Characterization of an Antibacterial Peptide from Calf Thymus.* (22728)

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Many investigators have succeeded in extracting antimicrobial substances from various normal tissues during the past several decades. Some of these factors have been shown to be basic proteins or peptides. Bloom *et al.*(1) obtained an anthracidal substance from calf thymus and other tissues. This material was found to be stable to acid and heat, of polypeptide nature, and basic due to the presence of a large amount of lysine. That this antibacterial peptide was derived from a histone has been suggested(16). A mycobactericidal thymus peptide has been described in a series of papers by Dubos and Hirsch (5,7,8). Upon chemical analysis it was shown to be basic, possessing large amounts of lysine and arginine, and having an isoelectric point of 10-11.

This investigation is concerned with further studies and characterization of the basic thymus peptide of Bloom and co-workers(1).

Methods and materials. The tissue peptide was prepared from fresh calf thymus according to the method of Bloom and Prigmore(3) with one modification. The final S-IV product was brought to half saturation with ammonium sulfate at room temperature and pH of 6.0. The supernatant solution was dialysed free of salt and then lyophilized and weighed. Stock solutions of the thymus peptide were made up in distilled water at a concentration of 0.1%. Hyaluronic acid, prepared from type 28 streptococci, was furnished by Dr. Y. Ginzburg of this laboratory, glutamyl polypeptide of the anthrax bacillus was supplied by Dr. R. D. Housewright, and desoxyribonucleic acid was purchased from Nutritional Biochemicals Co. Stock solutions of the various acidic polymers were prepared in distilled water to contain 5 mg/ml. Blockage of antibacterial activity was examined when the acidic macromolecules were added to the test systems just prior to inoculation with the microorganisms. Turbidity readings were made every 2 hours for a 10 hour period, during which time the ability of any particular acidic polymer to reverse growth inhibition could be observed. Normal growth and growth inhibition controls were included with each experiment.

Preliminary experiments were carried out to examine differences in the antibacterial capacity of the thymus peptide against 2 encapsulated and non-encapsulated species. A Group A, type 28 streptococcus and a strain of *Bacillus anthracis* were tested. Thermostability of the peptide was tested in distilled water at acid, neutral and alkaline pH. The pH for greatest activity was determined using phosphate buffered broth at the pH values of 6.7 and 8.0. Electrophoretic mobility of the product was measured using cacodylate buffer (pH 7.0) at an ionic strength of 0.08. An ultraviolet absorption pattern was run using a 0.05% solution of the thymus peptide. An amino acid analysis was performed following the method of Stein and Moore(11,12). The Dowex 50-X4 resin (-400) was used in the column. The thymus peptide sample was weighed from the lyophilized state in the amount of 63.5 mg. The product was hydrolyzed in a vacuum-sealed glass tube with 3.5 ml of constant boiling hydrochloric acid for 48 hours at 110°C. The hydrolysate was carefully washed from the tube into a round bottom flask and vacuum distilled with 3 additions of distilled water. After the third distillation the residue was brought down to approximately a 1 ml volume and carefully washed into a 25 ml volumetric flask with citrate buffer, pH 3.1, and brought to volume with buffer and distilled water. The final pH of the diluted sample was 2.2. The sample was then filtered through a fiber glass pad and stored at 4°C until used. The total nucleic acid content of the thymus peptide was determined from the phosphorus value(6).

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Desoxyribonucleic acid was measured by a modification of the Dische test(14). The ribonucleic acid value was computed by subtraction of the Dische test result from the total nucleic acids. Total carbohydrate was measured by the anthrone test(13) after deproteinization with trichloroacetic acid, and was based on glucose equivalents. The total protein content of the thymus peptide was obtained from the amino acid assay and by the microKjeldahl method(9).

Results. The *in vitro* antibacterial potency of the thymus peptide was quite high. The final step in the preparation of the active fraction, which involved removal of precipitable materials at half saturation with ammonium sulfate, concentrated activity 2 to 3 times. The outgrowth of a staphylococcus was inhibited for 8 hours by 5 μg thymus peptide per ml of broth.

The acidic macromolecules blocked the antibacterial action of the tissue peptide when the ratio of acid to peptide was equal or greater. The addition of 0.5 to 1.0% NaCl to any of the test systems interfered with the capacity of the acidic polymers to block antibacterial activity of the tissue peptide. In the correlative study with encapsulated and non-encapsulated streptococci and anthrax bacilli it was found that the encapsulated forms were less strongly inhibited by the thymus peptide than the rough forms, particularly in the case of the anthrax organisms. However, the differences in degree of inhibition between smooth and rough streptococci were not always significant.

The peptide was found to be very active at pH 8.0 but its concentration had to be increased 10-fold to approach the same degree of antibacterial effectiveness at pH 6.7. From the electrophoretic determinations the material was found to migrate toward the negative pole at pH 7.0. The mobility was calculated to be 6.8 x 10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$.

The peptide was active against staphylococci, Group A streptococci and anthrax bacilli but not type II pneumococci. While it inhibited *Escherichia coli, Salmonella typhosa* and *Vibrio comma* were not affected markedly by its antibacterial action. The ultraviolet absorption pattern showed one peak at 264 mμ indicating a protein-nucleic acid complex. The material could be dialyzed in cellophane bags for prolonged periods without a detectable loss in antibacterial potency. It was very heat stable, withstanding boiling temperature for periods up to one hour at neutral or acid pH. However, most of its activity was lost in 15 minutes when boiled at pH 9.6. It was quite potent against a strain of *Micrococcus pyogenes*, var. *aureus* (5-10 μg per ml) in nutrient broth but in more complex media its activity was decreased somewhat. The factor was quite soluble in water at acid or neutral pH, forming tan colored solutions at concentrations of 1-2%.

TABLE I. Amino Acid Analysis of Thymus Peptide.

Amino acid	% amino acid of total wt	g amino acid/100 g protein
Arginine	1.64	2.1
Lysine	27.10	34.7
Phenylalanine	.63	.8
Tyrosine	.34	.4
Leucine	3.20	4.1
Isoleucine	.75	.9
Valine	3.50	4.5
Alanine	13.85	17.8
Glycine	3.48	4.5
Glutamic acid	3.98	5.1
Proline	8.22	10.5
Aspartic acid	2.27	2.9
Threonine	4.10	5.4
Serine	4.24	5.4
Ammonia	.84	1.1
Total	77.25%	99.2

Table I contains the result of the amino acid analysis of the thymus peptide. The basic amino acid, lysine, represented nearly 35% of the total weight of the protein portion. The concentration of arginine was small and histidine was absent. The total protein content of the tissue peptide was found to be approximately 78% by the amino acid analysis. An average value of 80% protein was obtained by microKjeldahl determinations. Hydrolysis of nucleic acids by this latter method may have accounted for the higher value.

From the phosphorus value of 0.69%, total nucleic acids were calculated to be 6.37%. The Dische test result for desoxyribonucleic acid was 4.0% and the ribonucleic acid con-

tent, determined by subtraction, was 2.37% of the total peptide. Total carbohydrate, as glucose equivalents, was shown to be 0.83% by the anthrone method.

Discussion. Certain of the characteristics of the thymus peptide which have been studied reaffirmed and enlarged upon the original work which showed that this tissue factor was a basic polypeptide(1). The acid and heat stability of the thymus material has been confirmed. The electrophoretic mobility at pH 7.0 was found to be almost the same as that reported in the original work. The lysine content of the peptide as first reported was 29.3% by microbiological assay and in this case it was 34.7% by column chromatography. Furthermore, thymus peptide antibacterial activity was blocked by the acidic polymers, hyaluronic acid, glutamyl polypeptide and nucleic acid. Such blockage by acidic macromolecules has been reported for the previously cited thymus peptide(2,10, 15). Furthermore, the thymus peptide reported here was found to be most active at an alkaline reaction and apparently more active against gram positive bacteria.

Crampton, Stein and Moore have recently performed amino acid analyses on three calf thymus histone fractions(4). One of the 3, histone Fraction A, was shown to contain a large amount of lysine as well as alanine and proline and to be lacking four amino acids, cystine, methionine, histidine and tryptophane. The thymus peptide studied here was also rich in lysine, alanine and proline and lacking in cystine, methionine, histidine and tryptophane. It seems very likely that this peptide is the same as the histone Fraction A reported by Crampton and co-workers.

The antibacterial thymus peptide investigated by Hirsch and Dubos(7) was shown to contain large amounts of lysine and arginine. From its amino acid analysis it appears that this thymus factor is similar to Fractions B and C of Crampton, *et al.* Since the peptide was dialyzable it is possible that it represented a lower molecular weight histone fraction. It is not difficult to envisage the formation of split histone products differing in molecular weight and amino acid composition when extracting these basic proteins by related chemical procedures. The work of Weissman and Graf(16) indicates that these histone derivatives occur as a result of the splitting of the tissue nucleoproteins.

Summary. The antibacterial thymus peptide has been characterized and strong confirmatory evidence has been presented to support the view that it has been derived from a histone present in thymus tissue.

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