CHEMICAL STUDIES ON A BASIC PEPTIDE PREPARATION DERIVED FROM CALF THYMUS

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The antimycobacterial activity of a substance extracted from calf thymus was described in the preceding communication (1). Final concentrations of 1 to 10 μg. of this material per ml. of culture medium inhibited the growth of mammalian mycobacteria under certain conditions *in vitro*, but did not exert a rapid bactericidal effect. Products manifesting similar suppressive effects on the multiplication of tubercle bacilli were prepared from certain organs other than the thymus by a procedure similar to that used for preparation of the thymus factor.

The present report deals with studies on the chemical nature of the substance derived from calf thymus. These studies indicate that this material contains a basic peptide or peptides which are responsible for the inhibitory microbiological activity.

RESULTS

Preparation of Thymus Factor.—The details of the procedure used for the preparation of the substance manifesting antimycobacterial properties were

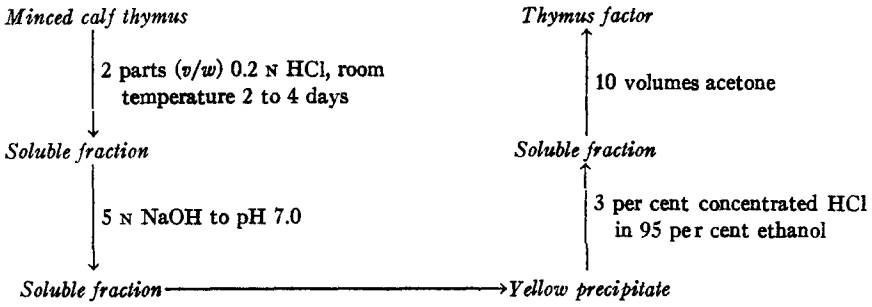

FIG. 1. Outline of the method for the preparation of thymus factor.

presented in the preceding paper (1). An outline containing the essential features of this procedure is presented in Fig. 1. The tan to white powder ob-

tained in the final step of this method will be referred to for the present as thymus factor.

Solubility of Thymus Factor.—Thymus factor was soluble in water at concentrations up to at least 1 per cent. Concentrated aqueous solutions were tan in color. In order to determine whether the solubility in water was dependent on the reaction, HCl and NaOH were added to 1 per cent solutions of thymus factor, and the solutions were observed for precipitation in various pH regions. These solutions remained clear at all acidic reactions studied. In contrast, small additions of alkali caused the solutions to become cloudy near pH 9.5, and to precipitate in the vicinity of pH 11. On the addition of further NaOH to pH 12, the precipitate dissolved to form again a clear tan solution.

A precipitate appeared in aqueous solutions of thymus factor at concentrations of ammonium sulfate higher than 80 per cent of saturation. Substances capable of inhibiting the growth of tubercle bacilli were no longer demonstrably present in the supernatant from solutions of thymus factor which had been precipitated with ammonium sulfate.

The solubility of thymus factor in organic solvents was studied by visual observations and microbiological tests.

Mixtures of thymus factor and various organic solvents were allowed to stand at room temperature overnight. Visual estimations of the degree of solution were made, and the supernatant phases were then separated and evaporated to dryness under reduced pressure. The residues were dissolved in distilled water and were examined for their capacity to inhibit the multiplication of mycobacteria, using techniques identical to those described in the preceding communication (1). For these experiments the pH of the culture medium was 7.0, and BCG-Phipps was used as the test organism in a final concentration of 10^{-3} of a fully grown stock culture.

These experiments showed that thymus factor was moderately soluble in methanol, ethanol, and *n*-propanol, but was insoluble in diethyl ether, acetone, chloroform, and benzene.

Stability of Thymus Factor.—Solutions of thymus factor were exposed to a variety of conditions, and the antimycobacterial activity was then assayed to determine whether the exposure had led to the destruction of the components responsible for this activity. The technique used for the microbiological tests was the same as that referred to and described in the section above.

As was reported in the preceding communication (1), autoclaving solutions of thymus factor buffered near pH 7.0 for 15 minutes at 15 pounds' pressure did not lead to detectable alteration in potency against tubercle bacilli. However, when solutions of thymus factor dissolved in 0.5 N HCl or in 0.5 N NaOH were autoclaved for 45 minutes at 15 pounds' pressure, all antimycobacterial activity was destroyed, indicating that the substance responsible for this activity was susceptible to hydrolysis or to other irreversible alteration on exposure to high temperatures in an acidic or an alkaline environment.

The results of studies inquiring into the effect of proteolytic enzymes on the antimycobacterial action of thymus factor are presented in Table I.

TABLE I

The Effect of Proteolytic Enzymes on the Antimycobacterial Activity of Thymus Factor

Solutions in which thymus factor was exposed to proteolytic enzymes before addition to the medium*	Growth of mycobacteria		
	Final concentration in the medium of thymus factor from solutions which were incubated at 38°C. for 2 hrs. under conditions listed in left column		
	10 μg. per ml.	1 μg. per ml.	None
0.1 per cent trypsin, 0.05 M mixed phosphate buffer at pH 8.0	++++‡	++++‡	++++‡
0.1 per cent trypsin, 0.05 M mixed phosphate buffer at pH 8.0, boiled for 1 min. prior to incubation	+	++	++++
0.05 M mixed phosphate buffer at pH 8.0	+	++	++++
0.1 per cent crystalline trypsin, 0.05 M mixed phosphate buffer at pH 8.0	++++	++++	++++
0.1 per cent crystalline trypsin, 0.05 M mixed phosphate at pH 8.0, boiled for 1 min. prior to incubation	+	++	++++
Beef spleen cathepisn (see text), 0.05 M citrate buffer at pH 3.5	++	+++	++++
Beef spleen cathepsin (see text), 0.05 M citrate buffer at pH 3.5, boiled for 1 min. prior to incubation	++	+++	++++
0.05 M citrate buffer at pH 3.5	+	++	++++
0.05 per cent 1:10,000 pepsin, 0.01 N HCl	+	++	++++
0.05 per cent 1:10,000 pepsin, 0.01 N HCl, boiled for 1 min. prior to incubation	+	++	++++
0.01 N HCl	+	++	++++

* See text for complete description of the experimental method used.

‡ Amount of growth after incubation at 38°C. for 10 days estimated by visual examination and graded from 0 (no growth) to ++++ (heavy growth).

The enzymes were obtained from the following sources: crude trypsin, Fairchild Bros. and Foster, New York City; crystalline trypsin, Worthington Biochemical Laboratory, Freehold, New Jersey; cathepsin, prepared from beef spleen according to Fruton and Bergmann (2); pepsin, Cuhady pepsin, 1:10,000, lot 1464.

The crystalline trypsin was dissolved in 0.01 N HCl at a concentration of 0.2 per cent and was dialyzed against several large volumes of 0.01 N HCl in order to remove salt adsorbed during the crystallization. The stock solution of cathepsin was a 0.2 per cent solution in saline of the wet filter cake obtained in the procedure outlined by Fruton and Bergmann. The activity of the trypsin and cathepsin solutions was established in preliminary tests.

For the experiments reported in Table I, thymus factor was dissolved at a concentration of 0.1 per cent in each of the following solutions: (*a*) 0.1 M mixed phosphate buffer at pH 8.0, (*b*) 0.1 M sodium citrate-HCl buffer at pH 3.5, and (*c*) 0.01 N HCl. The phosphate buffer solutions were mixed with an equal volume of a solution containing 0.2 per cent trypsin in water and also with an equal volume of 0.2 per cent dialyzed cystalline trypsin in 0.01 N HCl. The citrate buffer solutions were mixed with an equal volume of a 1:2 dilution of the stock solution of beef spleen cathepsin. The thymus factor in 0.01 N HCl was mixed with an equal volume of a 0.1 per cent solution of 1:10,000 pepsin in 0.01 N HCl. These mixtures were divided into 2 equal parts, 1 of which was promptly boiled for 1 minute to inactivate the enzymes and thus serve as a control. Other control tubes included those in which the enzyme was omitted entirely, and those in which thymus factor was omitted but the appropriate buffer and enzyme solutions were used.

These mixtures were then incubated at 38°C. for 2 hours. After clarification by centrifugation as necessary, they were added in various dilutions to culture medium and autoclaved along with it. The conditions used for the testing of these solutions on the growth of tubercle bacilli were the same as those described in preceding sections.

As is seen in Table I, the ability of thymus factor to inhibit the growth of tubercle bacilli was destroyed by incubation with crude or crystalline trypsin. Cathepsin prepared from beef spleen partially inactivated thymus factor, but it should be noted that this same effect was brought about by cathepsin solutions which had been boiled prior to the incubation. This finding suggested that the antagonistic effect of the cathepsin preparation was not due to its proteolytic enzymatic activity, but rather to its content of heat-stable substances which are capable of partially neutralizing the antimycobacterial activity of thymus factor (see reference 3). Under the conditions of the test, incubation with pepsin did not alter the biological action of solutions of thymus factor.

Qualitative Chemical Examination of Thymus Factor.—The results of qualitative chemical tests performed on thymus factor and on acid-hydrolyzed thymus factor are presented in Table II.

For the acid hydrolysis 200 mg. of thymus factor was dissolved in 5 ml. of 33 per cent sulfuric acid. The tube was covered with an aluminum cap and placed in a stream of flowing steam overnight. The following day the solution was autoclaved for 1 hour at 15 pounds' pressure. The 5 ml. of acid solution was then mixed with 95 ml. of a hot solution containing 9.96 gm. of $Ba(OH)_2 \cdot 8H_2O$. The barium sulfate precipitate was removed by filtration through fine paper, and the filtrate was examined for its qualitative chemical reactions.

The data in Table II demonstrate that thymus factor was composed of a substance or substances in the general classification of proteins, and that it contained no carbohydrate or phosphorus. In addition, the reactions indicated that the amino acids arginine, tryptophan, and tyrosine were probably present.

Fig. 2 shows the ultraviolet absorption spectrum of a sample of thymus factor dissolved in water at a concentration of 0.4 per cent. This examination was performed in the Cary continuous recording spectrophotometer through the courtesy of Dr. Herbert Jaffe. As illustrated in the figure, the absorption pattern was typical of substances containing aromatic amino acids.

TABLE II

Qualitative Tests on Thymus Factor

Test*	Thymus factor		Probable significance
	Whole	Hydrolyzed	
Xanthoproteic	+	+	Benzene nucleus present
Biuret	+	−	Amide linkage present prior to hydrolysis
Millon's	+	+	Tyrosine present
Ninhydrin	+	+	α-amino carbonyl pattern present
Ehrlich's diazo	+	+	Tyrosine or histidine present
Sakaguchi	+	+	Arginine present
β-Nitroso-α-naphthol	+	+	Tyrosine present
Ehrlich's *p*-dimethylamidobenzaldehyde	+	−	Tryptophan present
Molisch	−	−	No carbohydrate
Phosphorus	−	−	No phosphorus

* These tests were performed using the techniques outlined in reference 4.

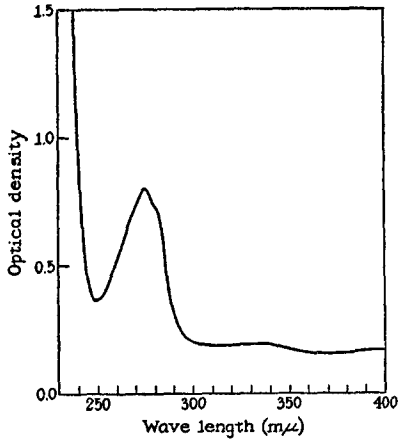

FIG. 2. The absorption of ultraviolet light by a solution of thymus factor. This pattern was obtained in a Cary recording spectrophotometer through the courtesy of Dr. Herbert Jaffe. A cell 1 cm. in length was used, and this cell was filled with a 0.4 per cent solution of thymus factor dissolved in distilled water.

Molecular Size of Thymus Factor.—In numerous experiments employing the microbiological activity of thymus factor as a detection system, it was found that this substance passed readily through cellulose casings at acidic, neutral, or alkaline reactions. This finding suggested that the molecular weight of the substance concerned in the effect on the growth of tubercle bacilli was less than 10,000. No actual studies of molecular weight were done, but the behavior on

dialysis, the solubility in certain organic solvents, and the formation of a stable picrate derivative (see reference 1) all indicated that the size of the thymus factor molecule was, for a protein-like compound, relatively small.

It thus became apparent that the preparation called thymus factor contained a peptide or peptides which accounted for the antimycobacterial activity. The qualitative chemical tests, the inactivation by acid or alkaline hydrolysis or by trypsin digestion, and the precipitation by concentrated solutions of ammonium sulfate established clearly the protein nature of this substance, and the permeability of cellophane membranes to it led to the conclusion that the compound(s) had a molecular weight placing it in the peptide classification. The name thymus factor, which had been applied at first, was accordingly discarded in favor of the more descriptive term thymus peptide.

Purity of Thymus Peptide.—One of the major difficulties encountered in further study of the chemical nature of thymus peptide was that of establishing the purity (or impurity) of the preparation at hand.

Repeated attempts to crystallize thymus peptide met with failure. Among the conditions employed were variations in the concentration of thymus peptide, temperature, alkalinity or acidity of the solution, the use of certain organic solvents and solvent mixtures, and the use of various salts. Although precipitation could be varied in its rapidity and degree, crystals were never obtained.

During the course of these attempts to crystallize thymus peptide, solutions of it were of course divided into several fractions under a variety of conditions. In many instances these fractions were recovered and examined in quantitative fashion for their antimycobacterial activity. In no case was any of these fractions appreciably more or less active against tubercle bacilli than was the parent thymus peptide preparation. These findings suggested that the thymus peptide powder was of reasonable purity, but in view of the relatively low degree of sensitivity of the microbiological test, they did little to establish purity in a chemical sense.

The behavior of thymus peptide under the influence of an electrical field was studied in the hope that some evidence of homogeneity or heterogeneity would be obtained. Dr. Henry Kunkel kindly examined a sample of this substance by the zone electrophoresis technique (5). In this apparatus thymus peptide migrated as a strongly basic compound and as a single spot with a small amount of tailing on the paper. This observation indicated that the preparation was fairly homogeneous, but it was not possible to decide if the tailing was due to impurities or to adsorption phenomena between the basic peptide and the paper. Dr. Lewis Longsworth also graciously studied a sample of thymus peptide to determine its behavior under conditions of free electrophoresis. Here again the substance migrated as a strongly basic compound and the main peak was followed by a considerable amount of tailing. Since the preparation could not be dialyzed prior to the electrophoretic run, and since experience is

limited in the examination of relatively low molecular weight substances in free electrophoresis, it was not possible to decide with any degree of assurance whether the pattern indicated that the thymus peptide preparation was pure or impure.

Finally, a series of studies was done to determine whether thymus peptide could be purified, or at least analyzed for purity, using column chromatographic techniques. The kindness and wise counsel of Drs. William Stein and Stanford Moore were of great assistance during these experiments.

Basic compounds of protein nature have been purified by column chromatography using the carboxylic acid resin IRC-50. The behavior of thymus peptide was therefore studied in the presence of IRC-50 under a variety of conditions.

The preparation of the resin and the conditions used for the determination of distribution coefficients were identical to those described by Hirs, Moore, and Stein (6). Chemical tests used for quantitation of the concentration of thymus peptide in solution were the ninhydrin reaction (7) and the new Sakaguchi reaction for arginine (8). The method described by Sakaguchi was modified in that the optical densities of the solution were determined in a Coleman Junior photoelectric colorimeter at a wave length of 500 mμ. It was also necessary to construct calibration curves for this determination, since the optical density at 500 mμ was not strictly proportional to the concentration of arginine.

Distribution coefficients for thymus peptide–IRC-50 mixtures were determined in a variety of buffer systems (dibasic sodium phosphate, sodium borate, sodium carbonate–sodium bicarbonate) at reactions between pH 7.0 and pH 10.5, and at ionic concentrations varying from 0.1 to 0.5 molar. With any one of these buffer systems, conditions were found wherein thymus peptide was partially adsorbed to the resin and thus gave a finite distribution coefficient; however, on reequilibration with fresh buffer of the same composition there was no redistribution of the peptide bound to the resin. These test tube experiments thus indicated that attempts to purify thymus peptide on columns of IRC-50 would be to no avail.

The adsorption of thymus peptide on IRC-50 was employed in another manner in order to gain some insight into the purity of this preparation. We are indebted to Dr. William Stein for suggesting these experiments.

For these experiments, and for the quantitative analysis and the studies of amino acid composition to be reported below, a sample of thymus peptide was used which had been dissolved in methanol and reprecipitated with acetone. 500 mg. of thymus peptide powder was mixed with 100 ml. of reagent grade absolute methanol. After stirring at room temperature for 15 minutes, the small amount of undissolved residue was removed by filtration through paper. The filtrate was mixed with 200 ml. of reagent grade acetone and 3 ml. of chemically pure concentrated HCl. On mixing a heavy white precipitate appeared. This precipitate was collected on hard filter paper and was then washed repeatedly with reagent grade acetone and air-dried. The yield was 110 mg. of a white powder. Although this powder was not significantly more active than the original thymus peptide preparation in inhibiting the multiplication of tubercle bacilli, it was thought that the solution in methanol and precipitation with acetone might have removed or reduced in amount any contaminating inorganic materials.

A batch of IRC-50 was thoroughly equilibrated with 0.2 M Na_2HPO_4 at pH 8.9. This resin was washed with 0.2 M Na_2HPO_4 in a graduated 50 ml. centrifuge tube and manipulated so that the volume of packed resin measured 25 ml. The supernatant was poured off and replaced by 25 ml. of a 0.2 per cent solution of thymus peptide (treated by methanol extraction and acetone precipitation as described above) dissolved in the same 0.2 M Na_2HPO_4 buffer. This mixture was then agitated for 10 minutes during which time the thymus peptide became distributed between the resin and the buffer solution. After centrifugation the supernatant was removed, filtered through fine paper, and saved for subsequent examination. The resin in the centrifuge tube was then washed ten times with 25 ml. portions of 0.2 M Na_2HPO_4 in order to remove all the thymus peptide except that actually bound to the resin. Next 25 ml. of 0.36 M Na_2HPO_4 buffer was added to this washed resin and the mixture was agitated for 10 minutes. Since 25 ml. of packed resin retains approximately 15 ml. of liquid, the final ionic concentration of the liquid phase was about 0.3 M in Na_2HPO_4. After equilibration the supernatant was once more removed, filtered, and saved for analysis. The resin pack was then washed with 10 volumes of 0.3 M Na_2HPO_4. 25 ml. of 0.62 M Na_2HPO_4 was then mixed with the resin in order to produce a fluid phase approximately 0.5 M in Na_2HPO_4. After equilibration for 10 minutes, the liquid phase was again collected and saved for tests.

The assay for antimycobacterial activity, ninhydrin color reactivity, and arginine content was done using techniques described in earlier sections. Nitrogen was determined by the standard micro-Kjeldahl method. Optical densities in ultraviolet light were measured in a Beckman spectrophotometer.

TABLE III

Heterogeneity of Thymus Peptide Preparation as Indicated by Examination of Eluates from Material Adsorbed on Ion Exchange Resin

Supernatant solution in system* at equilibrium containing thymus peptide and	Ratios‡ of values obtained in testing for				
	Antimycobacterial activity	Ninhydrin color	Sakaguchi color	Nitrogen	Absorption at 275 mμ
0.2 M Na_2HPO_4 only (starting solution)	8	6	6.5	4.2	—
0.2 M Na_2HPO_4 + IRC-50	1	2.6	3.4	2.4	3.8
0.3 M Na_2HPO_4 + IRC-50	1	1	1	1	1
0.5 M Na_2HPO_4 + IRC-50	2	1.8	0.9	1.2	0.9

* See text for details of the experimental procedure.

‡ The value obtained for the supernatant in 0.3 M Na_2HPO_4 + IRC-50 was taken as unity, and the ratios of the other values were then calculated.

Preliminary experiments had shown that thymus peptide which was adsorbed onto IRC-50 at a given ionic concentration was at least partially eluted from the resin when the ionic concentration of the buffer was increased. By the procedure outlined above the following samples were obtained: (*a*) a solution of thymus peptide not exposed to IRC-50, that is, the starting solution; (*b*) a solution which contained thymus peptide not adsorbed to IRC-50 in 0.2 M Na_2HPO_4 buffer; (*c*) a solution of thymus peptide adsorbed to the resin in 0.2 M Na_2HPO_4 buffer but eluted from the resin in 0.3 M Na_2HPO_4 buffer; and (*d*) a solution containing thymus peptide held on the IRC-50 in 0.2 and in 0.3 M Na_2HPO_4 but eluted in 0.5 M Na_2HPO_4 buffer. These samples were then an-

alyzed for antimycobacterial activity, nitrogen content, arginine reactivity, ninhydrin reactivity, and adsorption of ultraviolet light at 275 mμ. If the thymus peptide preparation were indeed homogeneous, it would be expected that the ratio between these various values would be the same in all specimens. However if two or more compounds were present in the original preparation, it would be predicted that the ratios would differ, since even closely related compounds have different distribution characteristics on ion exchange resins under differing conditions.

As is shown in Table III, the ratios between the four solutions were in general similar, but there were also apparent differences. Since these differences in many instances exceeded the calculated maximal variation which might result from the manipulations and from the methods used, it was concluded that the thymus peptide preparation was heterogeneous. Further evidence in support of this conclusion is presented in a later section dealing with studies of the amino acid composition of this substance.

Since the thymus peptide preparation is soluble in certain organic solvents, it is possible that it might be purified using countercurrent distribution techniques. This possibility has not as yet been investigated.

Quantitative Analysis of the Thymus Peptide Preparation.—Despite the evidence of impurity of the thymus peptide preparation, quantitative chemical studies were done to characterize its composition more thoroughly. For these quantitative tests, material was used which had been dissolved in methanol and reprecipitated with acetone (see preceding section). These tests, done by Mr. Theodore Bella, gave the following results:

Ash	None
Carbon	45.27 per cent
Hydrogen	7.25 per cent
Nitrogen	18.42 per cent
Chlorine	11.78 per cent
Sulfur	0.7 per cent
Oxygen	16.58 per cent (by difference)

Thymus peptide was recognized to be a strongly basic organic compound, and since it was prepared by precipitation from a medium containing an excess of HCl, the substance analyzed (and used in all the investigations reported in the previous sections) was undoubtedly the hydrochloride salt of an organic base. If one corrects the above results of the quantitative analysis for the calculated HCl content, the values for the free base become:—

Carbon	51.5 per cent
Hydrogen	8.2 per cent
Nitrogen	20.9 per cent
Sulfur	0.8 per cent
Oxygen	18.6 per cent (by difference)

Amino Acid Composition of Thymus Peptide.—In order to define the chemical nature of the thymus peptide preparation more completely, studies were done of the amino acid composition of this substance.

In the first series of experiments on this point, thymus peptide was hydrolyzed with HCl (see previous section for details) and the hydrolysate was then subjected to analysis by paper strip chromatography using techniques outlined by previous workers (9). These studies indicated that arginine, glycine, alanine, leucine, and lysine were present in the hydrolysate, and suggestive evidence was obtained that glutamic acid and phenylalanine were also present. However, the paper strip chromatographic technique was found to be unsatisfactory for clarifying one aspect of special interest, namely the quantitative basic amino acid composition of thymus peptide.

Drs. William Stein and Stanford Moore again kindly offered their assistance, and determined the amino acid composition of a sample of thymus peptide hydrolyzed with HCl. These determinations were performed by column chromatography on ion exchange resins, using the general techniques developed in the laboratory of Drs. Moore and Stein (10), with modifications described in detail in a forthcoming publication (11).

For these studies, a sample of thymus peptide preparation was used which had been dissolved in methanol and precipitated with acetone (see previous section). 9.907 mg. of material which had been dried *in vacuo* at 105°C. was dissolved in 2 ml. of glass distilled 6 N HCl in a glass tube. This tube was then sealed and heated at 110°C. for 20 hours. No humin appeared. The fluid was transferred quantitatively to a flask and evaporated to dryness *in vacuo*. The residue was dissolved in water and the evaporation to dryness was repeated. The residue was then taken up in a small amount of water and transferred quantitatively to a 10 ml. flask. 0.2 ml. of N NaOH was added, bringing the pH to a level higher than 8.0, and the solution was allowed to stand at room temperature for 4 hours to insure reconversion of cystine. Then 0.2 ml. of N HCl was added and the volume brought to 10 ml. with water. 2 ml. of this solution was used for the amino acid analysis by the column chromatographic technique (10, 11).

The results of the analysis of the thymus peptide preparation for its amino acid composition are presented in Table IV. Several features of these results deserve comment. First of all, the total yield of amino acid residues (*i.e.* the sum of the yield of each amino acid less the calculated portion of its weight due to water added during the hydrolysis) was very near the weight of the starting material, thus indicating that the thymus peptide preparation was not contaminated with appreciable amounts of non-nitrogenous substances, or with large amounts of free amino acids. With the technique used, all the common amino acids were determined with the exception of tryptophan, which was destroyed by the acid hydrolysis. Considering the yield of amino acid residues, it seems unlikely that much tryptophan was present in the thymus peptide preparation, even though a positive qualitative test for tryptophan was obtained (see earlier section). Also noteworthy was the complete absence of

cystine, indicating that the thymus peptide preparation was free of the many proteinaceous substances containing this amino acid. The most striking feature of the amino acid pattern was, of course, the high content of the basic amino acids lysine and arginine, a finding in keeping with the earlier impression that thymus peptide was strongly basic in nature.

TABLE IV

*The Amino Acid Composition of the Thymus Peptide Preparation**

Amino acid	Amount of amino acid residue‡ per 100 gm. thymus peptide (free base) hydrolyzed	Molar ratios, assuming 1 molecule of aspartic acid residue per molecule of thymus peptide (free base)
	gm.	
Aspartic acid	2.7	1.0
Glutamic acid	5.8	1.9
Glycine	7.0	5.4
Alanine	9.8	5.9
Valine	3.9	1.7
Leucine	4.0	1.5
Isoleucine	2.5	1.0
Serine	4.2	2.1
Threonine	4.7	2.0
Methionine	0.3	0.1
Proline	7.0	3.1
Phenylalanine	1.0	0.3
Tyrosine	1.9	0.5
Histidine	3.4	1.1
Lysine	22.8	7.6
Arginine	17.7	4.9
Total	98.7	

* The results in this table are based upon a single analysis of a 20 hour hydrolysate, with approximate corrections applied for the partial destruction of serine and threonine.

‡ $\left[\begin{array}{c}\text{Gm. amino acid obtained per 100} \\ \text{gm. thymus peptide (free base)}\end{array}\right] \times \frac{\text{Molecular weight amino acid} - 18}{\text{Molecular weight amino acid}}$; correction made to compensate for H_2O added on hydrolysis of peptide chain to liberate free amino acids.

In the second column of Table IV, the amino acid yields are converted into molar ratios. For this purpose it was arbitrarily assumed that there might be 1 molecular residue of aspartic acid per molecule of thymus peptide, an assumption which, if correct, would signify a molecular weight of about 4300 for this peptide. It is seen in the table that a few of the amino acids were present in approximately integral molar ratios; however, the absence of such a whole number relationship between the molar ratios of many of the other amino acids added weight to the evidence presented earlier that the thymus peptide preparation was not homogeneous.

DISCUSSION

The findings reported in this communication establish that the antimycobacterial activity of a substance prepared from calf thymus is due to a peptide or peptides. Since these peptides have not been isolated in pure form it is not possible to make categorical statements about their other chemical characteristics. However, in view of all the evidence, including the method of preparation, it seems safe to conclude that this peptide fraction is basic, with a high content of lysine and arginine.

Although it was not possible to ascertain the degree of impurity of the thymus peptide preparation, the amino acid analysis suggests that this material is a mixture of peptides, and that the complexity of this mixture is probably not great. This suggestion is based on the observation that there is good agreement, in terms of whole number molar ratios, between some of the amino acids, and also on the fact that cystine, a common constituent of proteins and peptides, is completely absent. More reliable information about the composition of thymus peptide must obviously await its purification.

If indeed the thymus peptide preparation is a mixture of two or more closely related compounds, perhaps more than one of these compounds possesses the capacity to inhibit the growth of tubercle bacilli under certain conditions *in vitro*. Since many of the attempts to fractionate and to purify thymus peptide were evaluated solely by assay of the antimycobacterial activity of the products, it is thus possible that at least some measure of purification, in a chemical sense, was achieved but went unrecognized.

The quantitative analysis of the thymus peptide preparation revealed that it contained 0.7 per cent sulfur, yet from the amino acid analysis only about one-tenth of this sulfur was accounted for. Although this disparity is at present unexplained, it might be pointed out that another series of experiments (3) has suggested that thymus peptide has some special affinity for sulfate ions. Perhaps then, a small amount of sulfate was bound by the peptide during its preparation or manipulation.

From the findings obtained thus far thymus peptide appears to differ from other basic peptides previously prepared from animal tissue. Polylysine peptide, first extracted from calf thymus by Bloom and coworkers (12) is similar in some regards to thymus peptide; however thymus peptide and polylysine are notably different in their behavior on dialysis, in their arginine content, and in their ultraviolet light absorption patterns. Also, as reported in an accompanying communication (3), the thymus peptide preparation has somewhat greater antimycobacterial activity than polylysine.

It is of course possible that thymus peptide does not occur as such in animal tissues. Since the extraction was performed at an acid pH, thymus peptide may be a product of partial hydrolysis of histones, basic proteins present in large

amounts in thymus. It should also be pointed out that, in all likelihood, compounds similar or identical to the thymus peptide preparation may be extracted from organs other than calf thymus. In an accompanying communication (1) it was demonstrated that products exhibiting potent antimycobacterial activity under certain conditions *in vitro* could be extracted from many bovine organs and from sheep thymus using techniques similar to those developed for the preparation of thymus peptide. No study has been made, however, of the chemical nature of these products from sources other than calf thymus.

Nothing is known concerning the function or the biological activity of these peptides derived from the thymus. Up to 10 mg. of thymus peptide has been injected intraperitoneally into Swiss mice, with no discernible change appearing in the mice in the following 24 hours. It is therefore unlikely that thymus peptide bears any similarity, in terms of a hormone-like effect or of toxicity, to other basic peptides prepared from the pituitary gland or from other animal tissues.

SUMMARY

A substance possessing antimycobacterial activity under certain conditions *in vitro* has been prepared from aqueous extracts of calf thymus. Chemical studies have demonstrated that the activity of this substance is due to a basic peptide or a mixture of basic peptides. Although this thymus fraction has been shown to be essentially free of compounds other than peptides, it has not been obtained in a homogeneous state.

The thymus peptide preparation is soluble in water and in the lower alcohols. Its solubility is minimal between pH 10 and 11, suggesting that its isoelectric point may be in this vicinity. The microbiological activity of thymus peptide is destroyed by acid or alkaline hydrolysis and also by trypsin digestion, but is unaffected by pepsin digestion. Cellulose membranes are permeable to thymus peptide.

The most noteworthy finding concerning the amino acid composition of thymus peptide is the preponderance of the basic amino acids lysine and arginine, which together account for about 40 per cent of the weight of this substance. No cystine, and only trace amounts of other amino acids containing sulfur, are present in the thymus peptide preparation.

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