Antimicrobial Activity of a Natural and a Synthetic Polypeptide.* (19769)

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In an attempt to isolate factors concerned in the natural resistance of experimental animals to infection with *Bacillus anthracis*, Bloom, *et al.*(1) isolated an antibacterial basic protein characterized by a high content of lysine. Active fractions were isolated from polymorphonuclear leucocytes, caecum, thymus, and the pancreas of various species of animals. Later, Bloom and Blake(2) found the active fraction in thyroid and more recently it has been isolated from spleen(3). In addition to *Bacillus anthracis*, these fractions are active against *Escherichia coli*, *Staphylococcus aureus*, *beta*-hemolytic streptococci and *Bacillus megatherium*(2,3). Impressed with the high lysine content of these tissue polypeptides, Weissman and Graf(4) compared the antibacterial activity of a histone containing 16.4% lysine with a tissue polypeptide which had a lysine content of 30%. Expressing their results in terms of milliequivalents of lysine per unit of antibacterial activity, they found a correlation between the content of lysine and the antibacterial activity. These results led Stahmann *et al.*(5) to synthesize lysine polypeptides for the purpose of studying their antimicrobial activity. As predicted, these synthetic lysine poylpeptides had high antimicrobial activity when tested *in vitro*(5,6).

The purpose of this communication is to present evidence which indicates that the antimicrobial activity of the tissue polypeptide resides in lysine residues within the molecule. These results were obtained by comparing the antibacterial and antiviral activity of the natural tissue peptide with that of a synthetic lysine polypeptide. Further evidence was obtained by studying their neutralization and complex formation with desoxyribonucleic acid (DRNA) and ribonucleic acid (RNA).

Methods. The antimicrobial activity of these substances was tested with a system comprising bacteriophage (bacterial virus) type A and its host *Clostridium madisonii* McCoy, strain 16. The assay method, including the composition of the medium, the preparation of the stock virus and methods for cultivating the host are given in detail elsewhere(7). The tissue polypeptide was prepared by the method of Bloom, *et al.*(1) from calf thymus, while the synthetic lysine polypeptide† was prepared by a modification of the method of Katchalski, *et al.*(8). Although both the hydroiodides and the hydrochloride salts were used, all of the work reported here was carried out with the hydrochloride. The concentrations were based on the weight of the free base. This particular sample of synthetic polypeptide contained, on the average, 85 lysine residues. The DRNA was prepared by the method of Mirsky and Pollister(9) with the protein extraction of the nucleoprotein as reported by McCarty(10). The DRNA contained 13.4% nitrogen and 8.0% phosphorus; the RNA contained 13.6% nitrogen and 8.5% phosphorus. These materials were dissolved in distilled water and the pH adjusted to 7.0 with a dilute solution of NaOH. Since *Clostridium madisonii* was employed in the assay of virus, it was necessary to determine the influence of two peptides on this particular host. In previous studies (1,2), the antibacterial activity was determined by measuring inhibition of aerobic respiration. In the present investigation, the inhibition of cellular multiplication was de-

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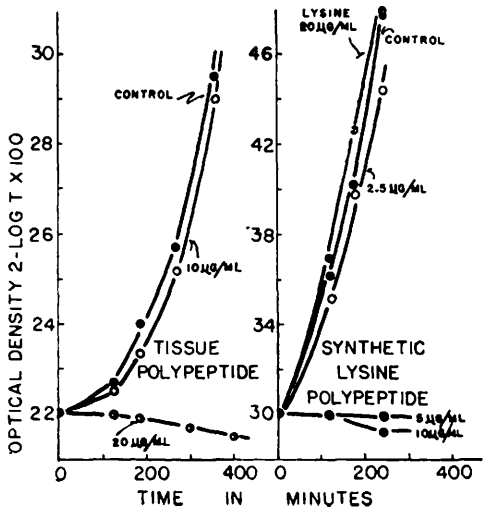

FIG. 1. Determination of relative antibacterial activity of tissue and synthetic polypeptides and lysine employing *Clostridium madisonii*.

termined as an index to the amount of antibacterial activity of the peptides. This was accomplished by the determination of optical density using a Coleman "junior spectrophotometer" in a manner previously described(7).

Experimental. In Fig. 1, the antibacterial activity of the tissue polypeptide and synthetic lysine polypeptide are compared, employing the bacteriophage susceptible host, *Clostridium madisonii*. The conditions and methods used in this experiment relative to the composition of medium and preparation of inocula were similar to those used in the viral assay (7). The initial concentration of bacterial cells was in the order of 1.0 to 1.5 x 10^8 organisms per ml; these were added to several tubes each containing 18 ml of sucrose medium adjusted to pH 6.5 and maintained at a temperature of 34°C. To each of these tubes was added the indicated concentrations of tissue polypeptide and synthetic lysine polypeptide. The degree of inactivation was followed by frequent determinations of the changes in optical density. It can be seen that the quantity of tissue polypeptide required to inactivate completely this concentration of cells falls somewhere between 10 and 20 micrograms per ml. The amount of synthetic lysine polypeptide, however, required to inactivate the same quantity of cells was between 2.5 and 5 µg per ml. From these results, one can conclude that the synthetic lysine polypeptide has approximately 4 times the activity of the natural tissue polypeptide.

Antiviral activity of tissue and synthetic lysine polypeptide. The comparative antiviral activity of these preparations was determined in a solution consisting of 3 g of $(NH_4)_2HPO_4$ and 6 g of KH_2PO_4 per liter of distilled water adjusted to pH 6.5. Eight ml of this solution was placed in each of a series of tubes. To each of these tubes was added 1 ml containing 1 x 10^8 units of virus; these tubes were then divided into 3 groups. As indicated in Fig. 2, tissue polypeptide, synthetic lysine polypeptide and lysine were added at the indicated concentrations to each of the tubes within each group. The tubes were then incubated at 34°C for 30 minutes. At the end of this period, a portion of the mixture was assayed for active virus. It may be seen from Fig. 2 that the synthetic lysine polypeptide was highly antiviral, 10 µg inactivating in excess of 99% of the added virus. If one examines the slopes of the inactivation curves, it is immediately apparent that the synthetic lysine polypeptide is approximately 4 to 5 times more active than the tissue polypeptide on a weight basis. Lysine, run as a control, at concentrations in excess of 30 µg per ml did not inactivate this bacterial virus.

The neutralization of synthetic lysine polypeptide and tissue polypeptide by DRNA and

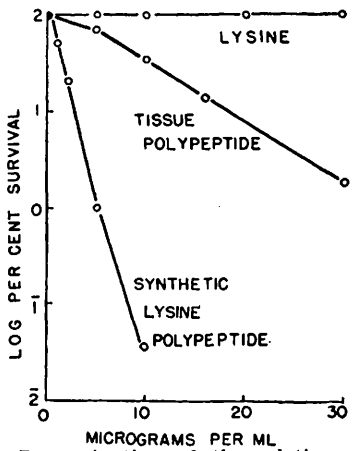

FIG. 2. Determination of the relative antiviral (bacteriophage) activity of tissue and synthetic lysine polypeptides and lysine. Initial concentration of virus, 1 × 10^8 units per ml. Exposed for 30 min at 34°C.

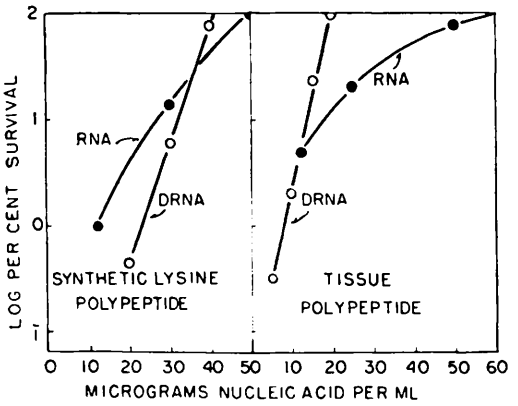

FIG. 3. Neutralization of synthetic lysine polypeptide (10 μg per ml) and tissue polypeptide (30 μg per ml) by DRNA and RNA as determined by the inhibition of antiviral effect.

RNA. Previous investigators have reported that DRNA combines with the tissue polypeptide stoichiometrically, while RNA combines in multiple proportions, depending upon the relative concentrations of the reactants (11). It was of interest, therefore, to determine the ability of RNA and DRNA to neutralize the synthetic lysine polypeptide and to compare the results in a similar experiment with tissue polypeptide. The experiment comprised 4 series of tubes divided into two groups. Into each tube of the first group was placed 8 ml of medium containing 10 μg per ml of synthetic lysine polypeptide. Likewise, 30 μg per ml of tissue polypeptide was placed into each tube of the second group. RNA, at the concentrations indicated in Fig. 3, was placed in the first series of Groups 1 and 2, while the given concentrations of DRNA were placed in the second series of Groups 1 and 2. The contents of the tubes were mixed thoroughly and bacterial virus was added to each tube to give a final concentration of 1×10^8 units per ml. The tubes were then incubated at 34°C for 30 minutes. At the end of this period, aliquots were removed from each tube and assayed for the amount of active virus remaining. The results given in Fig. 3 are plotted as log per cent survival of virus against the number of micrograms of nucleic acid, either DRNA or RNA, added to each tube. The amount of free virus remaining is a sensitive indicator of the concentration of uncombined polypeptide as previously shown in Fig. 2. Results given in Fig. 3 show that 40 μg of nucleic acid (DRNA) neutralize completely 10 μg of synthetic lysine polypeptide. On the other hand, about 18 μg of nucleic acid (DRNA) neutralize 30 μg of tissue polypeptide. The linearity of these curves indicates that the DRNA is combining with both the tissue polypeptide and synthetic lysine polypeptide stoichiometrically. The nonlinearity of the RNA curves might indicate that this nucleic acid does not combine with either the tissue polypeptide or the synthetic lysine polypeptide stoichiometrically, but rather in multiple proportions depending upon the relative concentrations of the two reactants. As noted previously (11) and in these experiments, when increasing concentrations of RNA is added to constant amounts of synthetic lysine polypeptide, a precipitate forms which increases to a maximum and then sharply declines as the ribonucleic acid concentration is increased. The results of such an experiment are given in Fig. 4 where the precipitate is recorded as optical density and plotted against the number of μg of RNA added to each of a series of tubes, each containing 10 μg of the synthetic lysine polypeptide. As the RNA is increased beyond 25 μg per ml the complex formed between RNA and the peptide either dissociates or forms soluble complexes. To test these two possibilities, aliquots were removed from each tube after a suitable incubation period and tested for free virus. The amount of virus remaining is a test for the amount of free peptide. It can be seen in Fig. 4 that as the RNA increases, the amount of free peptide decreases, until at approximately 50 μg of RNA, 100% of the virus is recovered, indicating that nearly all of the peptide has been neutralized. It can also be seen that at concentrations of RNA where there is a minimum of precipitate formed, all of the peptide must be incorporated in soluble complexes because nearly 100% of the virus is recovered in these tubes. This would indicate, therefore that in the presence of excess RNA a soluble complex is formed which results in the complete neutralization of the synthetic lysine polypeptide.

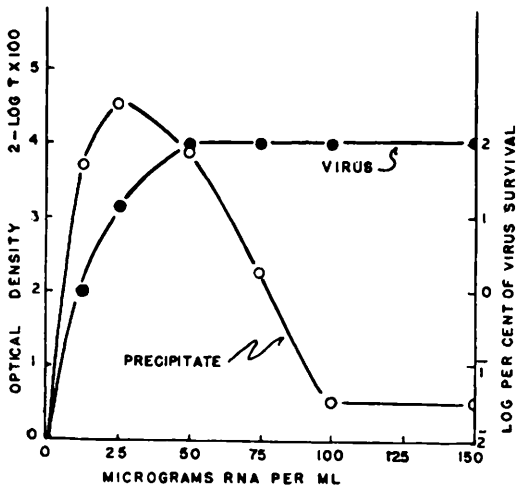

FIG. 4. Quantitative titration of synthetic lysine polypeptide (10 μg per ml) with RNA and the demonstration of insoluble and soluble complexes as determined by optical density and the inhibition of antiviral effect.

Discussion. These comparative studies with synthetic lysine polypeptide and tissue polypeptide lend significance to the high content of lysine in the natural tissue polypeptide. The fact that the synthetic lysine polypeptide is approximately 4 to 5 times more active than the tissue polypeptide when compared on a weight basis, but of comparable activity when compared on the number of meq. of lysine within the molecule suggests that the activity of the natural peptide resides in the lysine portion of the molecule. The comparable types of interaction between DRNA and RNA with the two polypeptides lends further support to this implication of lysine as the active portion of the natural peptide. These findings are not in conflict with the previous postulation on the possible mechanism of action(12). These polypeptides have high isoelectric points and are, therefore, positively charged at pH 6.5; on the other hand, substances such as DRNA and RNA are negatively charged. It can be assumed, therefore, that the phosphoral groups within DRNA combine stoichiometrically with the epsilon amino group of the lysine residues within these peptides. The interaction between RNA and these peptides seems somewhat more complicated and more difficult to explain. This latter interaction seems to be quite comparable to an antigen-antibody reaction. The curve in Fig. 4 representing the amount of precipitate formed when varying amounts of ribonucleic acid are added to constant amounts of synthetic lysine polypeptide is quite comparable to that obtained when antibody concentration is maintained constant, increasing the quantity of antigen added. In both cases it appears that soluble complexes are formed. The similarity goes further, in that Lewis, *et al.*(11) have shown that if one determines the nitrogen-phosphorus ratio of the resulting precipitate at varying places along a similar curve, one finds that the ratio varies depending upon the relative concentration of the reactants. A comprehensive study of this phase of the investigation comparable to that reported by Bjornesjo and Teorell(13) for the DRNA-oval-albumin and DRNA-histone systems is anticipated.

The efficacy of the tissue and the synthetic polypeptides when tested for their antimicrobial activity *in vivo* has not been promising (1,6). This is not surprising when one considers their high affinity for such substances as RNA, DRNA and other negatively charged high molecular weight substances commonly found in tissues. This fact, however, does not preclude the importance of the tissue peptide in the natural resistance of the host to microbial infections as originally postulated (14). It is possible that as a result of the host-parasite interaction and the resulting tissue damage, the peptide is liberated, destroying the parasite within the immediate environment of the necrotic tissue.

If one assumes that this tissue peptide is liberated as a result of tissue necrosis, it could also function in the formation of fibrinoid in a manner postulated by Altshuler and Angevine(15). This tissue polypeptide combines with the acidic polysaccharides, hyaluronic acid and heparin, to form insoluble complexes. Whether these insoluble complexes have the chemical and physical properties of fibrinoid has not been determined.

Summary. The antibacterial and antiviral properties of a tissue polypeptide were compared with the activity of a synthetic lysine polypeptide. On a weight basis, the activity of the synthetic peptide was approximately

4 times greater than that of the natural peptide. From the fact that the tissue peptide contains 30% lysine, it is possible to conclude that the activity resides within the lysine portion of the molecule. Additional evidence to support the implication of lysine was obtained from a comparative study on the mode of inactivation of these peptides by desoxyribonucleic acid (DRNA) and ribonucleic acid (RNA). Both peptides combine stoichiometrically, at pH 6.5 with DRNA but not with RNA; it is apparent that both peptides combine with RNA in multiple proportions depending upon the relative concentrations of the reactants. In this respect, the similarity between the RNA-peptide interaction and antigen-antibody reactions is pointed out. A mechanism by which the tissue polypeptide could take part in the natural resistance of animals to infection and the possible role of these basic peptides in fibrinoid degeneration is discussed.

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Use of Radioactive Iodinated Human Serum Albumin as Tracer Agent in Study of Cerebral Disorders.* (19770)

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During the past 4 years diiodofluorescein (DIF)(1-7), radioactive potassium(8,9), iodinated human serum albumin (RIHSA)(10), sodium iodide(11), and radioactive copper (12) have been used in the study of intracranial lesions by the isotope-encephalometric technic. An increased radioactivity at a particular site has been used as evidence of the possible presence of a neoplasm. The reported accuracy of localization of tumors has varied from 5%(13) to 95%(5). Technics have varied with advances in the instruments used. Emphasis has been placed on the study of patients with known or suspected tumors. In previous studies with DIF by the authors (14) increases in uptake above 10% were observed in 21 of 25 cases of cerebral neoplasm, diagnosed by operation or autopsy. However, wide variations in uptake on repeated studies were observed in some of the patients with neoplasms. Accurate studies of control groups were difficult due to the rapid excretion of

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