THE ANTIMYCOBACTERIAL ACTIVITY OF A PEPTIDE PREPARATION DERIVED FROM CALF THYMUS

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(Received for publication, August 25, 1953)

In the course of experiments concerned with the effect of the local biochemical environment in tissues on the fate of tubercle bacilli *in vivo*, it was noted that some basic proteins, for example histones, protamines, and lysozyme, inhibited the growth of mycobacteria under certain conditions *in vitro* (1). Considerable differences were observed in the antimycobacterial activity of the various samples of these substances tested. It was found furthermore that the extraction of calf thymus with aqueous ethanol yielded fractions differing from histones in chemical nature, and which possessed high activity against the growth of acid-fast bacteria. The present report deals with the characteristic features of the antibacterial action of this material. An accompanying communication (2) will describe chemical studies which indicate that the active compound is of a peptide nature.

Methods

The microbiological tests were performed in a liquid medium of the following composition: asparagine, 0.2 per cent; KH_2PO_4, 0.1 per cent; $Na_2HPO_4 \cdot 12H_2O$, 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-80 was included. All ingredients were dissolved in distilled water, and the medium was adjusted to pH 6.8.

Screw cap culture tubes measuring 16 × 100 mm. were used. Into these tubes were introduced 1.6 ml. of the medium described above and 0.2 ml. of serial dilutions in water of the product to be tested for antimycobacterial activity. The tubes were then capped and the contents were sterilized by autoclaving at 15 pounds' pressure for 15 minutes. A set of preliminary experiments established that the active factor in thymus extracts was stable under these conditions.

Except as otherwise noted in the text, the inoculum was made from stock cultures of an attenuated strain of tubercle bacillus (BCG-Phipps) maintained in standard tween-albumin medium. A 10 to 14 day old stock culture was 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. To each of the tubes in the test 0.2 ml. of this bacterial suspension was then added aseptically, yielding a final concentration of 0.1 per cent albumin, 0.5 per cent glucose, and 10^{-3} of the fully grown stock culture.

The tubes were incubated at 38°C. in the upright position. The final volume being small (2 ml.), aeration was adequate, and abundant growth developed in the control tubes in 7 to 10 days. Under these conditions the bacilli grew in the form of flakes or large clumps which could be partially dispersed by shaking. Readings of the degree of growth were made by visual

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examination, and were graded according to an arbitrary scale from 0 (no growth) to ++++ (heavy growth).

RESULTS

Fresh calf thymus was extracted in acidified water, and numerous fractionation procedures were applied to the soluble portion. The antimycobacterial activity of the resulting fractions was then assayed. Finally, a procedure was developed for the preparation of a product of reasonable purity and potent antimycobacterial activity. This procedure is described in detail below.

Ten pound of fresh calf thymus was obtained from the slaughter house. After removal of superficial fat, the thymus (3.5 kilos) was cut into small pieces (approximately ½ inch cubes) and was mixed with two parts (7 liters) of 0.2 N HCl. The mixture was stirred well and allowed to stand at room temperature for 2 to 4 days. At the conclusion of the extraction period the reaction of the mixture was approximately pH 2.5. The supernatant fluid was removed by gentle siphoning, and additional liquid was removed from the residue by filtration through one layer of gauze supported on a wire screen. The semisolid cake was discarded. The liquid portions were pooled, mixed with filter cel, and filtered through one layer of coarse paper by gravity in a large funnel. The clear or slightly opalescent filtrate was brought to pH 7.0 by the addition of 5 N NaOH. On standing at room temperature for 2 hours or more, the flocculent white precipitate which had formed was removed by filtration through paper. The filtrate was a tan, clear solution. This filtrate was mixed with an equal volume of a saturated solution of picric acid in water, at which time a massive yellow precipitate appeared. The mixture was then adjusted to pH 7.0 by the addition of 5 N NaOH, and was allowed to stand at room temperature overnight. As much of the supernatant orange-yellow liquid as possible was then removed by suction, and the precipitate was collected in the centrifuge. The precipitate was suspended in 600 ml. of 3 per cent concentrated HCl in 95 per cent ethanol and mixed well overnight at room temperature. The insoluble material was removed by filtration through paper and was discarded. The filtrate was then mixed with 10 volumes of acetone, with the formation of a flocculent white precipitate. After standing at room temperature for 2 hours or more, the supernatant was removed so far as possible by suction, and the precipitate was collected by filtration or centrifugation. This white precipitate was washed repeatedly with reagent grade acetone and air-dried. The yield from 3.5 kilos of calf thymus varied between 1.5 and 5 gm. on different occasions.

No precipitate was obtained when the technique was altered so that the original extraction took place at a pH higher than 2.5. It was also found that no material could be isolated when the thymus was boiled prior to extraction, even when the extraction was performed at pH 2.5 or lower.

The white or faintly tan powder obtained by the procedure outlined above was at first referred to as thymus factor. When later chemical studies (2) indicated its peptide nature, it was called thymus peptide. The designation thymus peptide will be used for this material hereafter in this report.

Thymus peptide was readily soluble in water, slightly soluble in methanol and ethanol, and insoluble in acetone and diethyl ether. Preliminary experiments revealed that the inhibitory activity of this substance was not altered when it was autoclaved with the medium instead of being added aseptically to sterilized medium. The dry thymus peptide powder retained its activity

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when stored at room temperature for at least 1 year, but there was some indication that solutions of it slowly lost some of their antimycobacterial activity even when stored at 10°C.

Range of Antibacterial Activity of Thymus Peptide.—The results of studies of the effect of thymus peptide on the growth of various strains of mycobacteria are presented in Table I.

TABLE I

Inhibition of Growth of Various Strains of Mycobacteria by Thymus Peptide

Final concentration of thymus peptide	Growth of mycobacteria						
	H37Rv virulent human	Vallée virulent bovine	R1Rv attenuated human	BCG-Phipps attenuated bovine	H37Ra avirulent human	Sigerson avian	Smegmatis saprophyte
µg. per ml.							
300	0*	0*	0*	0*	0*	+*	0*
100	+	+	+	0	+++	++	0
30	+	+	+	+	++++	++	+++
10	++	++	+++	+	++++	+++	+++
3	+++	++++	++++	+	++++	++++	++++
1	++++	++++	++++	++	++++	++++	++++
0.3	++++	++++	++++	++++	++++	++++	++++
None	++++	++++	++++	++++	++++	++++	++++

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

The medium used for these tests was that described in the section on methods except that the reaction was adjusted to pH 7.0. Serial dilutions of thymus peptide dissolved in water were added to this medium to give the desired final concentrations, and this mixture was then sterilized in the autoclave. Dilutions of stock cultures of various strains of mycobacteria maintained in standard tween-albumin medium were made in sterile solutions of bovine albumin and glucose, and were added to the sterilized medium to give final concentrations of 0.1 per cent albumin, 0.5 per cent glucose, and 10^{-3} of the fully grown stock cultures.

As is seen in Table I, the different strains of mycobacteria varied considerably in their sensitivity to the growth inhibition caused by thymus peptide. An attenuated strain of bovine tubercle bacillus, BCG-Phipps, was the most sensitive organism of those examined, being retarded in its multiplication by as little as 1 µg. of thymus peptide per ml. of medium. The virulent human and bovine strains tested were approximately 10 times more resistant than BCG-Phipps to the action of thymus peptide. Even larger amounts of this substance, 30 to 100 µg. per ml., were required to inhibit the growth of the avirulent human strain H37Ra, an avian strain, and a saprophytic mycobacterium.

The effect of thymus peptide on the growth of non-acid-fast microorganisms was also studied.

The medium used for these tests was the same as that employed for the cultivation of tubercle bacilli except that a small amount of yeast extract ("vegex," final concentration 0.03

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per cent) was added to enhance bacterial growth. It was established that this concentration of yeast extract did not affect the action of thymus peptide on tubercle bacilli.

When tested under the conditions described above, high concentrations (300 μg. per ml.) of thymus peptide inhibited the growth of a strain of *Micrococcus pyogenes* var. *aureus* and of *Bacillus brevis*, but numerous other bacteria grew normally even in a medium containing 1 mg. of thymus peptide per ml. Among the organisms insensitive to the action of this material were *Klebsiella pneumoniae*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Escherichia coli*, *Proteus vulgaris*, *Streptococcus hemolyticus*, and *Streptococcus fecalis*.

Influence of the Size of the Inoculum on the Antimycobacterial Activity of Thymus Peptide.—The experiments summarized in Table II were designed to determine whether the antimycobacterial activity of thymus peptide was affected by the numbers of microorganisms present in the original inoculum.

TABLE II

Influence of the Size of the Inoculum on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps) from following inocula indicated as final concentration of full grown culture			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
μg. per ml.				
30	0*	0*	0*	0*
10	++	+	0	0
3	++	+	+	+
1	++++	+++	++	++
0.3	++++	++++	++++	++++
None	++++	++++	++++	++++

* Symbols the same as in Table I.

The techniques used were similar to those described in a preceding section. The pH of the medium was 7.0. Dilutions of a stock culture of BCG-Phipps were added to the medium to yield the final concentrations of bacillary inoculum indicated in Table II.

As appears from the results in Table II, the activity of thymus peptide was essentially the same when the final concentration of inoculum was 10^{-3}, 10^{-4}, or 10^{-5} of a fully grown culture of tubercle bacilli. With larger inocula the activity of thymus peptide was moderately reduced.

Effect of the Composition of the Medium on the Antimycobacterial Activity of Thymus Peptide.—The influence of the reaction of the medium on the ability of thymus peptide to inhibit the growth of tubercle bacilli is shown in Table III.

The medium used was the same as that described previously except that the pH was adjusted to the values listed in Table III by the addition of 1 N NaOH or 1 N HCl. The pH values were measured with an industrial model Beckman pH meter. The tubes contained a final concentration of 0.1 per cent albumin, 0.5 per cent glucose, and 10^{-3} of a fully grown stock culture of BCG-Phipps as in earlier experiments.

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TABLE III

The Influence of pH of the Medium on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps) in a medium at pH:		
	6.4	6.8	7.3
µg. per ml.			
50	0*	0*	0*
25	++	0	0
12.5	++++	+	+
6.25	++++	+	+
3.125	++++	++	+
1.56	++++	++	+
None	++++	++++	++++

* Symbols the same as in Table I.

The results shown in Table III demonstrate that the antimycobacterial activity of thymus peptide increased with increasing alkalinity of the medium. At pH 7.3 it was approximately 20 times higher than at pH 6.4. These findings contrast with those reported for other tissue substances (3) which exhibit greater activity against the growth of tubercle bacilli the higher the hydrogen ion concentration.

The addition to the medium of enzymatic hydrolysate of casein or of beef heart infusion broth brought about a marked reduction in the antimycobacterial activity of thymus peptide, as is shown in Table IV.

TABLE IV

Antagonistic Effect of Hydrolysate of Casein and of Beef Heart Infusion Broth on the Antimycobacterial Activity of Thymus Peptide

Final concentration of thymus peptide	Growth of tubercle bacilli (BCG-Phipps)		
	Basal medium*	Basal medium + 0.1 per cent hydrolysate of casein	Basal medium + 0.1 per cent beef heart infusion broth
µg. per ml.			
1000	0‡	0‡	+‡
300	0	++	++
100	+	+++	++++
30	+	++++	++++
10	+	++++	++++
3	+	++++	++++
1	+++	++++	++++
0.3	++++	++++	++++
None	++++	++++	++++

* See text for a description of the basal medium.
‡ Symbols the same as in Table I.

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The composition of the medium and inoculum of tubercle bacilli were the same as in the preceding experiment. The pH of the medium was 7.0. Enzymatic hydrolysate of casein or beef heart infusion broth were added to the medium and autoclaved along with it. The preparation of casein used was Sheffield N-Z amine, type B. The beef heart infusion broth contained a standard beef heart infusion base, 1 per cent Pfanstiehl peptone, and 0.5 per cent NaCl.

A final concentration of 0.1 per cent hydrolysate of casein or of 10 per cent beef heart infusion broth in the medium reduced the power of thymus peptide to inhibit the growth of tubercle bacilli approximately 100-fold under the conditions of the test. The factors in these preparations which may account for their antagonistic effect are discussed in an accompanying communication (4).

Tween-80, which is often added to liquid media to produce dispersed growth of tubercle bacilli, did not alter the antimycobacterial activity of thymus peptide.

Of special interest was the fact that the inhibitory action of thymus peptide was essentially the same in media which contained a final concentration of 0.1 per cent or 0.5 per cent bovine albumin, or in media in which crystallized bovine albumin was used in place of plasma fraction V. The fact that thymus peptide was active in media containing crystallized bovine albumin as the only protein constituent eliminated the possibility that the inhibitory effect on the growth of mycobacteria was due, in totality or in part, to spermine. This substance, which might have been extracted from tissues by the technique used for the preparation of thymus peptide, has been shown to exert an inhibitory effect on tubercle bacilli only in the presence of spermine oxidase, an enzyme present in bovine plasma fraction V but not in crystallized bovine albumin (5).

The Effect of Thymus Peptide on the Survival of Tubercle Bacilli.—It was of interest to determine whether the effect of thymus peptide on tubercle bacilli was primarily bacteriostatic or bactericidal.

Cultures of various strains of tubercle bacilli were suspended in the same medium as that described in the first section of this report. The reaction of the medium was pH 6.8, the final concentration of thymus peptide in all tubes was 100 μg. per ml., and the bacilli were added so as to give a final concentration of 10^{-4} of culture. Tween-80 was included in the medium in a final concentration of 0.05 per cent. The suspensions were incubated at 38°C., samples were removed at intervals of time, and dilutions of them were inoculated on oleic acid–albumin agar plates in order to determine the numbers of surviving microorganisms.

The results presented in Table V reveal that thymus peptide did not exert a rapid bactericidal effect on the bacilli under the conditions of the test. After exposure for 5 days to a concentration of this material substantially higher than that which suffices to inhibit growth, the numbers of viable organisms were essentially unchanged. It is not possible to state whether the reduction in numbers of organisms observed after longer periods of exposure to thymus peptide was due to a delayed bactericidal effect, or to non-specific death of the bacteria in an environment which precluded their multiplication.

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TABLE V

The Effect of Thymus Peptide on the Survival of Mycobacteria

Strain of mycobacterium	Approximate numbers of surviving microorganisms after incubation in the presence of 100 µg. per ml. of thymus peptide for					
	1 hr.	1 day	5 days	9 days	16 days	23 days
Vallée (virulent bovine)	10^3	10^3	10^3	10^2	$<10^2$	0
H37Rv (virulent human)	"	"	"	"	"	"
R1Rv (attenuated human)	"	"	"	"	"	"
BCG-Phipps (attenuated bovine)	"	"	10^2	0	0	"
H37Ra (avirulent human)	"	"	10^3	10^2	$<10^2$	"

The Occurrence in Various Organs of Substances Resembling Thymus Peptide.—In order to determine whether the peptide with antimycobacterial properties was organ- or species-specific in its occurrence, the following experiments were done.

TABLE VI

The Occurrence of Substances Resembling Thymus Peptide in Various Organs

Organ extracted	Weight of product per 500 gm. wet organ extracted	Color of product	Concentration required to produce inhibition of growth of tubercle bacilli (BCG-Phipps)
	mg.		*µg. per ml.*
Calf spleen	110	Brown	3
Sheep thymus	190	White	3
Beef lymph nodes	60	Tan	3
Calf thymus	320	White	3
Calf lung	None	—	—
Calf liver	Trace	—	—
Calf pancreas	60	Brown	3

Fresh organs obtained from the slaughter house were cleaned, cut into small pieces, and weighed. A volume of tap water corresponding to twice the weight of each organ was added, and 1 ml. of concentrated HCl was added for each 60 ml. of total (organ plus water) volume. After 3 days at room temperature, the pH of each batch was measured and found to be 1.0 ± 0.1. From this point forward, the technique used for the fractionation and preparation was identical to that described for thymus peptide in an earlier section.

The products obtained were tested for their antimycobacterial activity using techniques similar to those described previously. The culture medium was adjusted to pH 7.0, and BCG-Phipps was employed as the test organism in a final concentration of 10^{-3} of a fully grown culture.

As is seen in Table VI, some active material was obtained from calf spleen, sheep thymus, beef lymph nodes, calf thymus, and calf pancreas, using extrac-

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tion and purification procedures similar to those for the preparation of thymus peptide. However the yield obtained from the various organs was in all cases smaller than that obtained from calf thymus under the same conditions. It is noteworthy that each of these products manifested the same degree of antimycobacterial activity as that demonstrated by thymus peptide. When calf liver and calf lung were extracted and the soluble fractions processed in identical fashion, no product was obtained, indicating either that these organs contain none of this peptide, or that different procedures are required for its isolation from them.

It is thus likely that peptides similar or identical to thymus peptide are constituents of many organs and tissues. Further studies are required to determine more thoroughly the distribution of these substances in animal tissues.

DISCUSSION

There is much evidence that the fate of tubercle bacilli *in vivo* is determined in part by biochemical conditions prevailing in the local environment. Recent studies in this laboratory and elsewhere have demonstrated that many substances or conditions which occur under physiological circumstances are capable of inhibiting the multiplication of tubercle bacilli *in vitro*, and of bringing about their death (for a recent review, see reference 6).

The observations reported in this and the accompanying communication (2) add compounds of another type, namely peptides, to the list of those which may occur in animal tissues and which are capable of suppressing the multiplication of acid-fast bacteria under certain conditions *in vitro*. Certain basic peptides derived from animal tissues have been noted to possess antibacterial activity by previous authors (7), but no reports of the effect of these or similar substances on tubercle bacilli have been encountered.

The state in which thymus peptide exists in the intact gland is, of course, unknown. Since it is strongly basic in nature (2), it is probably associated with some acidic molecule such as nucleic acid. It might be postulated that the procedure used for the extraction of thymus gland in this study leads to the acid hydrolysis of certain proteins, and that the peptide is a product of this hydrolysis rather than a natural constituent of the tissue. Although studies bearing directly on this possibility have not been done, it may be pointed out that the conditions which result in the liberation of thymus peptide are no more drastic than those commonly used in the preparation of many other strongly basic proteins.

Whether or not thymus peptide occurs as such in tissues, its possible function as one of the substances which affect the fate of tubercle bacilli *in vivo* is apparent. It is not unlikely that this peptide or related compounds are released as a result of the autolytic processes accompanying inflammation or necrosis, and thus may account, at least in part, for the limitation of growth of tubercle

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bacilli often observed in certain caseous areas. The amount of this peptide potentially available in thymus appears to be of the order of 1 per cent by dry weight, a concentration considerably higher than that sufficient to inhibit the multiplication of tubercle bacilli under certain conditions *in vitro*. However, it has not yet been shown that peptides of this nature actually occur in necrotic areas, and that they can exert *in vivo* an inhibitory effect similar to that observed *in vitro*. Their role as components of the tissue environment which affect the course of the infectious process must therefore remain speculative until further information is available.

SUMMARY

A stable, water-soluble substance which possesses potent antimycobacterial activity under certain conditions *in vitro* has been prepared from calf thymus. This substance has been tentatively named thymus peptide. In final concentrations of 1 to 10 μg. per ml. of an albumin medium it inhibits the growth of various strains of mammalian mycobacteria, but manifests only little or no inhibitory activity against a variety of other microbial species.

The ability of thymus peptide to inhibit the multiplication of tubercle bacilli diminishes when the inoculum is large, or when the medium is acidic. It is also markedly antagonized by addition of enzymatic hydrolysate of casein or beef heart infusion broth to the culture medium.

Thymus peptide does not exert a rapid bactericidal action on tubercle bacilli, but organisms exposed to this compound for longer than 2 weeks could not be made to multiply in ordinary culture media.

Substances similar or identical to the thymus peptide preparation could be extracted from calf spleen, sheep thymus, beef lymph nodes, and calf pancreas, but not from calf lung or calf liver.

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