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Isolation of a Low-Molecular-Weight Antibacterial System from Human Amniotic Fluid

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A low-molecular-weight antibacterial system has been isolated from human amniotic fluid. The bacterial inhibitor requires the metal cation zinc and a peptide with a molecular weight of 630. The peptide component was purified using ultrafiltration, gel filtration, and ion-exchange chromatography. It can be inactivated by digestion with carboxypeptidase. The amino acid composition of the peptide is: 3 glutamine-glutamic acid, 2 glycine, and 1 lysine. Removal of zinc from the peptide has been shown to remove bacterial inhibitory activity.

Human amniotic fluid (AF) has been shown to inhibit bacterial growth (2, 13, 14, 18, 21, 24, 38). The inhibitory activity is due to the presence of an active antibacterial component and not to the absence of nutrients required for bacterial growth (24). The antibacterial activity of the inhibitor is reversed by the addition of small amounts of phosphate (25) and can be removed by preincubation of AF with the cation exchanger bentonite (39). The inhibitory component is heat stable (39) and resistant to inactivation by Pronase and trypsin (37).

The metal cation zinc is required for the antibacterial activity of AF (36). However, zinc alone at the concentration found in AF was unable to effect bacterial killing. The presence of an organic component associated with zinc appeared to be required to inhibit bacterial growth. The organic component has not as yet been purified, but it is not one of the known bacterial inhibitors present in AF (37). Preliminary experiments suggested that the organic component may be a peptide with a molecular weight of less than 5,000 (37). Further, the organic component appeared to be associated with the sensitivity to phosphate. In addition, AFs with a phosphate-to-zinc ratio of less than 100 were bactericidal and those with a ratio between 100 and 200 were bacteriostatic. AFs with a phosphate-to-zinc ratio greater than 200 were noninhibitory (36). This study was undertaken to purify and characterize the phosphate-sensitive organic component of AF.

MATERIALS AND METHODS

AF. Samples of AF were obtained either by transabdominal amniocentesis or by needle aspiration at the time of cesarean section. Fluids containing blood or from patients receiving antibiotics were not used. Prior to use, each AF was passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and then assayed for bacterial growth-inhibitory activity using a semimicro viable plate count technique (38). Phosphate reversal of inhibitory activity was determined as previously described and was used to establish inhibitor identity (36).

Bacteria. *Escherichia coli* type O6 was obtained from the clinical microbiology laboratory at the University of Iowa Hospitals, Iowa City, and served as the test organism. Starter cultures of *E. coli* were grown in Trypticase soy broth (BBL, Becton, Dickinson, and Co., Cockeysville, Md.) until the logarithmic phase of growth, diluted in sterile distilled water to give a final inoculum of approximately 10^3 cells/ml, and inoculated into 1-ml volumes of test material.

Solutions. The standard phosphate solution used was that contained in the chemically defined medium described by Larsen et al. (25). The solution consisted of 0.18 M potassium phosphate (180 μg of phosphate/10 μl) (Fisher Scientific Co., Fair Lawn, N.J.).

Metal solutions were prepared and contained the following metal concentrations. A solution of $ZnNO_3 \cdot 6H_2O$ (Fisher Scientific Co.) was prepared in sterile distilled water and contained 13 μg of zinc/10 μl, as determined by atomic absorption spectrophotometry. Standard copper and magnesium solutions ($CuSO_4 \cdot 5H_2O$, $MgCl_2 \cdot 6H_2O$) were prepared in sterile distilled water and contained 6.5 μg of metal/10-μl volume. The effect of each metal on antibacterial activity was determined by adding 10-μl volumes of varying metal concentrations to 1 ml of test material and assaying for bacterial growth.

Biochemical and chemical assays. Protein was measured either by the method of Lowry et al. (26) or by the microbiuret procedure described by Zamenhof (44). Carbohydrate was measured by the technique of Dubois et al. (10). Ribonucleic acid was determined using the orcinol assay (8). Deoxyribonucleic acid was measured using the diphenylamine

procedure described by Dische (8). Lysozyme was measured according to the procedure described by Larsen et al. (22). Bovine serum albumin was purchased from Pentex Inc. (Kankakee, Ill.). Yeast ribonucleic acid and deoxyribonucleic acid and cells of *Micrococcus lysodeikticus* were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lysozyme (3 × crystallized) was purchased from Worthington Biochemicals Corp. (Freehold, N.J.).

Phosphate concentrations were measured using a modification of the method of Youngburg and Youngburg (43); the modifications are as described. One milliliter of AF to be tested was mixed with 4 ml of trichloroacetic acid. After room temperature incubation for 10 min, the precipitate was pelleted by centrifugation (2,000 × *g*, 15 min), and then 1 ml of the sulfuric acid-sodium molybdate solution was added. After mixing, 0.5 ml of the stannous chloride solution was added. Phosphate concentrations were determined by measuring absorbance at 660 nm after 2 min. Standard solutions consisted of dilutions of the standard phosphate solution in deionized, distilled water, with phosphate concentrations ranging from 0 to 200 μg/ml.

Zinc levels were quantified using a Perkin-Elmer model 303 atomic absorption spectrophotometer (Norwalk, Conn.).

Noninhibitory control media. Samples of AF obtained at 15 to 20 weeks of gestation were pressure dialyzed using an Amicon Diaflo cell equipped with a UM10 membrane (Amicon Corp., Lexington, Mass.), which excluded compounds of greater than 10,000 molecular weight. A 10% concentration of the ultrafiltrates (UM10 filtrate) from these fluids served as noninhibitory control media (NCF). A chemically defined medium (CDM) contained: 10^{-4} M phosphate, 4×10^{-4} M magnesium ($MgCl_2 \cdot 6H_2O$), 10^{-4} M glucose, 4×10^{-5} M sodium chloride, 2×10^{-5} M ammonium sulfate (pH 6.7). Both noninhibitory media contained similar concentrations of phosphate, magnesium, and glucose. Zinc was not present in either control medium. Lysozyme activity was not present in the NCF.

Proteolytic enzyme treatment. Carboxypeptidase was obtained from Worthington Biochemicals Corp. The enzyme was purified, and its concentration was determined by the method described by Narita (30). One hundred microliters of the carboxypeptidase solution, containing 100 μg of enzyme, was added to 0.5 ml of test material, which contained 325 μg (15× concentrated) of protein as determined by the microbiuret procedure. Control tubes contained 325 μg of protein, but no enzyme was added. Test and control tubes were then incubated for 7 or 15 h at 37°C. After the incubation period, 100 μg of carboxypeptidase was added to each control tube. All tubes were immediately placed in an ice-water bath, and 5 ml of ice-cold, sterile, distilled water was added. The material in each tube was then pressure dialyzed using an Amicon Diaflo cell equipped with a UM10 membrane. The retentate material was washed twice with 5 ml of sterile distilled water and again pressure dialyzed. The filtrates from respective tubes were pooled and then lyophilized. The lyophilized preparations were reconstituted to 1 ml with distilled water, and 2.2 μg of zinc was added. The samples were passed through 0.45-μm membrane filters. This final material was 7.5× concentrated. Bacterial growth-inhibitory activity of carboxypeptidase-digested and control samples was measured in the CDM.

Ultrafiltration. As the first step in the purification of the organic inhibitory component, samples of inhibitory AF were pressure dialyzed using an Amicon Diaflo cell equipped with a UM10 membrane.

Chromatographic procedures. Samples of UM10 filtrates were concentrated by lyophilization and reconstituted to 5 ml with distilled water. Particulate material was removed by centrifugation (2,000 × *g*, 15 min). The supernatant fluids were fractionated on a Bio-Gel P-2 column (2.5 by 90 cm; Bio-Rad Laboratories, Richmond, Calif.) equilibrated with distilled water at a flow rate of 30 ml/h. No more than 5 ml of UM10 filtrate, which had been concentrated from a 30-ml sample, was applied to the column. Effluent material was collected in 3-ml volumes and was monitored for absorbance at 280 nm. The pooled fractions of each 280-nm-absorbing peak were concentrated by lyophilization and then dissolved in sufficient distilled water to bring the volume to one-tenth the volume of the original unchromatographed UM10 filtrate. After filtration through 0.45-μm membrane filters, 280-nm-absorbing peaks were assayed for bacterial growth-inhibitory activity in the NCF and CDM using the semimicro plate count technique. The absorbance spectrum of each peak was determined using a Perkin-Elmer model 124 double-beam spectrophotometer.

Peak material that absorbed at 280 nm was further fractionated on a diethylaminoethyl (DEAE)A-25 Sephadex column (1.5 by 50 cm; Pharmacia Fine Chemical Co., Piscataway, N.J.) with a flow rate of 30 ml/h. The column was equilibrated with distilled water (pH 6.7). Material was eluted from the column using a 0 to 0.3 M NaCl gradient. Effluent material was collected in 3-ml fractions and was monitored for absorbance at 280 nm. Pooled fractions of peaks that absorbed at 280 nm were concentrated by lyophilization and then reconstituted to 5 ml with distilled water. Salt was removed from the peak material by gel filtration three times on Bio-Gel P-2. Peak material that absorbed at 280 nm, after desalting, was assayed for bacterial growth-inhibitory activity in the NCF and CDM using the following procedure. Peak material at one-half physiological concentration was added to the NCF and CDM, to which 0.3 μg of zinc per ml had been added. Controls consisted of the NCF and CDM with 0.3 μg of zinc per ml added. Bacterial inhibitory activity was measured using the semimicro plate count technique.

Amino acid analysis. The amino acid composition of the organic inhibitory component from the DEAE column was determined using an amino acid analyzer (JEOL, Tokyo, Japan). The samples to be analyzed were obtained from the Bio-Gel P-2 column and contained 1 mg of peptide, as determined by the microbiuret procedure. Acid hydrolysis of the peptide material was performed as follows. The samples were evaporated to dryness under a nitrogen atmosphere in an 18- by 150-mm Pyrex tube. Constant

boiling HCl (1.5 ml of 6 M) was added, and each tube was sealed under high vacuum. Acid hydrolysis was allowed to proceed for 6, 8, or 12 h at 150°C. Each tube was then opened, and the mixtures were evaporated to dryness. The dryed material was dissolved in 2.2 ml of 0.1 M sodium citrate buffer, and 2 ml was added to the amino acid analyzer. The amino acid composition was determined by comparison to standards. Tryptophan was determined before acid hydrolysis by the absorbance method of Edelhoch (11).

Polyacrylamide gel electrophoresis. Peptides were analyzed by polyacrylamide gel electrophoresis according to the methods of Ornstein (31) and Reisfeld et al. (33). The gel columns were subjected to electrophoresis at 4 mA/tube at 4°C. Bromophenol blue or basic fuchsin served as the tracking dye. Gels were fixed in trichloroacetic acid (10%) for 2 h and then stained with 0.25% Coomassie brilliant blue R. Destaining was accomplished by a hot 5.6% acetic acid procedure (56°C).

Dithizone chelation of zinc. Zinc was removed from fractions using the water-insoluble cation chelator dithizone (diphenylthiocarbazone). Material was obtained from 10 ml of UM10 filtrate and was concentrated to a volume of 2 ml. The material was then mixed with 2 mg of dithizone and incubated at 4°C for 24 h with occasional shaking. At the conclusion of the incubation period, dithizone was removed by centrifugation (2,000 × *g*, 15 min). The supernatant fluid was filtered through an 0.45-μm membrane filter and assayed for bacterial growth-inhibitory activity. Phosphate and zinc concentrations were determined before and after dithizone treatment.

RESULTS

Samples of inhibitory AF were initially fractionated by pressure dialysis through UM10 membranes. The UM10 filtrates were shown to contain the organic inhibitory component, and these were used in further fractionation procedures.

Samples of inhibitory UM10 filtrates were chromatographed on a Bio-Gel P-2 column. Shown in Fig. 1 is a summary of elution profiles obtained. Peaks 1 and 2 were present in all inhibitory UM10 filtrates studied. Peaks 3 through 8 were occassionally present. By comparison to the bromophenol blue standard, the molecular weights of peak 1 and 2 material were estimated to be 700. Lysozyme used as a standard eluted at fraction 44 (132 ml), and tryptophan eluted at fraction 150 (450 ml). All 280-nm-absorbing peaks obtained from the Bio-Gel P-2 column were assayed for inhibitory activity in physiological concentration (assuming 100% recovery) in both the NCF and CDM. Peak 1 material, when added to the NCF, was bactericidal, and the anti-bacterial activity was reversed by the addition of 10 μl of the standard

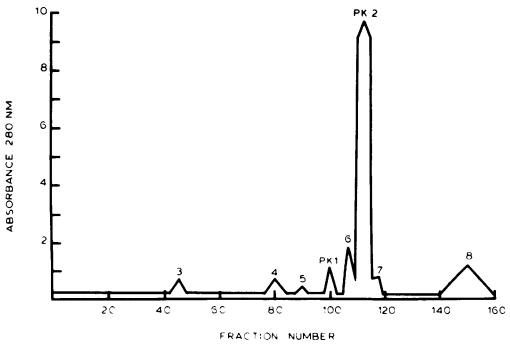

FIG. 1. *Summary elution profile of 280-nm-absorbing material from inhibitory UM10 filtrates chromatographed on Bio-Gel P-2 (2.5- by 90-cm column) equilibrated with distilled water. Fraction size, 3 ml. Standards: lysozyme (molecular weight, 14,000) eluted at void volume (fraction 44); bromophenol blue (670) eluted at fraction 105; tryptophan (204) eluted at fraction 150.*

phosphate solution. (Fig. 2) The bacterial inhibitory activity of peak 1, added to the NCF, may be due to zinc, since previous research has shown that the addition of as little as 0.08 μg of zinc to 1 ml of NCF resulted in phosphate-sensitive antibacterial activity (36). The organic component of the phosphate-sensitive inhibitor was already present in the NCF. Peak 1 material, when added to the CDM, was also bactericidal, and the bactericidal activity was reversed by the addition of phosphate. The inhibitory activity of peak 1, added to the CDM, suggested that both zinc and the organic component were contained in the peak, since it was previously shown that the zinc concentration contained in AF was not sufficient to inhibit bacterial growth in the CDM (37).

Peak 2 material from the Bio-Gel P-2 column, when added to the NCF, was bactericidal (Fig. 3) but, when added to the CDM, was noninhibitory. The inhibitory activity of peak 2 material added to the NCF was reversed by addition of 10 μl of the standard phosphate solution. The inhibitory activity obtained when peak 2 material was added to the NCF is consistent with that obtained when zinc alone is added to the NCF.

Peaks 3 through 7 were consistently bacterial growth promoting when added to either the NCF or CDM. Peak 8 was present in only one AF sample and was bacteriostatic when added to the CDM, but was noninhibitory when added to the NCF. No further work has been done to characterize the material contained in peaks 3 through 8.

Shown in Table 1 is a summary of the biochemical properties of peaks 1 and 2 from an

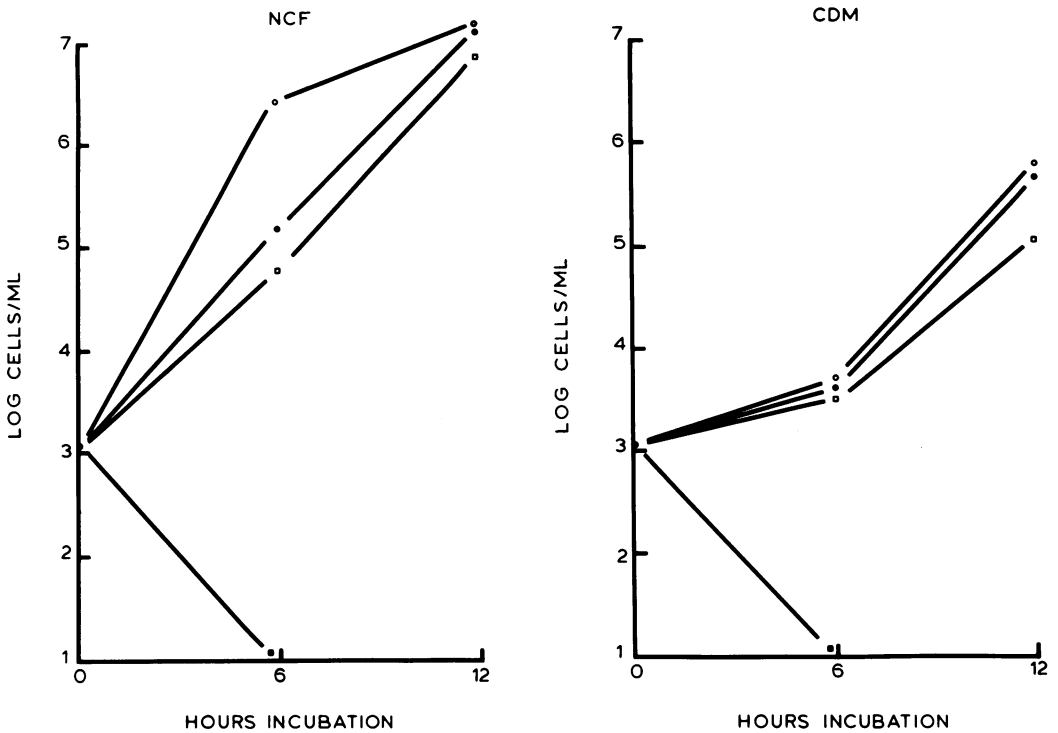

FIG. 2. *Bacterial growth-inhibitory activity of Bio-Gel P-2 peak 1 in physiological concentration added to the NCF and CDM. Symbols: NCF or CDM (●); NCF or CDM + 180 μg of phosphate per ml (○); NCF or CDM + peak 1 (■); NCF or CDM + peak 1 and 180 μg of phosphate per ml (□).*

inhibitory UM10 filtrate. The concentrations of components of each peak are at physiological concentration, assuming 100% recovery of material from the Bio-Gel P-2 column. Both peaks contained biuret-positive material suggestive of peptide components. Peak 1 contained six times as much peptide when the microbiuret assay was used as compared to the Lowry assay, suggesting that peak 1 contained few aromatic amino acids. Peak 1 also contained a significant amount of carbohydrate. No deoxyribonucleic acid or ribonucleic acid was detected in either peak. Each peak contained approximately one-half the zinc contained in the unchromatographed UM10 filtrate. Peak 1 contained no phosphate, whereas peak 2 contained 4.6 μg/ml. Since the NCF and CDM contained 9.5 μg of phosphate per ml, when peak 1 was added to either medium a phosphate-to-zinc ratio of 53 was obtained consistent with the bactericidal activity obtained. Peak 2 added to either NCF or CDM resulted in a phosphate-to-zinc ratio of 67. The concentration of peptide contained in peak 1 varied considerably in different fluids, ranging from 25 to 260 μg/ml with an average of approximately 50 μg/ml.

Neither peak absorbed in the visible range at physiological concentration. However, peak 1 absorbed slightly at 610 nm when concentrated 20 times. Ultraviolet absorbance spectra of peaks 1 and 2 are shown in Fig. 4. Peak 1 had absorbance maxima at 272 and 210 nm. Peak 2 had absorbance maxima at 290, 245, 235, and 190 nm. The absorbance spectrum of peak 1 was unaffected by adding 1 μg of zinc per ml in a 10-μl volume or by adding 186 μg of phosphate per ml in a 10-μl volume.

The purity of peak 1 from the Bio-Gel P-2 column was determined using polyacrylamide gel electrophoresis. One stainable peptide band was obtained when 200 μg of peptide (microbiuret) was subjected to electrophoresis towards the anode. This band rapidly destained and could not be fixed permanently in the gel column. No bands were observed when peptide samples were subjected to electrophoresis towards the cathode.

The P-2 peak was treated with carboxypeptidase to determine if the activity of the peak was due to a peptide. After 7 h of digestion, partial reversal of inhibitory activity was obtained (Fig. 5). Complete reversal of inhibitory activity was obtained after 15 h of digestion.

Final purification of the organic inhibitory

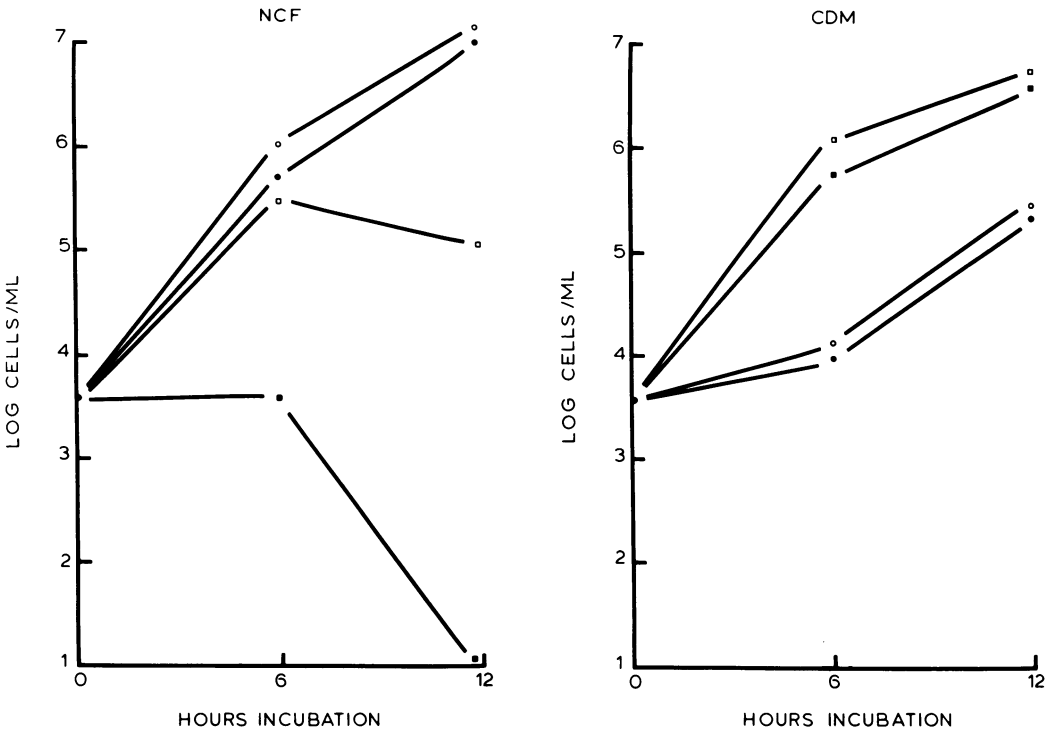

FIG. 3. *Bacterial growth-inhibitory activity of Bio-Gel P-2 peak 2 in physiological concentration added to the NCF and CDM. Symbols: NCF or CDM (●); NCF or CDM + 180 μg of phosphate per ml (○); NCF or CDM + peak 2 (■); NCF or CDM + peak 2 and 180 μg of phosphate per ml (□).*

TABLE 1. *Biochemical properties of Bio-Gel P-2 peaks 1 and 2 at physiological concentration*

Property	Peak 1	Peak 2
Peptide, microbiuret (μg/ml)	50	186
Peptide, Folin (μg/ml)	8	180
Carbohydrate (μg/ml)	50	ND[a]
Deoxyribonucleic acid	0	0
Ribonucleic acid	0	0
Phosphate (μg/ml)	0	4.6
Zinc (μg/ml)	0.18	0.2
PO_4/Zn	0	22
PO_4/Zn in NCF and CDM	53	67
Estimated molecular weight	700	700

[a] ND, Not done.

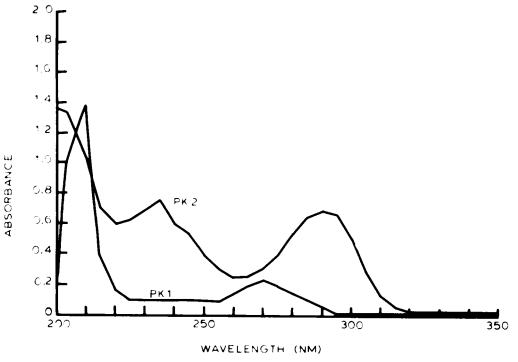

FIG. 4. *Ultraviolet absorbance spectra of Bio-Gel P-2 peak 1 and peak 2. Peak 1 contained 82 μg of peptide (microbiuret) in a 0.5-ml volume; peak 2 contained 62 μg of peptide (microbiuret) in a 2-ml volume.*

component contained in the UM10 filtrate was accomplished using ion-exchange chromatography. Peak 1 obtained from Bio-Gel P-2 was chromatographed on DEAE A-25 Sephadex (Fig. 6). One 280-nm-absorbing peak was obtained and contained 92% of the peptide material (microbiuret) applied to the column. The major 280-nm-absorbing peak eluted at pH 6.7 using 0.17 M NaCl in distilled water. The zinc concentration of the peak was below detection at physiological concentration. No phosphate or carbohydrate was detected.

The DEAE A-25 Sephadex 280-nm-absorbing peak was partially deslated and then assayed for bacterial inhibitory activity at one-half physiological concentration in the NCF and CDM (Fig. 7). Because zinc was removed from

the peptide during ion-exchange chromatography, 0.3 μg of zinc per ml was added to both noninhibitory media (PO_4/Zn = 32) prior to adding peak material. The addition of 0.3 μg of zinc per ml in the absence of the peptide peak material was sufficient to be bactericidal in the NCF but not in the CDM (Fig. 7). The peptide material was required to obtain inhibitory activity in the CDM. The antibacterial activity observed in either medium after the addition of the peptide peak was effectively reversed by the addition of phosphate.

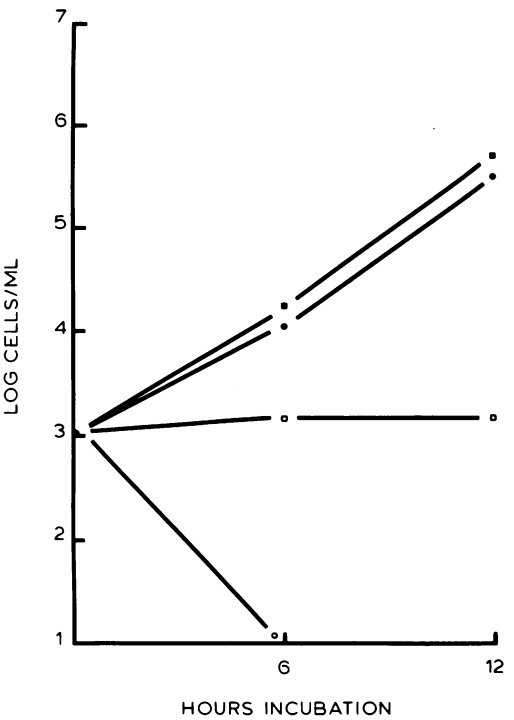

FIG. 5. *Effect of carboxypeptidase digestion of Bio-Gel P-2 peak 1 on bacterial inhibitory activity in the CDM. Symbols: CDM (●); peak 1 material added in physiological concentration to the CDM (○); carboxypeptidase-treated (7 h) peak 1 material added to the CDM (□); carboxypeptidase-treated (15 h) peak 1 material added to the CDM (■).*

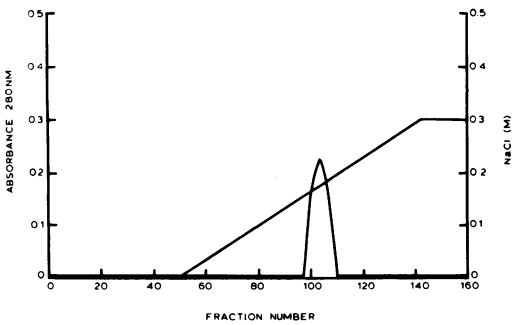

FIG. 6. *Elution profile of 280-nm-absorbing components of Bio-Gel P-2 peak 1 chromatographed on DEAE A-25 Sephadex (1.5- by 50-cm column) equilibrated with distilled water (pH 6.7). NaCl gradient: 0 to 0.3 M.*

The requirement of zinc for bacterial growth inhibition by Bio-Gel P-2 peaks 1 and 2 was tested by attempting to reverse the bacterial inhibitory activity of each peak by chelation of zinc. The data in Fig. 8 show that zinc was required for peak 1 bactericidal activity. Chelation of either the NCF or the CDM at physiological concentration reversed the inhibitory activity. Readdition of zinc restored bactericidal activity. The phosphate-to-zinc ratio of peak 1 in the NCF and CDM before dithizone treatment was 41 (9.5/0.23), and after treatment with dithizone it was greater than 200. Chelation of zinc contained in Bio-Gel P-2 peak 2 effectively reversed the bacteriostatic activity of the peak added to the NCF at physiological concentration (Fig. 9). Readdition of 0.3 μg of zinc restored the antibacterial activity. The phosphate-to-zinc ratio of peak 2 added to the NCF before dithizone treatment was 230 (30/0.23), and after treatment it was greater than 300.

The amino acid composition of Bio-Gel P-2 peak 1 is shown in Table 2. The data suggest that 1 lysine, 3 glutamine-glutamic acid, and 2 glycine were present. A molecular weight of 630 at 71% purity was obtained.

Both copper and magnesium are present in AFs (3, 19). To test whether these metals could replace zinc in the phosphate-sensitive antibacterial system, varying concentrations of each were added to the NCF and CDM and antibacterial activity was determined. The data were then compared to controls to which varying concentrations of zinc had been added. The data obtained are summarized in Table 3. The minimum inhibitory concentration of zinc in the NCF was 0.40 μg/ml. Zinc was not inhibitory to bacterial growth when added to the CDM. Copper did not appear to inhibit bacterial growth in the same way as zinc. Although 0.40 μg of copper added to the NCF resulted in bacterial inhibition, as little as 0.05 μg of copper per ml was inhibitory when added to the CDM. Copper at a concentration of 1.62 μg/ml or greater, when added to the NCF, was bactericidal. Copper at a concentration of 0.1 μg/ml or greater was bactericidal when added to the CDM. In contrast, the addition of zinc to the noninhibitory media did not result in bactericidal activity. Magnesium was not inhibitory to bacterial growth.

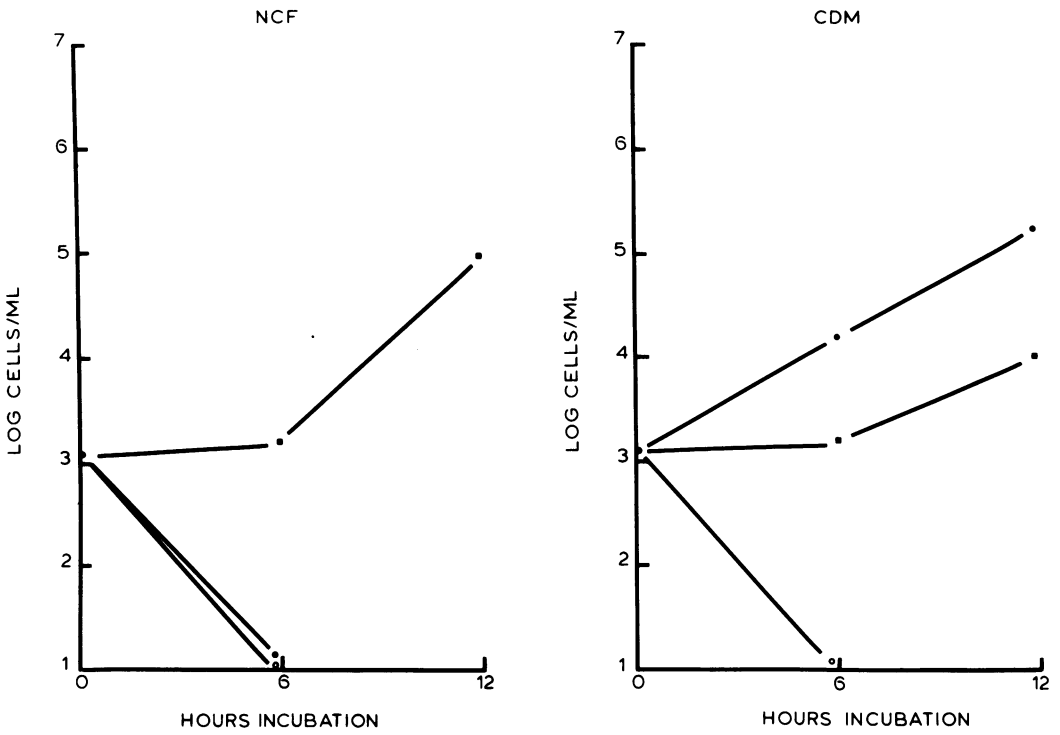

FIG. 7. *Bacterial growth-inhibitory activity of the DEAE A-25 Sephadex peptide peak added to the NCF and CDM. Symbols: NCF or CDM containing 0.3 μg of zinc per ml prior to adding peak material (●); the peptide peak added to NCF or CDM at one-half physiological concentration (○); peak and 180 μg of phosphate added to NCF or CDM (■).*

DISCUSSION

Several antimicrobial factors have been shown to be present in AF (5, 7, 15, 18, 22, 23, 28, 42). These include β-lysin, lysozyme, peroxidase, transferrin, and 7*S* immunoglobulin. Each of these protein bacterial inhibitors has a molecular weight of greater than 5,000 (9, 12, 17, 35, 40) and, as reported earlier, probably have limited function in the inhibition of the growth of *E. coli* in AF (37).

In this study, it was shown that a major bacterial inhibitor present in AF consists of two components: a peptide with a molecular weight of 630 and the cation zinc. Purification of the peptide component of the phosphate-sensitive inhibitor was accomplished using ultrafiltration, gel filtration, and ion-exchange chromatography. Two peptide peaks were consistently obtained when inhibitory UM10 filtrates were chromatographed on a Bio-Gel P-2 column. Both peptides were estimated to have molecular weights of 700 when compared to the standard, bromophenol blue. Peak 1 from the Bio-Gel P-2 column was shown to contain both zinc and the peptide component of the phosphate-sensitive inhibitory. The zinc content of the peptide peak alone was sufficient to account for the inhibitory activity obtained in the NCF, since previous research has demonstrated that the addition of zinc alone to the NCF results in antibacterial activity (36). But, both zinc and the peptide component were required for inhibitory activity in the CDM. The inhibitory activity of the zinc-containing peptide in both noninhibitory media was effectively lost by the addition of phosphate, demonstrating the presence of the phosphate-sensitive inhibitor.

Confirmation that zinc is required for the inhibitory activity of the peptide component of peak 1 was obtained by removal of the inhibitory activity of the peptide in CDM after chelation of the zinc. Confirmation that the peptide is required for inhibitory activity was obtained by removal of the inhibitory activity of the peptide in CDM after carboxypeptidase digestion.

In contrast to peak 1, which contained both zinc and the peptide component of the phosphate-sensitive inhibitor, peak 2 contained zinc but lacked the peptide component. Confirmation that the zinc contained in peak 2 was re-

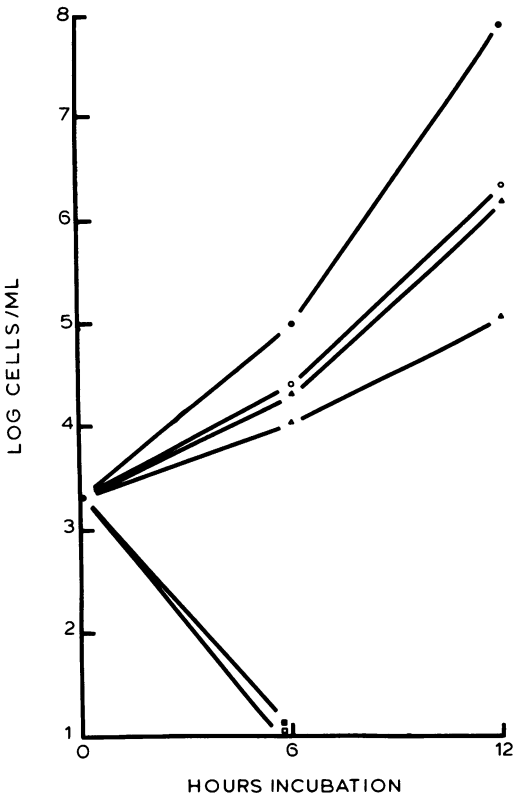

FIG. 8. *Effect of dithizone on Bio-Gel P-2 peak 1 bacterial inhibitory activity in the NCF and CDM. Symbols: NCF (●); CDM (○); NCF + peak 1 (■); CDM + peak 1 (□); NCF + dithizone-treated peak 1 (▲); CDM + dithizone-treated peak 2 (△).*

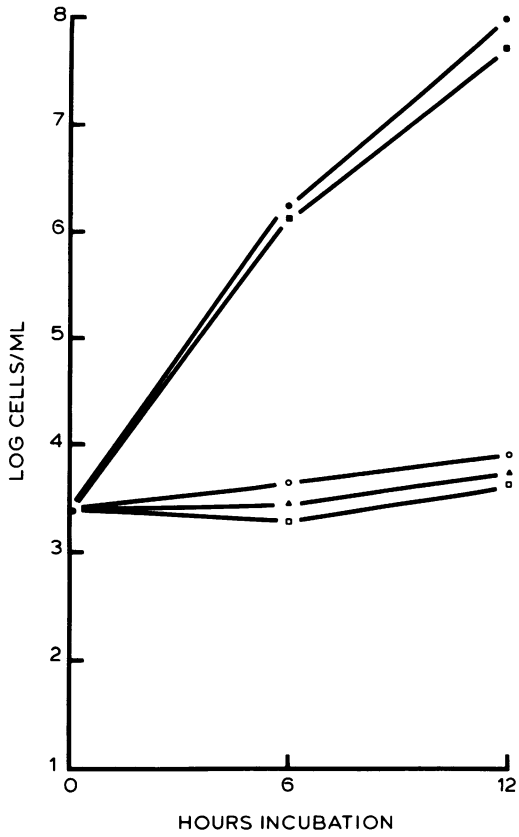

FIG. 9. *Effect of dithizone on Bio-Gel P-2 peak 2 bacterial inhibitory activity in the NCF. Symbols: NCF (●); NCF + peak 2 (○); NCF + dithizone-treated peak 2 (■); NCF + 0.3 μg of zinc per ml (□); NCF + dithizone-treated peak 2 + 0.3 μg of zinc per ml (▲).*

sponsible for the antibacterial activity of the peak was obtained by removal of the inhibitory activity in the NCF after chelation of the zinc.

Peak 1 material was applied to the DEAE A-25 Sephadex column to determine whether the peptide component was free of other peptides and to determine whether the carbohydrate contained in the peak was either bound to the inhibitory peptide or was a contaminant. Only one peptide was eluted from the DEAE column, and this peptide contained the phosphate-sensitive bacterial inhibitory activity. In addition, only one peptide band was detected by acrylamide gel electrophoresis. At pH 8.6 the peptide migrated toward the anode almost as fast as bromophenol blue. This indicates a peptide with a low molecular weight and net negative charge. The peptide band obtained in the gel column consistently diffused out of the 15% gel. The peptide was not precipitable by trichloroacetic acid, and it could not be stained using the perchloric acid-Coomassie blue procedure of Reisner et al. (34).

TABLE 2. *Amino acid analysis of Bio-Gel P-2 peak 1*

Residue	No./ molecule[a]
Lys	1
Glx[b]	3
Gly	2

[a] Based on lysine as one residue.
[b] Glx includes glutamine and glutamic acid.

The carbohydrate contained in the peak 1 material from the Bio-Gel P-2 column was separated from the peptide by ion-exchange chromatography. This is significant since it demonstrates that the carbohydrate does not stabilize the peptide and make it resistant to heat and to Pronase and trypsin inactivation as has previously been shown to occur with glycoproteins (6, 27).

The peptide obtained from the ion-exchange

TABLE 3. *Specificity of the zinc-peptide antibacterial system for zinc*

Metal concn (μg/ml)	Inhibition by:					
	Zinc		Copper		Magnesium	
	NCF	CDM	NCF	CDM	NCF	CDM
0.05	−[a]	−	−	+	−	−
0.10	−	−	−	++	−	−
0.20	−	−	−	++	−	−
0.40	+[b]	−	+	++	−	−
0.81	+	−	+	++	−	−
1.62	+	−	++[c]	++	−	−
3.25	+	−	++	++	−	−
6.50	+	−	++	++	−	−

[a] −, Not inhibitory.

[b] +, Inhibition present; log cells per milliliter of NCF or CDM minus log cells per milliliter of NCF or CDM + metal is greater than 1 after 12 h of incubation.

[c] ++, Bactericidal.

column was shown to be inhibitory when added to the CDM to which 0.3 μg of zinc per ml had been added. The inhibitory activity of the peptide in the CDM was partially lost by the addition of phosphate. Failure to completely destroy the inhibitory activity may be due to the toxic effects on *E. coli* of the NaCl used in the elution gradient. The peptide could not be completely separated from NaCl using the Bio-Gel P-2 column. To minimize the toxic effects of NaCl, the peptide material was added to the CDM at one-half physiological concentration.

Amino acid analysis of the peptide component of the phosphate-sensitive inhibitor suggested that glutamine-glutamic acid, glycine, and lysine were present. Glutamine and glutamic acid cannot be distinguished after acid hydrolysis. Since the peptide migrates rapidly toward the anode at pH 8.6, there may be more glutamic acid present than glutamine. Tryptophan, which is completely destroyed by acid hydrolysis, was not present. It is presently unclear what compound was responsible for the 272-nm absorbance peak of the peptide.

The peptide is heat stable (39), and its activity is resistant to Pronase and trypsin digestion (37). Similar properties are associated with lysozyme when the metal cation manganese is bound (16). The removal of manganese renders the enzyme susceptible to inactivation by heat and proteolytic enzymes. Zinc bound to the inhibitory peptide from AF may stabilize the structure of the peptide in a similar way. Alternatively, the peptide may be cleaved by the proteolytic enzymes. But the cleaved structure may still retain activity. Evidence against this second possibility, however, was provided by carboxypeptidase inactivation of the peptide inhibitory component. Since carboxypeptidase destroyed the inhibitory activity, the intact peptide molecule or at least carboxyl end amino acids may be required for inhibitory activity. Since trypsin does not destroy inhibitory activity, the lysine contained in the peptide may be the carboxyl terminal amino acid (1). It is possible that heat treatment would not extensively denature the peptide in the absence of zinc since the low molecular weight of the peptide would limit the formation of a tertiary structure.

The 6 to 1 ratio of peptide concentration using the microbiuret and Folin assays is consistent with the observation that the peptide probably does not contain aromatic amino acids. Protein measured by the Lowry assay depends largely on the presence of aromatic amino acids (20), which are often absent or in low concentration in low-molecular-weight peptides (4, 29). In contrast, the microbiuret procedure measures biuret content ($H_2NCONHCONH_2$) and, therefore, depends on the presence of peptide bonds. The microbiuret assay should then be the more accurate measure of peptide content.

The molecular weight of the peptide component of the phosphate-sensitive inhibitor was estimated to be 630 based on the amino acid analysis. The 630-dalton value corresponds closely with the 700-dalton value obtained by gel filtration and, therefore, is likely the correct molecular weight estimate. Based on the 630-value for the peptide, the amino acid content would be: 3 glutamine-glutamic acid, 2 glycine, and 1 lysine. With this amino acid composition, the peptide was determined to be 71% pure. Final determination of amino acid content and molecular weight requires sequence determination of the peptide. Synthesis of the peptide after sequence determination and subsequent demonstration of phosphate-sensitive inhibitory activity in the presence of zinc would provide conclusive evidence that the peptide is the second component of the phosphate-sensitive inhibitor.

Zinc and the peptide appear to be the only two components required for bacterial inhibitory activity. The mechanism by which these two components interact to form the antibacterial system is unclear at present. The amino acids contained in the peptide would provide a good environment for binding zinc (32). Both glutamine and lysine contain nitrogen atoms not involved in peptide bond formation, and glutamic acid has an additional carboxyl group. At physiological pH values, there should be sufficient lone-pair electrons present on nitrogen and oxygen atoms to complex zinc in a way

similar to histidine-containing peptides (41). Since copper and magnesium do not appear to be able to replace zinc, a very precise structure may be required to effect bacterial killing.

The interaction of phosphate with the zinc-peptide complex is also unclear. Phosphate does not appear to be tightly bound by the zinc-peptide complex since it is removed by gel filtration. Another possibility is that peaks consisting of peptide-zinc-phosphate, peptide-phosphate, and peptide alone may have been present but, because of their phosphate content or lack of zinc, would be noninhibitory. These combinations of components would not be detected by the techniques used.

In summary, the phosphate-sensitive inhibitor contained in human AF was shown to consist of zinc and a very low-molecular-weight peptide with a probable amino acid composition of: 3 glutamine-glutamic acid, 2 glycine, and 1 lysine. Its mechanism of antibacterial activity is not known but merits investigation.

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