity were heparin, thiamin, methionine, and taurine. None of these organic molecules was as potent as the inorganic sulfate salts in counteracting thymus peptide. There were differences in the antagonistic effects of various organic compounds containing the same sulfur radical, for example sulfonic acids, thus suggesting that the capacity of these substances to antagonize the inhibitory action of thymus peptide may be related to the ability of the tubercle bacillus to metabolize the particular compound and utilize the sulfur.

The Antimycobacterial Activity of Various Peptides Derived from Animal Tissues.—It was of interest to determine whether other basic peptides found in animal tissues also inhibited the growth of tubercle bacilli under certain conditions *in vitro*.

The basic peptides derived from animal tissues were obtained from the following sources. A sample of polylysine peptide prepared from beef spleen was kindly supplied by Dr. Dennis Watson. Several samples of pituitary adrenocorticotropic hormone were tested, including ACTHAR, produced by Armour and Co., Chicago, and specimens of corticotropic hormone of varying degrees of purity which had been prepared by chromatography and countercurrent

distribution in the laboratories of the Lederle Division of American Cyanamid Corporation, Stamford, Connecticut, and which were obtained through the courtesy of Dr. Bell of that firm and of Dr. Henry Kunkel of The Rockefeller Institute for Medical Research. The posterior pituitary hormones were prepared in the laboratories of Dr. Vincent DuVigneaud, and we are indebted to Dr. DuVigneaud and to Dr. Kunkel for samples of these.

These peptides were studied for their antimycobacterial activity in duplicate tests. In one case solutions of them in sterile N/20 HCl were added aseptically to sterilized medium; in the other case, solutions of the peptides in water were added to the medium and sterilized along with it in the autoclave. The results of the tests using these two procedures were identical.

The conditions for the test and the microorganism used were the same as those outlined in the section on experimental methods.

TABLE VI

The Antimycobacterial Activity of Various Basic Peptides

Final concentration of basic peptide	Growth of tubercle bacilli (BCG-Phipps)				
	Basic peptide added to the medium				
	Thymus peptide	Spleen polylysine*	Pituitary‡ corticotropin (ACTH)	Posterior pituitary§ pressor	Posterior pituitary§ oxytocic
μg. per ml.					
100	0‖	0‖	0‖	+++‖	++++‖
30	0	+	+	++++	++++
10	+	+	+	++++	++++
3	+	++++	++	++++	++++
1	++	++++	++++	++++	++++
0.3	++++	++++	++++	++++	++++

* Kindly supplied by Dr. Dennis Watson.

‡ ACTHAR, pituitary adrenocorticotropic hormone, Armour and Co., Chicago.

§ Purified posterior pituitary hormones prepared in the laboratory of Dr. Vincent DuVigneaud, and obtained through the courtesy of Dr. DuVigneaud and Dr. Henry Kunkel.

‖ Symbols the same as in Table I.

Table VI shows the results of the examination of several basic peptides for their antimycobacterial activity. Polylysine and pituitary adrenocorticotropic hormone exhibited activity only slightly less marked than that of thymus peptide. The basic peptide hormones prepared from the posterior pituitary gland did not inhibit the growth of tubercle bacilli under the conditions of the test.

The activity of pituitary corticotropin in suppressing the multiplication of tubercle bacilli *in vitro* was studied further. Since the commercial preparation of ACTH is known to be impure, the possibility existed that the observed effect was due to a substance or substances other than the hormone itself. Tests for antimycobacterial activity were therefore made on a series of samples of pituitary adrenocorticotropic hormone which had been purified to varying

degrees using chromatographic and countercurrent distribution techniques. All these samples were active in the test system, and there was no indication that purification was accompanied by a loss of the capacity to inhibit the growth of tubercle bacilli. It thus appeared likely that the hormone molecule itself was responsible for the observed effect. This finding in turn suggested the possibility that the inhibition of growth of tubercle bacilli under these conditions might be of use as a microbiological method for the assay of pituitary corticotropic hormone. It was found, however, that preparations of ACTH which had been boiled at an alkaline reaction and thus rendered inactive in so far as hormonal effect is concerned, still possessed the same degree of antimycobacterial activity as the parent preparation which had not been treated. It was concluded, therefore, that the portion or portions of the ACTH molecule which account for the effect on the growth of tubercle bacilli are different in structure or stability from those responsible for the hormonal activity on the adrenal cortex.

Further experiments were also carried out to determine whether polylysine and corticotropin acted on tubercle bacilli through mechanisms similar to those involved in the action of thymus peptide. Both of these basic peptides exhibited the same pattern of inhibition as thymus peptide when tested against various strains of acid-fast bacteria (see reference 1). Also, like thymus peptide, the activity of polylysine and of ACTH diminished with increasing acidity of the culture medium. Finally, the antimycobacterial effect of these peptides was partially neutralized by the addition of sulfate ions to the medium. It thus seems likely that polylysine and pituitary adrenocorticotropic hormone suppress the multiplication of tubercle bacilli through mechanisms which are similar to or identical with those involved in the action of thymus peptide.

DISCUSSION

The experimental findings reported in this communication show that certain chemicals possess the property of antagonizing the antimycobacterial action of thymus peptide, polylysine peptide, and pituitary corticotropic hormone. The one feature common to all these antagonists is that they contain sulfur. In view of these findings, a hypothesis can be formulated to explain the inhibitory activity of these basic peptides on the growth of tubercle bacilli under certain conditions *in vitro*. Sulfur is undoubtedly essential for the multiplication of tubercle bacilli, and the only source of sulfur in the culture medium used in these experiments was a relatively small amount of sulfate ion. Therefore, the addition to the medium of any substance capable of binding this sulfate, or of otherwise rendering it unavailable to the microorganisms would result in inhibition of growth of the bacteria. It would then be expected that an increase in the concentration of sulfate in the medium, or the addition of other compounds containing sulfur available to the tubercle bacilli, would antagonize or

abolish the inhibition of growth. That thymus peptide and certain other basic peptides do indeed form complexes with the sulfate ion remains to be established; however, on theoretical grounds it does seem reasonable to speculate that such may be the case. Much has been done and written about the cation-chelating character of similar compounds in relation to the mediation of their biological action, but little information is available dealing with complex formation between peptides or proteins and negatively charged radicals.

Although the hypothesis presented above seems to be the most reasonable explanation for the antimycobacterial activity of these basic peptides, it should be pointed out that other possibilities exist, especially in view of the fact that even large amounts of sulfate do not completely neutralize the effect on the growth of tubercle bacilli. This antibacterial effect may, of course, be due at least in part to mechanisms as yet unrecognized. And, even if the sulfate-binding hypothesis is accepted, the recent studies of Albert *et al.* (5) have demonstrated that the antimicrobial action of chelating compounds does not always depend on simple binding activity with consequent elimination of elements essential for the growth of the microorganisms. In some cases at least, the activity of metal-binding substances appears to be related to complex formation which affects the solubility of the chelating agent and, in turn, determines whether or not it may penetrate the bacterial cell and exert a toxic action therein. It is possible that the relationship between sulfate ions and the antimycobacterial activity of certain peptides is due to a similar set of circumstances.

The high degree of antimycobacterial activity under certain conditions *in vitro* of some basic peptides derived from animal tissues, and the lack of similar activity of other basic peptides, is undoubtedly related to differences in their composition or structural configuration. The precise structural elements of importance in this regard are totally unknown.

The findings reported here do little to clarify speculation concerning the likelihood that some basic peptides may exert an influence on the fate of tubercle bacilli *in vivo*. Although the concentration of these peptides in normal and in inflamed or necrotic tissues is unknown, it seems likely that this concentration may reach levels which are, at least in some situations, higher than those which exert an inhibitory effect on the growth of pathogenic tubercle bacilli under certain conditions in the test tube. However, it is possible that some of the elements in the complex chemical environment provided by tissues may partially or completely neutralize the inhibitory activity. Compounds containing sulfur may exert an antagonistic effect *in vivo* as they do *in vitro*, but so little is known about the sulfur metabolism of mycobacteria and about the chemical make-up of inflammatory and necrotic tissue that no conclusion can be drawn.

SUMMARY

The antimycobacterial activity of thymus peptide under certain conditions *in vitro* can be partially neutralized by increasing the concentration of sulfate ions in the medium, and to a lesser extent by the addition of certain organic compounds which contain sulfur. It is suggested that thymus peptide suppresses the growth of tubercle bacilli by interfering with the normal sulfur metabolism of these microorganisms.

Polylysine peptide and pituitary adrenocorticotropic hormone, other basic peptides derived from animal tissues, also inhibit the multiplication of tubercle bacilli *in vitro*, and their antimycobacterial activity is also antagonized by sulfate ions. Basic peptide hormones prepared from the posterior pituitary gland do not affect the growth of acid-fast bacteria under the conditions of the test.

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