# Antibacterial Peptide from Normal Rabbit Serum. 1. Isolation from Whole Serum, Activity, and Microbicidal Spectrum†

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ABSTRACT: A procedure is described for purification of the primary bactericidal component of normal rabbit serum active in vitro against *Bacillus subtilis*. A 65 000-fold increase in specific bactericidal activity per milligram of serum protein was obtained, yielding a low molecular weight, heat-stable polypeptide fraction (PC-III) exhibiting biological activity at protein concentrations below 10 ng/mL. This preparation appeared homogeneous as judged by column chromatography and analytical $NaDodSO_4$–polyacrylamide gel electroporesis; recovery of serum bactericidal activity was routinely greater than 80%. Analysis of dansylated or $^{125}I$-labeled samples in peptide-resolving polyacrylamide gels revealed a single band with an $M_r$ of 1800. Optimal antibacterial activity of PC-III against *B. subtilis* occurred at an ionic strength of 0.24 and was absolutely dependent upon divalent cations; calcium was the most effective. Under optimum conditions, 4 ng/mL of PC-III reduced the viability of *B. subtilis* test innocula by 90% within 10 min at 37 °C. *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella typhimurium* were all sensitive to the action of PC-III, but higher bactericide concentrations were required to produce similar reductions in viability as observed with *B. subtilis*. All strains were killed by PC-III concentrations well below 1 μg/mL, roughly that found in normal serum. The activity of PC-III preparations was significantly reduced by pretreatment with trypsin or proteinase K but not by neuraminidase or periodate.

The microbicidal properties of normal mammalian sera against Gram-positive bacteria were first described nearly 100 years ago (Fodor, 1887; von Behring, 1888), yet little is known concerning the nature, number, and action of the agents involved. Numerous attempts to isolate these components from serum (e.g., Myrvik & Leake, 1960; Johnson & Donaldson, 1968) and platelets (Amano et al., 1952; Jago & Jacox, 1961; Weksler & Nachman, 1971), the presumed cellular source in rabbits, have met with only limited success. Problems usually encountered in characterizing these preparations have included significant losses of activity following preliminary fractionation as well as alterations in the physical and biological properties of these agents when compared to the sources from which they were isolated. Thus, as a result of numerous difficulties in purification and their apparent minute concentrations in serum, the role of humoral bactericides in infection–immunity remains unclear.

These serum factors, examples of what may be termed constitutive host defense mechanisms, are of biological interest because they may not only play a role in the delineation of saprophyte and pathogen but also represent a class of naturally occurring, antibiotic-like agents capable of affecting microbial viability in vivo. Recent investigations (Carroll & Martinez, 1979b) have demonstrated that the kinetics of action, cellular source, and relative concentrations of the serum bactericides, termed *β-lysins* in past literature (Pettersson, 1926), vary between animal species. Thus, characterization of the physical properties and molecular mechanisms of these agents may prove useful from both an experimental and a therapeutic standpoints.

Normal rabbit serum, a fluid which exhibits high levels of antibacterial activity (Mackie & Finkelstein, 1932; Myrvik & Weiser, 1955), contains at least two components responsible for its action in vitro against *Bacillus subtilis* (Carroll & Martinez, 1979b). The secondary component, lysozyme, has been purified to homogeneity (Carroll & Martinez, 1979a) and accounts for only a small fraction of the observed *serum* microbicidal action. In contrast, the primary rabbit serum bactericide appears to be of low molecular weight, is "released" from platelets in response to coagulation (Hirsch, 1960) or immune injury (Roberts et al., 1976) (events which result in aggregation and degranulation of platelets), and exhibits optimal activity at ionic strengths higher than that of normal blood fluids (Matheson et al., 1972; Carroll & Martinez, 1979b).

In this report, we describe a rapid, two-step purification scheme for isolation of the primary bactericide directly from normal rabbit serum. Sufficient yields of active, homogeneous material have been obtained to allow characterization of biological activity and optimal reaction conditions as well as chemical analysis on a microscale. Subsequent papers (Carroll & Martinez, 1981a,b) describe the composition and possible action of the purified serum bactericide. Taken together, these studies indicate that the biologically active serum component (i) is a cationic peptide composed of less than 20 amino acid residues, (ii) is a potent inhibitor of electron transport at concentrations below 2 nM, and (iii) exhibits a broad range of action against both Gram-positive and Gram-negative bacteria.

## Experimental Procedures

*Purification of the Rabbit Serum Bactericide.* The collection of whole blood and the production of partially purified bactericidal fractions from the serum of healthy New Zealand white rabbits have been described (Donaldson et al., 1964; Carroll & Martinez, 1979b). Purification of the rabbit serum component was achieved by modification and extension of the above techniques. In the procedure routinely employed, 150 mL of normal rabbit serum was allowed to drip through a cellulose–asbestos sterilizing filter (Hercules ST-L6) previously washed with 50 mM potassium phosphate buffer, pH 7.4, at 4 °C. Filtration was repeated twice under gentle suction. After two washes with 50 mL of phosphate buffer, the filter was inverted and replaced in the filtration apparatus, and a

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fraction containing bactericidal activity was eluted with 20 mL of 1.2 M phosphate buffer, pH 7.4. At no time was the filter allowed to dry out under suction. Contaminating blood cells and debris in the filter eluate fraction were removed by filtration through a 0.45-$\mu$m Millipore filter.

The filter eluate fraction was diluted to 0.4 M phosphate (1:3, 60 mL final volume) at 4 °C and applied to a 1.3 × 5 cm column of Whatman P11 cellulose phosphate (phosphocellulose). Prior to sample application, the ion exchanger was cycled with HCl and NaOH (Dixon & Thompson, 1968), followed by a 1.5 M phosphate wash and equilibration at 4 °C in 0.4 M phosphate buffer (pH 7.4), and then cast in a plastic cylinder equipped with a nylon wool plug. The effluent was collected batchwise until the entire sample had been applied by using a flow rate of 20 mL/(cm$^2$·h); washing of the column was performed at the same flow rate with 10 column volumes (60 mL) of equilibration buffer, and 4-mL fractions were collected. When the absorbance at 280 nm of wash fractions approached zero, the flow rate through the column was reduced to 12 mL/(cm$^2$·h). A linear 0.4–1.2 M phosphate gradient (70 mL total volume) was applied, and 0.75-mL fractions were collected, with monitoring at 215, 225, and 280 nm. High concentrations of phosphate buffer (above 0.4 M) had slight absorbance at 215 and 225 nm; values presented have been corrected by comparison with a standard curve. Column fractions were subsequently examined in the bactericidal assay (see below) for determination of activity. The single, biologically active peak eluting after the gradient was initiated (PC-III; see text) was pooled and stored in plastic tubes at −20 °C.

*Protein Determination and Iodination.* Protein concentrations were determined fluorometrically by using modifications of published procedures (Carroll & Nelson, 1979; Lai, 1977; Robrish et al., 1978). Samples and standards were placed in ignition-cleaned (500 °C, 3 h) 10 × 75 mm disposable glass tubes and made to 0.1 mL with high-performance liquid chromatography grade water (Baker). A 0.1-mL sample of 1.0 N NaOH was added with mixing, the rack was covered with aluminum foil, and the samples were hydrolyzed by placing the covered rack in an autoclave at 121 °C for 30 min. After cooling to room temperature, the samples were neutralized by adding 0.4 mL of 0.25 N HCl, followed by 1.0 mL of 0.4 M sodium borate buffer, pH 9.7, containing 0.1% 2-mercaptoethanol and 0.1% Brij 35 (Pierce Chemical Co.). A 0.4-mL sample of 0.04% *o*-phthalaldehyde (Fluoropa, Durram Chemical Corp.) in $H_2O$ (diluted from a 4% stock solution in Sequanal grade methanol, Pierce) was then added, and the incubation mixture (final volume 2.0 mL) was allowed to stand at room temperature for 10 min. Relative sample fluorescence was determined by using an Aminco J4-7439 Fluoro-Colorimeter as described (Carroll & Nelson, 1979). Hen egg white lysozyme (LZM)[1] (Worthington Biochemicals) dissolved in phosphate buffer was used as the standard at an $E^{1\%}_{1cm}$ (280 nm) of 25.5 (Praissman & Rupley, 1968).

Individual or pooled column samples were iodinated by using a modification of the method described by Fraker & Speck (1978). To a 0.1-mL sample in 0.6–0.8 M phosphate buffer at 4 °C was added 0.2 mCi of carrier-free $^{125}$I (Amersham), and the mixture was transferred to a precooled test tube previously coated with 10 $\mu$g of Iodogen (Pierce). The tube was kept on ice and agitated gently for 10 min, at which time 1 $\mu$L of KI (10 mg/mL) was added to quench the reaction. Radioactivity was monitored by mixing aliquots with 2 mL of ACS counting fluor (Amersham) in small beta/gamma vials (Research Products International) and counting in a Beckman LS-100C scintillation counter using the full $^3$H–$^{14}$C window. Pooled peak fractions labeled in this manner had specific activities of about 2 × 10$^5$ cpm/$\mu$g of protein.

The processing of small samples (up to 100 $\mu$L) to remove high levels of phosphate, or reaction products following iodination, was accomplished by gel filtration on a 0.8 × 25 cm column of Sephadex G-10 (Pharmacia). Elution solvents were 30% acetic acid or 50 mM phosphate buffer containing 0.5 M NaCl. Dialysis was performed in Spectrapor dialysis membranes (Spectrum Medical Industries) against several 1-L volumes of 50 mM phosphate buffer at 4 °C.

*Electrophoresis and Autoradiography.* Analytical sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gradient slab gel electrophoresis, pH 8.3, and native polyacrylamide gel electrophoresis, pH 4.5, were performed as previously described (Carroll & Martinez, 1979b). For molecular weight estimates of peptides in $NaDodSO_4$ gels, the ratio of acrylamide to bis(acrylamide) was decreased to 10:1 (12.5% and 1.25%, respectively), and urea was added to a final concentration of 8 M (Swank & Munkres, 1971). Dansylation reaction mixtures contained 1.0 nmol of sample, 0.1 M $NaHCO_3$, 2% $NaDodSO_4$ (BDH Chemicals, Ltd.), 8 M urea (Ultra pure, Schwarz/Mann), and 5 mM dansyl chloride (Dns-Cl) (Pierce). Hen egg white lysozyme ($M_r$ 14 300), cytochrome *c* ($M_r$ 12 300), basic trypsin inhibitor ($M_r$ 6518), and bacitracin ($M_r$ 1400) (all from Sigma) were used as standards [see Kato & Sasaki (1975)]. Relative mobilities of standards and samples were calculated from migration distances compared to that of Dns-OH.

Immediately following electrophoresis, gels to be autoradiographed were immersed in 30% methanol for up to 2 h with several changes and then transferred to a solution containing 30% methanol and 2% glycerol. One hour later, the gel was placed in a vacuum gel-drying chamber and dried flat onto a piece of Whatman No. 1 filter paper. Kodak X-Omat RP film was sandwiched between the dried gel and a Cronex Lighting-Plus intensifying screen (Du Pont) and exposed at −70 °C (Swanstrom & Shank, 1978).

*Test Organisms. Bacillus subtilis* 168 (*trp*$^-$, *fla*$^-$) was cultured in Spizizen (1958) minimal medium and used for most routine assays of bactericidal activity. *Listeria monocytogenes* SC-44-144 was cultured and plated in Brucella broth (BBL), while *Escherichia coli* ML 35 and *Salmonella typhimurium* 1783 were cultured in nutrient broth (Difco). All strains were grown at 37 °C, harvested in mid log phase, and resuspended in 50 mM phosphate buffer, pH 7.4, containing 1% glucose (glucose–phosphate). The cells were stored in ice until used.

*Bactericidal Assay and Activity Expression.* The antibacterial potency of serum or column fractions were measured in two ways. Initial experiments (Figures 1 and 2) followed the kinetics of reduction in colony-forming units (CFU) of *B. subtilis* over a 20-min period at 37 °C (Carroll & Martinez, 1979b). Experiments examining the susceptibility of other test organisms (Figure 9) were performed in a similar manner by using the indicated organisms. For these reactions, 0.1% BSA was included in the incubation mixtures, and both incubation and dilution buffers contained 0.25 mM $Ca^{2+}$ and 0.5 mM $Mg^{2+}$. Aliquots were removed at the times indicated, diluted up to 10 000-fold, and plated on the appropriate media. CFU

[1] Abbreviations used: LZM, hen egg white lysozyme; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; CFU, colony-forming units; Dns-Cl, 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride); Dns-OH, 5-(dimethylamino)naphthalene-1-sulfonic acid (dansyl hydroxide).

were determined following incubation at 37 °C for 18–24 h; variations between duplicate plates was <10%. Results are plotted as a function of the initial incubation period.

Subsequent quantitative analyses were performed by using *B. subtilis* in a single-point assay. Reaction mixtures (prepared in duplicate) contained various sample concentrations as indicated in the text, 0.8 mM $Ca^{2+}$, and 0.1% BSA. The ionic strength of each sample was adjusted to 0.24 with 1.5 M NaCl in buffer, and the volume made to 0.9 mL with glucose–phosphate. Following a 2-min preincubation at 37 °C, the reaction was initiated by adding 0.1 mL of the bacterial cell suspension, resulting in a final cell concentration of $2 \times 10^7$ per mL. At appropriate times (see text), samples were removed, serially diluted in glucose–phosphate containing 0.4 mM $Ca^{2+}$, and plated. Killing activity is expressed as a logarithmic function of the number of cells killed during the period of incubation per unit test sample volume or protein, i.e., log $(N_c/N)$ $(5\ \text{min})^{-1}$ $\text{ng}^{-1}$, where $N_c$ is the number of cells surviving control incubations in buffer and $N$ those surviving incubation with the test samples. All experiments were repeated 3 or more times. The data reported are the results of individual representative experiments.

*Ionic Strength and Cofactor Requirements.* The effect of ionic strength on the antibacterial activity of PC-III was examined by adding increasing amounts of 1.5 M NaCl (dissolved in phosphate buffer) to mixtures containing 20 ng/mL of the serum factor. Reaction mixtures were incubated for 20 min and CFU determined as above. The viability of control samples was examined in the same manner, with calculations ($N_c$) based on the number of cells surviving incubation at an ionic strength of 0.13.

Several divalent cations and anions were examined for their influence on bactericidal action. All were of analytical grade (Mallinkrodt) and were dissolved in double-distilled water to 0.1 M. Reaction mixtures contained 10 ng/mL of PC-III and up to 1.5 mM added ions. Incubations were for 10 min or longer at 37 °C, with a final ionic strength of 0.24. Samples were then diluted into (i) glucose–phosphate, (ii) glucose–phosphate containing either $Ca^{2+}$ or $Ca^{2+}$ and $Mg^{2+}$ as indicated, or (iii) glucose–phosphate containing 1 mM disodium ethylenediaminetetraacetic acid ($Na_2EDTA$, Sigma) and plated. Results are expressed as percent of the maximally observed activity.

*Enzymatic and Chemical Modifications.* Samples of PC-III containing 15 ng/mL of protein were preincubated at 37 °C with trypsin (TPCK, Worthington Biochemicals), proteinase K (Boehringer-Mannheim), DNase 1 (Sigma), RNase A (type II-A, Sigma), and neuraminidase (type V, Sigma) at the concentrations and for times indicated in the text and then examined in the bactericidal assay. Control experiments showed that the enzymes per se had no bactericidal effect at the concentrations used in the assay mixtures. PC-III samples were also treated with 2% periodic acid (Matheson, Coleman and Bell) at 37 °C for 15 min and then desalted on Sephadex G-10. The antibacterial activity of these fractions was compared to samples treated in an identical manner without periodate.

## Results

*Preliminary Fractionation.* Normal rabbit serum can be depleted of bactericidal activity for *B. subtilis* by passage through a cellulose–asbestos sterilizing filter (Donaldson et al., 1964; Carroll & Martinez, 1979b). Subsequent elution of the filter with NaCl yields a partially purified bactericidal fraction (the filter eluate fraction) containing most of the original serum activity, but less than 0.1% of the serum protein,

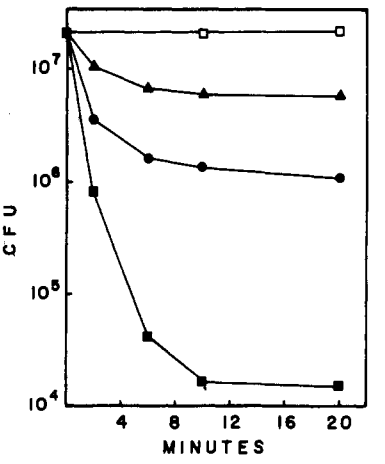


FIGURE 1: Effect of cellulose–asbestos filtration on the kinetics of killing of *B. subtilis* by normal rabbit serum. The bactericidal activity of serum fractions was examined following one (●) and two (or more) (▲) consecutive passages through the sterilizing filter. The viability of *B. subtilis* in buffer (□) was also examined. Incubations were performed in 50 mM phosphate buffer (pH 7.4) containing 1% glucose (glucose–phosphate), 0.5 mM $Mg^{2+}$, 0.25 mM $Ca^{2+}$, and 0.1% BSA. Following a 2-min preincubation of the samples at 37 °C, cells were added to a final concentration of $2 \times 10^7$/mL. Aliquots were removed at the times indicated, diluted in glucose–phosphate containing cations, and plated on nutrient agar. Colony-forming units (CFU) were determined after 18 h at 37 °C. All fractions were examined at the concentration equivalence of 2% (v/v) normal serum (■).

and was used as an initial enrichment for the rabbit serum component(s). Preliminary observations, however, indicated that a single filtration step did not remove all the nonlysozyme activity present in 150 mL of normal serum; varying the size of the filter or the amount of serum used did not significantly increase adsorption. Examination of serum (2% v/v) in the bactericidal assay following consecutive passages through the sterilizing filter (Figure 1) quantitates these observations. Roughly 85% of the nonlysozyme activity was removed by a single filtration; two or more passes reduced the serum activity to basal lysozyme levels [see Carroll & Martinez (1979b)]. This reduction in effluent activity was coincident with increases in the microbicidal activity of the filter eluate fraction. An increase in eluate activity was also observed when the filter was eluted with 1.2 M phosphate buffer, pH 7.4, instead of the previously used 1.5 M NaCl.

Figure 2 illustrates the effects of dialysis on the bactericidal activity of the filter eluate fraction. All samples were examined at concentrations comparable by volume to 2% serum [i.e., the material present in the filter eluate fraction had been concentrated 5-fold over that in serum and was examined at 0.4% (v/v)]. Dialysis of the filter eluate fraction in membranes which retain molecules greater than 12000–14000 (Spectrapor 2), 6000–8000 (Spectrapor 1), or 3500 daltons (Spectrapor 3) resulted in significant decreases in bactericidal activity. Increasing the ionic strength of reaction mixtures to compensate for lost electrolytes only partially reversed this effect, as did acetylation of membranes by pretreatment with 5% acetic anhydride in pyridine.

In contrast, dialysis of whole rabbit serum in Spectrapor 3 did not affect the bactericidal capacity of this fluid (data not shown), suggesting that the bactericide(s) may be firmly bound to a larger serum component. No evidence was found for the presence of dialyzable serum components; attempts to restore original levels of activity by adding lyophilized serum dialysates to dialyzed filter eluate fractions proved unsuccessful and did not alter their killing kinetics. Restoring physiological levels of rabbit lysozyme (removed during filtration step) to

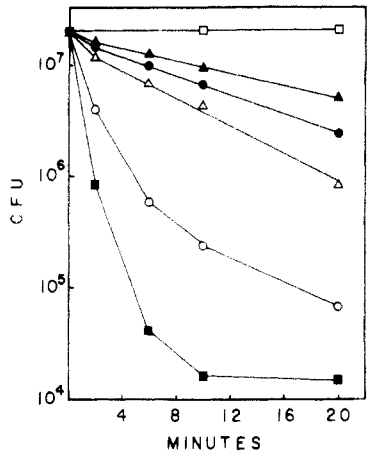


FIGURE 2: Effect of dialysis on the kinetics of killing of *B. subtilis* by the filter eluate fraction. Aliquots of the filter eluate were dialyzed overnight in Spectrapor 3 (△), 1 (●), or 2 (▲) against phosphate buffer (pH 7.4) at 4 °C. Samples were then examined for bactericidal activity compared to that of the undialyzed filter eluate fraction (○). Reactions were performed as described in Figure 1. All samples were examined at the concentration equivalence of 2% serum (■). (□) Buffer control.

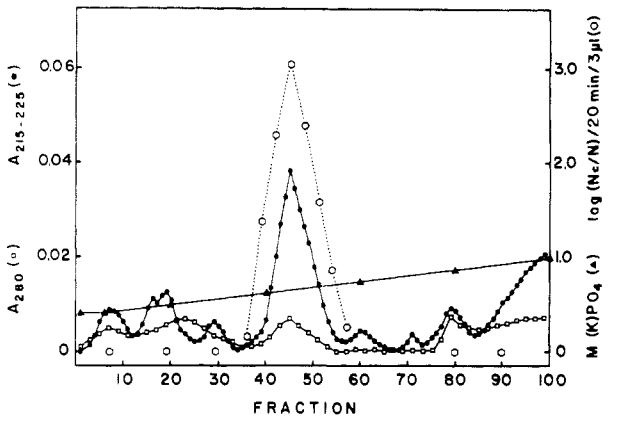


FIGURE 3: Chromatographic profile of the filter eluate fraction (diluted 1:3) on phosphocellulose. The undialyzed filter eluate was diluted with cold distilled water and applied to a 1.3 × 5 cm column of phosphocellulose equilibrated in 0.4 M phosphate buffer (pH 7.4). That material not absorbing to the column (column effluent) was collected as a single batch fraction, and then the column was washed with phosphate buffer. At fraction 1, a linear 0.4–1.2 M phosphate gradient (pH 7.4) was applied, and 0.75-mL fractions were collected. The absorbance of fractions at 280 nm (□) as well as the difference between absorbances at 215 and 225 nm ($\Delta A_{215-225}$) (●) was examined. The phosphate concentration of various fractions (▲) was determined by comparison of the conductivity to that of a standard curve. Four-microliter samples were examined in the bactericidal assay (hexagons), with the activities expressed as a log function of cells killed during the 20-min incubation (see Experimental Procedures and Figure 8).

the undialyzed filter eluate fraction resulted in killing profiles similar to those of 2% serum (data not shown), indicating virtually complete recovery of the primary bactericide(s) in this fraction. For a discussion of the accessory role of lysozyme in the rabbit serum bactericidal reaction, see Carroll & Martinez (1979b).

*Column Chromatography.* Bactericidal activity of the filter eluate fraction was completely neutralized by preincubation with calf thymus DNA (10-fold excess by weight) or phosphocellulose. This ion exchanger was thus used to fractionate the bactericidal serum component(s). Initially, the filter eluate was diluted to 50 mM phosphate (1:24), applied to a phosphocellulose column equilibrated in the same buffer, and eluted with a linear 0.05–1.5 M phosphate gradient (data not shown). Most of the filter eluate protein (~85%) was not adsorbed by the column; this column effluent fraction exhibited no antibacterial activity. With the exception of a broad peak following application of the phosphate gradient, the absorbance of eluted fractions at 280 nm exibited little variation. Four distinct peaks were resolved, however, when the absorbance of fractions was monitored at 215 and 225 nm ($\Delta A_{215-225}$; peptide bonds). Subsequent analysis of column fractions for bactericides using a single 20-min time point revealed that antibacterial activity coeluted with the third peptide peak (PC-III) at 0.7 M phosphate.

Significantly greater resolution was achieved by diluting the filter eluate to 0.4 M (1:3) prior to application and eluting the column with 0.4–1.2 M phosphate (Figure 3), effectively decreasing the slope of the elution gradient. Under these conditions, proteins comprising the first two peaks were not adsorbed to the column, and a single, biologically active peptide peak (refered to as PC-III) was eluted at ~0.63 M phosphate. When the phosphocellulose was precycled in 1.5 M phosphate prior to equilibration in the application buffer, much of the material having an absorbance at 280 or 225 nm could be eliminated. When chromatographed in this manner, more than 90% of the eluted material having an absorbance in the peptide region was found in the biologically active peak. Fluorometric protein analysis of column fractions with *o*-phthalaldehyde substantiated these results. Identical profiles of chromatography and elution were observed with freshly isolated serum or serum stored at −20 °C for up to 5 years. In contrast, citrated plasma serum processed in the same manner yielded a different pattern of elution (data not shown) with no detectable bactericidal activity.

*Analysis on Polyacrylamide Gels.* Electrophoretic analysis of serum and column fractions in an $NaDodSO_4$–polyacrylamide gradient slab gel is shown in Figure 4. Although filtration of whole serum through the sterilizing filter adsorbed most of the bactericidal activity, no apparent Coomassie blue staining bands were removed by this procedure (lanes 2 and 3). Similar results were obtained when the filter eluate fraction was passed through a phosphocellulose column. Batch collection of the column effluent (that major portion of the sample not retained by the column) followed by concentration and electrophoretic analysis yielded patterns indistinguishable from the filter eluate fraction (lanes 4 and 5). Furthermore, samples containing 10 μg of PC-III (lane 6) failed to show any bands, even though lower concentrations of rabbit lysozyme and bovine serum albumin (BSA) (5 μg each, lane 1) were readily detected. Labeling of PC-III with $^{125}I$ followed by electrophoresis and autoradiography resulted in a single, diffuse band (lane 7) which migrated faster than $^{125}I$-labeled lysozyme (lane 8) but slower than free $^{125}I$ (migrating at the dye front).

The homogeneity and apparent low molecular weight of the active fraction was further supported by electrophoresis of PC-III in peptide-resolving $NaDodSO_4$ slab gels containing 8 M urea. Here relative mobility was a linear function of log molecular weight for dansylated standards between 1400 and 14000 daltons [data not shown; see Kato & Sasaki (1975)]. Although dansylation increased the overall molecular weight of each sample, it did not alter the relative mobility of the standards and simplified the detection of low molecular weight peptides (i.e., bacitracin) necessary for calibration. In this system, dansylated or iodinated PC-III fractions exhibited a single band with an apparent $M_r$ of 1800.

*Antibacterial Activity of PC-III.* Previous investigations with the partially purified rabbit serum component indicated that activity against *B. subtilis* was enhanced at ionic strengths above 0.2 (Carroll & Martinez, 1979b). For determination

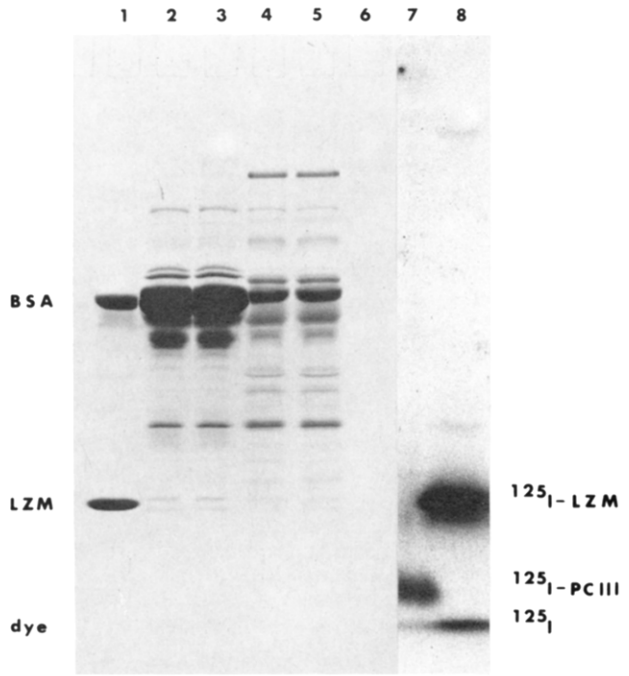


FIGURE 4: $NaDodSO_4$–Polyacrylamide gel electrophoretic analysis of serum and phosphocellulose column fractions. Samples were reduced with 2.5% 2-mercaptoethanol and then electrophoresed in a linear 7.5–20% polyacrylamide gradient–$NaDodSO_4$ slab gel (pH 8.3). (Lane 1) BSA and LZM (5 μg each); (lane 2) normal rabbit serum (50 μg); (lane 3) filtered normal rabbit serum (50 μg); (lane 4) filter eluate fraction (40 μg); (lane 5) phosphocellulose column effluent (40 μg); (lane 6) PC-III (10 μg); (lane 7) $^{125}$I-labeled PC-III; (lane 8) $^{125}$I-labeled LZM. Following electrophoresis, the gel was sliced between lanes 6 and 7. Lanes 1–6 were stained with Coomassie blue R-250 and destained as described (Carroll & Martinez, 1979b). Lanes 7 and 8 were immediately soaked in 30% methanol and 2% glycerol and then dried and autoradiographed.

of optimal conditions for antibacterial action, the effect of ionic strength was first examined by adding 20 ng/mL PC-III to reaction mixtures containing increasing amounts of NaCl and quantitating cell viability after a 20-min incubation. Values presented are relative to control cultures incubated at the same ionic strength. The results (Figure 5) showed that bactericidal activity increased dramatically between ionic strengths of 0.1 and 0.24, with a maximum observed at 0.24. Control cells were stable up to an ionic strength of 0.25. Between 0.25 and 0.3, viability in control incubations decreased slightly, while bactericidal activity decreased at a much faster rate. Raising the ionic strength above 0.3 significantly reduced the viability of control cultures as well as the activity of PC-III.

*Divalent Ion Requirements.* Several divalent cations and anions, including calcium (Jacox, 1950; Myrvik & Weiser, 1955) and bicarbonate (Jago & Jacox, 1961; Myrvik et al., 1958), have been implicated in serum bactericidal action. Consequently, the chloride salts of barium ($Ba^{2+}$), cadmium ($Cd^{2+}$), calcium ($Ca^{2+}$), and magnesium ($Mg^{2+}$) were examined for their ability to influence the action of PC-III. Zinc ions were not tested since under the conditions employed, they were lethal for *B. subtilis*. Incubation mixtures contained the indicated concentrations of cations, 10 ng/mL PC-III, and NaCl to a final ionic strength of 0.24. After 10 min at 37 °C, samples were diluted into glucose–phosphate without added ions and plated. As can be seen in Figure 6, an absolute requirement for divalent cations in the bactericidal reaction was observed. Calcium ions were most effective in enhancing the antibacterial activity of PC-III, followed by $Ba^{2+}$, $Cd^{2+}$, and $Mg^{2+}$. $MgSO_4$ produced the same profiles as $MgCl_2$. Above 1.5 mM, calcium phosphate precipitates formed and

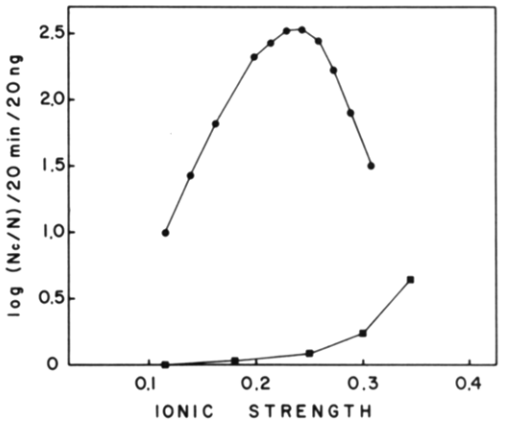


FIGURE 5: Effect of ionic strength on the killing kinetics of *B. subtilis* by PC-III. All reactions (1 mL) were performed in 50 mM phosphate buffer (pH 7.4) containing 1% glucose (glucose–phosphate), 0.1% BSA, 0.5 mM $Mg^{2+}$, and 0.25 mM $Ca^{2+}$ (final ionic strength 0.13). The ionic strength of reaction mixtures containing 20 ng/mL PC-III (●) or buffer controls (■) were increased by the addition of 1.5 M NaCl in phosphate buffer and made to 0.9 mL with glucose–phosphate. Following a 2-min preincubation at 37 °C, cells were added to a final concentration of $2 \times 10^7$/mL, and the incubation was continued. After 20 min, aliquots were removed, serially diluted into glucose–phosphate containing $Mg^{2+}$ and $Ca^{2+}$, and plated. Results are expressed as a log function of the number of cells killed during the 20-min incubation.

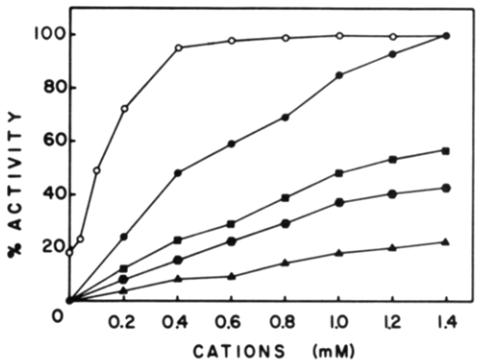


FIGURE 6: Divalent cation requirements in the bactericidal reaction of PC-III against *B. subtilis*. Reactions were performed in glucose–phosphate (pH 7.4) containing 0.1% BSA and sufficient NaCl such that following the addition of PC-III (10 ng/mL) and the indicated concentrations of cations, the final ionic strength was 0.24. Divalent cations examined were $Ba^{2+}$ (■), $Cd^{2+}$ (hexagons), $Ca^{2+}$ (●, ○), and $Mg^{2+}$ (▲). Following a 10-min incubation at 37 °C, aliquots were serially diluted into glucose–phosphate without added cations (closed symbols) or into glucose–phosphate containing 0.5 mM $Mg^{2+}$ and 0.25 mM $Ca^{2+}$ (open circles) and plated. Bactericidal activity was calculated by using the expression log $(N_c/N)/10$ min and had a maximal value of 1.45. Results are presented as percent maximal activity.

inhibited bactericidal activity.

Figure 6 also illustrates the effect of diluting identical reaction mixtures as above into glucose–phosphate containing $Ca^{2+}$ and $Mg^{2+}$. In contrast to dilution into cation-free glucose–phosphate, some killing of cells incubated with the bactericide in the absence of divalent cations was observed (Figure 6; open circles, no added cations). Moreover, the rate of killing increased sharply as the cation concentration in the incubation mixture was raised from 0 to 0.4 mM. Thereafter, a plateau was reached which extended up to 1.4 mM. The maximal killing rate observed under these conditions was equivalent to that seen with reaction mixtures containing 1.4 mM $Ca^{2+}$ and diluted into cation-free buffer (closed circles, Figure 6). The presence of $Mg^{2+}$ in reaction mixtures containing $Ca^{2+}$ had little effect on killing, nor did the inclusion of $Na_2CO_3$ up to 1 mM.

Table I: Effects of Enzymatic and Chemical Treatments on the Antibacterial Activity of PC-III [a]

| reagent | concn (μg/mL) | activity (%) |
|---|---|---|
| control | | 100 |
| trypsin | 10 | 17 |
| proteinase K | 10 | 23 |
| DNase I | 10 | 92 |
| RNase A | 10 | 99 |
| neuraminidase | 10 | 103 |
| periodate | 1 | 94 |

[a] Reactions contained 15 ng/mL of the bactericide and 0.8 mM $Ca^{2+}$ in glucose-phosphate (pH 7.4). Following the addition of the indicated concentrations of reagents, the mixtures were incubated at 37 °C for 10 min. At this time, BSA and NaCl were added to 0.1% and an ionic strength of 0.24, respectively, followed by the addition of cells. Remaining activity was determined as described in Figure 8. Results are expressed as percent activity in control incubations containing PC-III without additional reagents.

*Nature and Stability of the Active Component.* The effect of trypsin treatment on the antibacterial activity of PC-III (15 ng/mL) was examined. Samples were preincubated at 37 °C for 2 min with 0–20 μg/mL trypsin, and then BSA and NaCl were added to final concentrations of 0.1% and an ionic strength of 0.24, respectively, followed by the addition of cells. After incubation for 5 min, the reduction in CFU was determined. Antibacterial activity was found to decrease sharply with increased trypsin concentration to 1.2 μg/mL, at which point a plateau was reached (roughly 20% maximal activity) which extended beyond 5 μg/mL (data not shown). Identical results were obtained if the serum fraction was preincubated for increasing lengths of time with 500 ng/mL trypsin. Concentrations of trypsin as high as 100 μg/mL did not affect the viability of *B. subtilis*.

By use of similar methods, the effects of several other enzymes and periodate on antibacterial activity were examined. Table I presents the results of these experiments. Samples of PC-III were treated for 10 min prior to the addition of BSA and cells. As above, complete inhibition of activity was not observed with any agent, and only enzymes affecting protein structure significantly reduced antimicrobial action.

Because of the reported heat stability of serum β-lysin (Fodor, 1887; von Behring, 1888; Pettersson, 1926), the ability of PC-III to withstand elevated temperatures was examined. Samples were mixed with BSA to a total protein concentration of 70 mg/mL, heated at temperatures up to 80 °C for 10 min, and then tested for bactericidal activity. Both normal serum and the purified serum component were stable to temperatures above 60 °C and exhibited similar heat-inactivation kinetics (data not shown). Reducing the BSA concentration to 10 mg/mL resulted in rapid inactivation of PC-III above 40 °C. However, under both sets of conditions, BSA showed signs of denaturation at temperatures above 60 °C. From these results, the inherent heat stability of the bactericide appears highly dependent on total protein concentration.

The antibacterial activity of PC-III was most stable when stored in 0.8–1 M phosphate buffer, pH 7.4, at −20 °C. Significant losses of biological activity were observed in low ionic strength buffers as well as when samples were incubated between pH 3 and 7 for several hours at 0 °C, neutralized, and assayed.

*Bactericidal Kinetics and Expression of Activity.* Maximal killing occurred when reaction and dilution buffers contained 0.8 and 0.4 mM $Ca^{2+}$, respectively. The effect of increasing concentrations of PC-III on killing kinetics was examined by

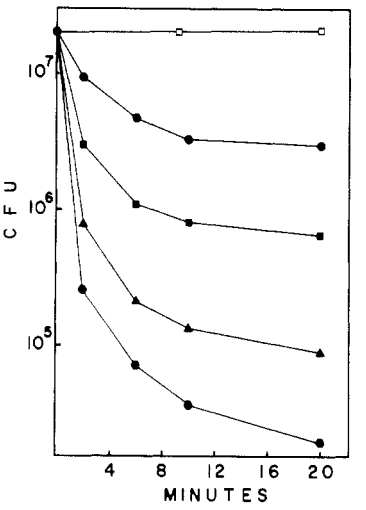


FIGURE 7: Kinetics of killing of *B. subtilis* in the presence of increasing concentrations of PC-III under standardized conditions. Reactions contained the indicated concentrations of PC-III, 0.8 mM $Ca^{2+}$, 0.1% BSA, and NaCl to an ionic strength of 0.24 in glucose–phosphate. As a function of time, aliquots were removed, serially diluted into glucose–phosphate containing 0.4 mM $Ca^{2+}$, and plated. (●) 4, (■) 8, (▲) 16, and (hexagons) 24 ng of PC-III/mL; (□) buffer control.

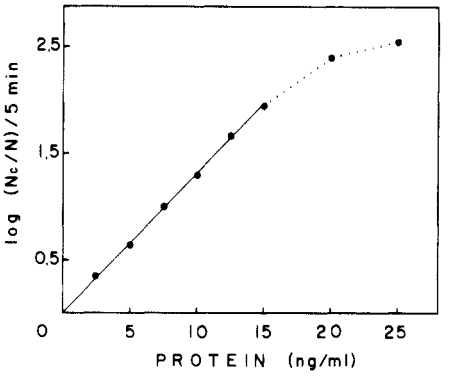


FIGURE 8: Rate relationship of the kinetics of killing of *B. subtilis* to PC-III concentration. Reactions were performed as described in Figure 7 and contained the indicated concentrations of PC-III. After a 5-min incubation at 37 °C, aliquots were removed, serially diluted, and plated. Results are expressed as a logarithmic function of the number of cells killed during the 5-min incubation. $N_c$, the number of cells surviving control incubations in buffer; $N$, the number of cells surviving incubations with the indicated concentration of PC-III.

using these standardized conditions of calcium concentration and ionic strength (0.24). The results (Figure 7) illustrate the potent killing potential of this material. Concentrations as low as 4 ng/mL reduced viability by ~90% within 10 min at 37 °C. Higher concentrations of PC-III resulted in increased rates and extents of killing. Similar results have been observed with partially purified preparations (unpublished data) and form the basis for the single-point assay used throughout these studies. Replotting the data from Figure 7 in terms of the logarithm of cells killed, i.e., log [cells surviving control incubations ($N_c$)/cells surviving incubation with samples ($N$)]

$$\log (N_c/N)$$

as a function of PC-III concentration resulted in a linear relationship for time points between 2 and 20 min, each with a different slope. Such a plot is presented in Figure 8, where reactions containing up to 25 ng/mL PC-III were examined after 5 min of incubation, and is similar to methods describing the action of colicins (Shannon & Hedges, 1967). It is clear that log cells killed after 5-min incubation was a linear function of PC-III concentration up to 15 ng/mL. Deviations from

Table II: Quantitation and Recovery Data of the Rabbit Serum Bactericide in Normal Rabbit Serum Fractions[a]

| sample | total protein (mg/mL) | spec bactericidal act.[b] | factor concn[c] (μg/mL) | volume (mL) | total factor[d] (μg) | % of act. in serum[e] |
|---|---|---|---|---|---|---|
| normal rabbit serum | 75.26 | 2.04 | 1.16 | 150 | 174 | 100 |
| filtered NRS | 75.11 | 0.04 | 0.01 | 150 | 3.5 | 2.0 |
| filter eluate fraction | 0.42 | $2.1 \times 10^3$ | 7.72 | 25 | 168 | 97 |
| PC effluent | 0.41 | 12.17 | 0.04 | 60 | 2.2 | 1.3 |
| PC peak III | 0.01 | $1.3 \times 10^5$ | 10.0 | 15 | 150 | 86 |

[a] The viability of *B. subtilis* was examined following a 5-min incubation with the various samples in reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% BSA, 0.8 mM $CaCl_2$, and NaCl to an ionic strength of 0.24. [b] Determined by using the relationship $\log (N_c/N)/5\ \text{min}^{-1}\ (\text{mg of protein})^{-1}$ (see text). [c] Concentration of the serum component was calculated from the reported specific activities by using the rate established for the purified factor. [d] Obtained by multiplying factor concentration by sample volume. [e] Represents the overall percent of serum activity recovered in each fraction.

linearity occurred beyond 99% cell death, a 2 log reduction. When recultured and tested in the bactericidal assay, cells surviving the above incubations were equally sensitive to the action of PC-III. That the above reductions in CFU were not the result of cellular agglutination was confirmed by microscopic examination of reaction mixtures throughout the incubation period.

*Recovery Data and Concentrations in Serum.* Using the type of analyses described above, we have quantitated the bactericidal factor in normal rabbit blood fluids. The results of several experiments are presented in Table II, along with average recovery data obtained for the isolation procedure. Pooled normal rabbit *serum* contained ~1.16 μg/mL of the bactericidal component, with the sera of individual rabbits varying between 0.7 and 1.4 μg/mL. The bactericidal component thus represents less than $^1/_{65000}$ of the total serum protein. Consecutive passes through the sterilizing filter removed more than 98% of this material, most of which was recovered in the filter eluate. On the average, about 85% of the original serum activity was found in PC-III after phosphocellulose chromatography, yielding ~1 μg of the purified component per mL of serum processed. Among the nine preparations examined to date, recoveries ranged between 75% and 90%. Normal rabbit *plasma* or *plasma serum* contained less than 5 ng/mL of this material.

*Microbicidal Spectrum.* The bactericidal activity of PC-III against other organisms was examined. Reactions were followed as a function of time and at an ionic strength of 0.13 to avoid possible errors resulting from variations in sensitivity. Figure 9 demonstrates that the microbicidal spectrum of PC-III is not restricted to Gram-positive bacteria. Both Gram-negative organisms tested, *Salmonella typhimurium* (Figure 9a) and *Escherichia coli* (Figure 9b), were rapidly killed when incubated with the factor. The rates and extents of killing, however, were much reduced when compared to those observed for the gram-positive organisms *B. subtilis* (Figure 7) and *L. monocytogenes* (Figure 9c). In order to produce a 1 log drop during the 20-min incubation, ca. 5, 50, 200, and 350 ng/mL of the purified component would be required for *B. subtilis*, *L. monocytogenes*, *E. coli*, and *S. typhimurium*, respectively. Thus, although Gram-negative organisms appear much more resistant to the serum component, viability was severely affected at factor concentrations well below 1 μg/mL. Variations in PC-III cation and ionic strength requirements as a function of test organism were not examined.

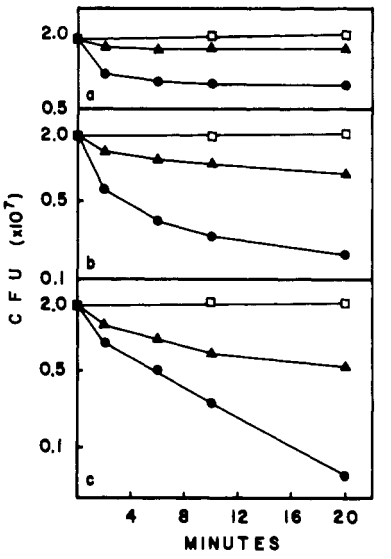


FIGURE 9: Kinetics of killing of Gram-positive and Gram-negative bacteria by PC-III. Reactions conditions were as described in Figure 1. Organisms and concentrations of PC-III examined were the following: (a) *S. typhimurium*, 80 (▲) and 200 (●) ng of PC-III/mL; (b) *E. coli*, 80 (▲) and 200 (●) ng of PC-III/mL; and (c) *L. monocytogenes*, 40 (▲) and 80 (●) ng of PC-III/mL. The viability of all organisms was examined in identical reaction mixtures without PC-III (□).

## Discussion

The constitutive microbicidal mechanisms of normal blood fluids have been recognized since the late 1880s [see Skarnes & Watson (1957)] and may represent one of the first lines of defense against invading microorganisms. In most cases, however, the significance and importance of these systems remain unclear. Only in one instance, i.e., that of human serum and plasma serum, has the primary bactericidin active against *B. subtilis* been isolated and identified as lysozyme (Selsted & Martinez, 1978). For rabbit serum, which contains even higher levels of antibacterial activity (Mackie & Finkelstein, 1932; Myrvik & Weiser, 1955), this enzyme contributes little to the bactericidal reaction (Carroll & Martinez, 1979b). A similar situation has been observed in rat serum (Jago & Jacox, 1961; Yotis & Ortiz, 1967), further demonstrating the heterogeneity of antibacterial mechanisms within mammalian sera.

Normal rabbit serum, due to its high levels of microbicidal action, has been used extensively in attempts to purify and characterize the active component(s). Several of these investigations (Jago & Jacox, 1961; Myrvik & Leake, 1960; Pettersson, 1926) have suggested that at least two components are required for activity. Others have provided evidence for multiple factors with altered microbicidal spectra (Johnson & Donaldson, 1968; Schultz & Wilder, 1971). Unfortunately, the homogeneity and purity of most preparations have not been carefully examined, resulting in considerable confusion.

The results presented here demonstrate that the primary bactericidal activity of normal rabbit serum active against *B. subtilis* resides in a single, low molecular weight, cationic polypeptide fraction designated PC-III. That other compo-

nents present in this fraction may have some influence on the bactericidal reaction, however, is possible since residual killing activity was observed following treatment with proteases. Alternatively, as both proteases used in these experiments have similar specificities, cleavage of the peptide at susceptible sites could release one or more fragments which retain a portion of the original antibacterial activity. Although PC-III also contains small amounts of carbohydrate (Carroll & Martinez, 1981a), additional support for the latter view came from examining the effects of carbohydrate-modifying reagents on bactericidal action. Treatment of PC-III with neuraminidase or *cis*-glycol cleavage with periodic acid had little influence on antibacterial activity, suggesting that sialic acid or defined carbohydrate structures are not necessary for antimicrobial action.

The purified rabbit serum bactericide requires divalent cations for activity (see Figure 6) and was unaffected by bicarbonate. Of the cations examined, calcium allowed the highest rates of bactericidal activity against *B. subtilis*. These results are in conflict with those of other investigators (Jago & Jacox, 1961), particularly those of Myrvik (1956) and Myrvik et al. (1958). In their studies, two nondialyzable rabbit serum components were found, both of which were necessary for activity. In contrast, our results indicate that a single factor may account for normal rabbit serum microbicidal activity. These discrepancies are difficult to reconcile.

Of further interest in this regard are the observations of Amano and co-workers (Kato et al., 1954; Higashi et al., 1966) concerning the effects of ionic cofactors on the action of *plakin*, a bactericidal substance fractionated from rabbit and horse platelets. Their studies demonstrated that plakin activity was absolutely dependent upon divalent cations, and as above, calcium was most effective. Combined with the fact that rabbit plasma serum contains substantially reduced levels of both antibacterial activity and PC-III, but identical levels of lysozyme (Carroll & Martinez, 1979b), these data strongly support the contention that platelets may be the source of the rabbit serum bactericide (Hirsch, 1960).

Weksler & Nachman (1971) have also reported the isolation of rabbit platelet components capable of killing *B. subtilis* in vitro. The primary component, which they suggested was responsible for serum activity, had a $M_r$ of 40 000 and was found localized within the platelet granules. This material not only is much larger than the factor we have recovered directly from serum but also exhibits a much reduced (up to 1000-fold) ability to kill similar test organisms. Although differences in bacterial strains and assay methods used may account for some discrepancies in results, the fact remains that the purified serum peptide exhibits a significantly higher specific activity than the platelet preparation. Both the serum and platelet components, however, are resistant to temperatures above 60 °C in protein-rich media but are unstable between pH 3 and 7 or in low ionic strength solutions.

Although other possibilities exist, the above observations may be explained by suggesting that a larger molecular weight platelet component (possibly plakin) serves as a precursor for the highly active serum peptide. Proteolytic processing of cellular components following their release during platelet aggregation could result in the fraction we have isolated. Since other similarities exist between PC-III and plakin [see Carroll & Martinez (1981b)], this suggestion is currently under investigation.

Within this context, it must be emphasized that although PC-III exibits potent antibacterial action, it may well have other physiological functions. Platelets release a number of components which influence coagulation and the induction of an inflammatory response. Included are a variety of vasoactive mediators (Nachman et al., 1970; Randive & Cochrane, 1968), platelet factor XIII, and phosphoglycerides (Kowalski & Niewiaroski, 1967). Thus, the identification of PC-III as a humoral bactericide does not preclude its possible role in other aspects of host defense.

Results from our laboratory (Carroll & Martinez, 1981b) as well as others (Gooch & Donaldson, 1974; Matheson & Donaldson, 1970; Matheson et al., 1972) make it reasonable to assume that the cytoplasmic membrane is the primary target of the rabbit serum bactericide. On the basis of this assumption, the greater resistance of Gram-negative bacteria (see Figure 7) may result from the reduced ability of PC-III to reach its target site. In addition to the cytoplasmic membrane and peptidoglycan layer found in Gram-positive organisms, Gram-negative bacteria also possess a semipermeable outer membrane (Nikaido, 1973). This structure, capable of regulating the accessibility of external compounds to underlying sites (Kahae & Nikaido, 1975), has been shown to function in resistance to penicillins and cephalosporins (Burman et al., 1972; Costerton & Cheng, 1976). Whether this structure serves a similar function in resistance to PC-III has yet to be examined.

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# Antibacterial Peptide from Normal Rabbit Serum. 2. Compositional Microanalysis†

Stephen F. Carroll* and Rafael J. Martinez

ABSTRACT: The composition and homogeneity of the primary bactericide (PC-III) isolated from normal rabbit serum were examined by microanalytical techniques. Dansylated acid hydrolysates of PC-III were analyzed by two-dimensional thin-layer chromatography on polyamide layers. Quantitation of the separated dansyl amino acids was achieved by elution into methanol, followed by determination of fluorescence in a filter fluorometer. The method proved inexpensive and reliable, allowing accurate analysis of peptide samples containing greater than 20 pmol of each amino acid. By use of this technique, an amino acid composition for PC-III is presented which suggests that the rabbit serum bactericide contains a single, 2000-dalton peptide composed of 17 amino acid residues, 24% basic and 35% nonpolar. One of the basic residues appears to be a modified amino acid, tentatively identified as $N^{\epsilon}$-methyllysine. Although small amounts of carbohydrate and lipid were also detected in PC-III by fluorescent and isotopic techniques, gel filtration or extraction with organic solvents removed much of this material without significantly affecting biological activity.

Normal rabbit serum contains a heat-stable, low molecular weight, cationic polypeptide component capable of killing *Bacillus subtilis* in vitro (Carroll & Martinez, 1981a). This material, designated PC-III,[1] is present in whole serum at ~1 μg of protein/mL, is not found in plasma serum, and appears to be "released" from platelets during coagulation (Hirsch, 1960) or immune injury (Roberts et al., 1977). Although this peptide fraction has been purified to homogeneity as evidenced by general chromatographic and electrophoretic behavior, it remains unclear whether it represents a single homogeneous component or a mixture of related molecules. Furthermore, whether PC-III exists preformed in the cellular elements of blood (presumably platelets) or is derived from a larger platelet component by proteolytic processing has yet to be established.

One approach to resolving these questions is compositional and *N*-terminal analysis, thus allowing correlation with other platelet and serum components. The low concentration of PC-III in rabbit serum (~1 μg/mL) suggests, however, that sufficient material for amino acid analysis by conventional analytical procedures would require the processing of several liters of blood. Although automated methods using the fluorogenic reagent *o*-phthalaldehyde in conjunction with

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[1] Abbreviations used: PC-III, biologically active, antibacterial rabbit serum fraction eluting from phosphocellulose [see Carroll & Martinez (1981a)]; CFU, colony-forming units; Dns-Cl, 5-(dimethylamino)-naphthalene-1-sulfonyl chloride (dansyl chloride); Dns-aa, dansyl amino acid; Dns-OH, 5-(dimethylamino)naphthalene-1-sulfonic acid (dansyl hydroxide); TLC, thin-layer chromatography.

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