Kowalski, E., & Niewiaroski, S., Eds. (1967) *Biochemistry of Blood Platelets*, Academic Press, New York.
Lai, C. Y. (1977) *Methods Enzymol. 47*, 236–243.
Mackie, T. J., & Finkelstein, M. W. (1932) *J. Hyg. 32*, 1–24.
Matheson, A., & Donaldson, D. M. (1970) *J. Bacteriol. 101*, 314–317.
Matheson, A., Kwong, M. C. M., & Chung, R. (1972) *Can. J. Microbiol. 18*, 1665–1670.
Myrvik, Q. N. (1956) *Ann. N.Y. Acad. Sci. 66*, 391–400.
Myrvik, Q. N., & Weiser, R. S. (1955) *J. Immunol. 74*, 9–16.
Myrvik, Q. N., & Leake, E. S. (1960) *J. Immunol. 84*, 247–250.
Myrvik, Q. N., Soto-Figueroa, E., Naff, G., & Henelt, E. (1958) *J. Immunol. 81*, 118–122.
Nachman, R. L., Weksler, B., & Ferris, B. (1970) *J. Clin. Invest. 49*, 274–281.
Nikaido, H. (1973) in *Bacterial Membranes and Walls* (Leive, L., Ed.) p 131–208, Marcel Dekker, New York.
Pettersson, A. (1926) *Z. Immunitaetsforsch. 48*, 233–296.
Praissman, M., & Rupley, J. A. (1968) *Biochemistry 7*, 2446–2450.
Randive, N. S., & Cochrane, C. G. (1968) *J. Exp. Med. 128*, 605–622.
Roberts, R. R., Tew, J. G., & Donaldson, D. M. (1976) *Infect. Immun. 15*, 485–490.
Robrish, S. A., Kemp, A. C., & Bowen, W. H. (1978) *Anal. Biochem. 84*, 196–204.
Schultz, L. D., & Wilder, M. S. (1971) *Infect. Immun. 4*, 703–708.
Selsted, M. E., & Martinez, R. J. (1978) *Infect. Immun. 20*, 782–791.
Shannon, R., & Hedges, A. J. (1967) *J. Bacteriol. 93*, 1353–1359.
Skarnes, R. C., & Watson, D. W. (1957) *Bacteriol. Rev. 21*, 273–294.
Spizizen, J. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 1072–1078.
Swank, R. T., & Munkres, K. D. (1971) *Anal. Biochem. 39*, 462–477.
Swanstrom, R., & Shank, P. R. (1978) *Anal. Biochem. 86*, 184–192.
von Behring, E. (1888) *Centralbl. Klin. Med. 9*, 681–690.
Weksler, B. B., & Nachman, R. L. (1971) *J. Exp. Med. 134*, 1114–1130.
Yotis, W. W., & Ortiz, J. S. (1967) *Can. J. Microbiol. 13*, 885–894.

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^{ϵ}-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

† From the Department of Microbiology, College of Letters and Science, University of California, Los Angeles, California 90024. *Received July 10, 1980; revised manuscript received June 2, 1981.* This investigation was supported by U.S. Public Health Service Grant AI 12835 from the National Institute of Allergy and Infectious Diseases. S.F.C. was the recipient of U.S. Public Health Service Traineeship AI 07116 from NIAID.

[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.

0006-2960/81/0420-5981$01.25/0

microbore columns (Benson & Hare, 1975) or high-performance liquid chromatography (Hill et al., 1979) have significantly increased the sensitivity of analysis, such instrumentation is not always available. A simplified manual technique for amino acid analysis which would still yield reliable results was thus felt desirable.

Of the current techniques for characterization of biologically active compounds on a microscale, quantitation of amino acids using isotopically labeled dansyl chloride (Dns-Cl) (Beart & Snodgrass, 1975; Briel & Newhoff, 1972; Brown & Perham, 1973; Burzgnshi, 1975) has gained wide acceptance. Several methods have also appeared which combine the separation of dansylated amino acids (Dns-aa) on silica gel, followed by fluorodensitometry (Spivak et al., 1971) or elution and determination of fluorescence (Crowshaw et al., 1967; Zanetta et al., 1970).

In this paper, we present data obtained from two-dimensional thin-layer chromatography (TLC) of Dns-aa on polyamide layers (Woods & Wang, 1967), coupled with methanol elution and determination of fluorescence, to quantitate the amino acid composition of PC-III. The method proved rapid and reliable, with better than 8% reproducibility. Our results indicate that the primary bactericide of normal rabbit serum is composed of a single peptide containing ~17 amino acid residues. In addition, small amounts of lipid and carbohydrate were also found in PC-III fractions when analyzed by microfluorometric and isotopic techniques which may prove useful in the characterization of other biological molecules.

Experimental Procedures

Amino Acid Analysis and Thin-Layer Chromatography. The primary bactericidal component, PC-III, present in normal rabbit serum was purified as described (Carroll & Martinez, 1981a). Phosphate buffer was removed by chromatography samples on phosphocellulose at 4 °C with equilibration and elution in 0.2 and 4 M ammonium acetate, respectively. Following lyophilization of desalted samples, the residues were redissolved in distilled water and twice lyophilized to remove additional traces of ammonia. Fractions containing up to 150 μg of protein and one drop of 5% phenol in 6 N HCl (200 μL) were hydrolyzed at 110 ± 1 °C for 16 h (Moore & Stein, 1963). After cooling to room temperature, the hydrolysates were extracted 3 times with 3 mL of light petroleum ether (bp 30–60 °C) and then dried in vacuo over NaOH and P_2O_5 at 40 °C. Residues were resuspended in 100 μL of 0.1 N HCl and stored at 4 °C. For analysis, a 10-μL sample was removed, dried in vacuo over NaOH, and dansylated essentially as described by Woods & Wang (1967). Dansylations were performed in the dark at 45 °C for 30 min by using a final concentration of 5 mM Dns-Cl (Pierce). Samples were then evaporated to dryness over H_2SO_4, resuspended in 2 μL of ice-cold Sequanal grade methanol (Pierce), and stored at −20 °C.

Dansylated amino acid hydrolysates were analyzed by two-dimensional TLC on double-sided polyamide sheets (5 × 5 cm, Schleicher & Schüll) (Brown & Perham, 1973; Hartley, 1970). Solvent systems (v:v) were (1) 1.5% formic acid, (2) benzene/acetic acid (6:1), (3) ethyl acetate/methanol/acetic acid (30:20:1), and (4) same as in solvent 3 but in the proportions 20:1:1 (Hartley, 1970). A sample of the unknown in methanol, approximately 0.1 μL, was spotted on the lower right-hand corner of the sheet, and after the sheet was air-dried, a similar volume of a dansylated Beckman amino acid standard (0.1–0.5 nmol each of 17 amino acids; Beckman Instruments Co.) was spotted in the same position on the reverse side of the sheet. Additional standard mixtures (including Asn and Gln) containing up to six dansylated amino acids were used to check the position of each amino acid. The sheets were placed in covered 250-mL beakers and developed in the first dimension by using 4 mL of solvent 1 until the entire sheet had been transversed. After air-drying in a fume hood for 10 min, the sheets were rotated 90° and developed in the second dimension with solvent 2. When dry, the chromatograms were examined under log wavelength UV light (366 nm) and cut vertically so as to bisect dansyl hydroxide (Dns-OH) but pass between Dns-Pro and Dns-Ala (Brown & Perham, 1973; see Figure 1b). The section not containing the origin was redeveloped with solvent 3 in the same direction as solvent 2. After the chromatograms were dried, 25-mm^2 sections of polyamide containing individual DNS amino acids were carefully removed and individually transferred to ignition-cleaned (500 °C, 3 h) 10 × 75 mm disposable tubes. Two milliliters of Sequanal grade methanol was added to each tube; the tubes were sealed with Parafilm, and samples were quantitatively eluted by rotary shaking for 30 min.

Fluorescence measurements were performed in an Aminco Fluoro-Colorimeter equipped with an 85-W mercury vapor lamp and adapted for use with 10 × 75 mm test tubes. A Corning 7-60 filter was used on the excitation side, and a Wratten 8 filter was used for emission (Carroll & Nelson, 1979). Samples were also examined in a Farrand Mark 1 spectrofluorometer by using excitation and emission wavelengths of 345 and 510 nm, respectively. For quantitative purposes, the relative fluorescence intensities of DNS-aa were compared to standards dansylated at the same time and removed from the same chromatogram. All preparations were chromatographed in triplicate. Less than 8% deviation of calculated residues was observed between runs. Photographs of UV-illuminated chromatograms were taken by using Polaroid Type 57 film with a Kodak Wratten 15 filter to block excitation light.

Amino acid hydrolysates were also analyzed by high-voltage paper electrophoresis at pH 1.9, followed by detection with the ninhydrin–cadmium acetate reagent as described by Glazer et al. (1975). Electrophoresis was carried out on 27 × 94 cm lengths of Whatman 3MM filter paper for 115 or 215 min at 4000 V. Mobilities were calculated in each lane by measuring amino acid migration distances relative to lysine or, in the longer runs, valine. Quantitation was achieved by removing 20-mm disks containing each spot, eluting the color into sealed tubes containing 2 mL of Sequanal grade methanol for 2 h, and reading the absorbance at 500 nm vs. a paper blank (Dreyer & Bynum, 1967). A Beckman Model 24 double-beam recording spectrophotometer was used for all absorbance measurements. The absolute amount of each amino acid recovered was determined by correlation with the color intensities of standard amino acids chromatographed on the same paper. All samples were run at least in triplicate; replicate values agreed within 2%.

The chromatographic and electrophoretic behavior of several unusual amino acids were examined as described above. Mono-, di-, and trimethyl derivatives of lysine modified on the ε-amino group were generous gifts from R. J. DeLange. 2- and 3-aminobutyric acids as well as 2,3-diaminobutyric acid were obtained from Nutritional Biochemicals. Hydroxylysine, hydroxyproline, and ornithine were from Sigma.

The presence of Trp was examined fluorometrically by comparison with Tyr fluorescence intensities in the native peptide. Samples of PC-III were dissolved in double-distilled water (pH 7.0) or 6 M guanidine hydrochloride (type 1, Sigma) at a concentration of 100 μg/mL and examined in a

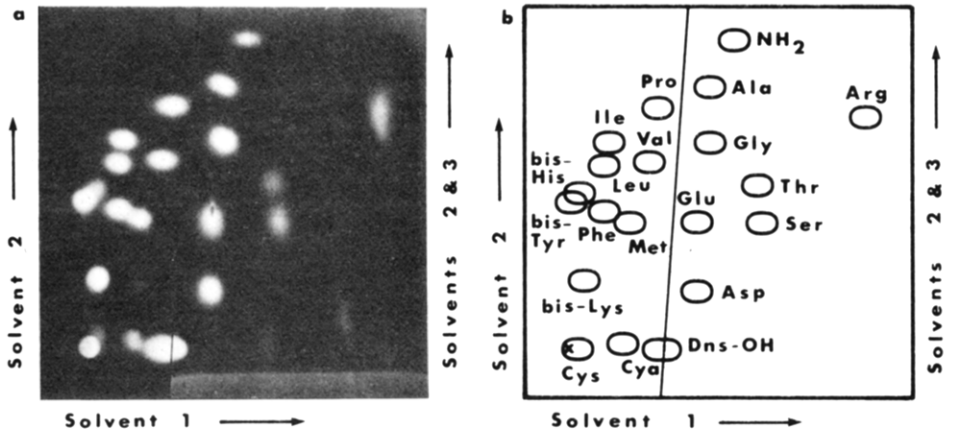

FIGURE 1: Two-dimensional thin-layer chromatography of dansylated amino acids on polyamide. A sample containing 0.2 nmol each of 17 amino acids was dansylated and chromatographed as described in the text. A photograph of the UV-illuminated chromatogram (a) is presented, along with a replica identifying the positions of individual Dns-aa spots (b). Solvents (v/v) were (1) 1.5% formic acid, (2) benzene/acetic acid (6:1), and (3) ethyl acetate/methanol/acetic acid (30:20:1).

spectrofluorometer with the excitation wavelength set at 275 nm. Relative emission intensities were determined vs. blank solutions at 310 (Tyr) and 350 nm (Trp). For the estimation of tryptophan, a molar Trp/Tyr ratio was calculated by using average quantum yields of 0.061 and 0.033 for tryptophan and tyrosine residues in low molecular weight peptides, respectively (Cowgill, 1963; Weber & Young, 1964), and multiplied by the average value for Tyr obtained from two-dimensional TLC analysis of dansylated derivatives.

N-Terminal Analysis. N-Terminal residues were dansylated and analyzed following slight modifications of the method described by Gray (1972). Aqueous samples of PC-III (10 μg) were dried in vacuo in ignition cleaned 6 × 50 mm test tubes and resuspended in 15 μL of 0.2 M $NaHCO_3$. Dansylation was performed by adding 15 μL of Dns-Cl in acetone (5 mg/mL) and heating at 45 °C for 30 min in the dark. The samples were again dried in vacuo, redissolved in 100 μL of 6 N HCl, flushed with N_2, and sealed. Protein hydrolysis was performed at 105 °C for 4 and 18 h. Samples were dried in vacuo over NaOH, resuspended in 2 μL of Sequanal grade methanol and analyzed by two-dimensional TLC on polyamide as described above.

Lipid Analysis. Petroleum ether extracts from PC-III samples hydrolyzed as above or hydrolyzed in 4 N HCl for 12 h at 100 °C were pooled in small screw-cap test tubes and dried under N_2. H_2O (0.5 mL) was added to each tube, followed by 0.1 mL of BF_3–methanol reagent [14% (w/v), Applied Sciences Laboratories]. Samples were methylated by heating the reaction mixtures in a boiling water bath for 2 min and then quickly cooling to room temperature. An additional 0.5 mL of H_2O was added, and the methyl esters were extracted 4 times with 0.5 mL of petroleum ether. After drying under N_2, the residues were redissolved in hexane and analyzed on a Perkin-Elmer 990 gas chromatograph as described by Thomasow & Rittenberg (1978).

Unhydrolyzed samples of PC-III were extracted with petroleum ether or 1-butanol and analyzed for lipids by TLC on 20 × 20 cm Whatman K5 silica gel G plates. Neutral lipids were resolved by the solvent system petroleum ether/diethyl ether/acetic acid (90:10:1) (Malins & Mangold, 1960). Polar lipids were resolved by chromatography in chloroform/methanol/acetic acid/water (50:30:8:4) (Privett et al., 1973). Total lipids were detected by spraying the plate with (i) 0.05% rhodamine 6G in 95% ethanol (Vroman & Baker, 1965) or (ii) 0.005% protoporphyrin in 50% ethanol [see Sulya & Smith (1960)], and examination under UV light (366 nm). Iodinated samples chromatographed on TLC plates were labeled and detected by autoradiography as described (Carroll & Martinez, 1981a).

Carbohydrate Analysis. A micromodification of the fluorometric method described by Rogers et al. (1966) was devised. Samples were placed in acid-washed 12 × 100 mm screw-capped test tubes and made to 0.1 mL with H_2O. Concentrated HCl (0.5 mL) was added, followed by 0.05 mL of resorcinol reagent [2.5 mg/mL in 66% (v/v) HCl]. The tubes were sealed with Teflon-lined caps and incubated at 122 °C for 30 min. After the samples were cooled to room temperature, 0.2-mL aliquots were removed and neutralized with 0.3 mL of 6.7 N NaOH, followed by 2.0 mL of 0.4 M sodium borate, pH 9.0. Relative fluorescence intensities were determined vs. appropriate buffer blanks in a Farrand Mark I spectrofluorometer by using the 20-nm slit arrangement and excitation/emission wavelengths of 485/510 nm. D-Glucose (Sigma) was used as standard, with results presented as glucose equivalents. This assay produced a linear relationship between relative fluorescence and carbohydrate concentration from 0.1 to 20 nmol in the original reaction mixture (i.e., less than a 0.2 μg/mL sample). Additionally, the absorbance of undiluted acidic samples at 435 nm was a linear function of concentration up to 60 nmol, while that of samples diluted 1:10 and examined at 390 nm was linear from 40 to 250 nmol.

Carbohydrates were also examined by thin-layer chromatography following acid hydrolysis. Samples were hydrolyzed in 1 N HCl for 2 h at 100 °C and then dried in vacuo over NaOH. Released sugars were chromatographed on phosphate-buffered silica gel (Applied Sciences Labs) with butanol/acetone/water (4:5:1) and detected with *p*-anisaldehyde in acid–ethanol (Stahl & Kaltenbach, 1961).

Results

Amino Acid Analysis by Two-Dimensional TLC. A mixture of dansylated amino acid standards was initially examined by the two-dimensional TLC technique to ascertain the workable limits of the procedure. Control experiments using standard mixtures of DNS-aa suggested that methanol was a more suitable solvent for application than acetone/acetic acid (3:2) (Woods & Wang, 1967) and that a benzene/acetic acid ratio of 6:1 for solvent 2 provided greater resolution than the more commonly used 9:1. If the diameter of the loading spot was less than 2 mm, samples containing up to 1 nmol each of 20 common amino acids could be readily separated and appeared uniform. Figure 1 illustrates the resolution achieved by

Table I: Elution Efficiencies of Dns-aa from Polyamide by Various Solvents[a]

solvent	RFI
ethanol	100
solvent 3 (two-dimensional TLC)	98
methanol	95
solvent 2 (two-dimensional TLC)	61
C/M/HOAc (7:2:2)	55
acetone/HOAc (10:1)	45

[a] Samples (0.1 μL) of the dansylated amino acid standard were spotted on polyamide TLC plates, and then fluorescent sections were removed and the Dns derivatives eluted with 0.5 mL of the solvents indicated. After 30 min, the eluates were diluted into methanol, and relative fluorescence intensities (RFI) were determined in a spectrofluorometer by using excitation and emission wavelengths of 345 and 510 nm, respectively. The intensity of ethanol-eluted Dns-aa was given a value of 100. C/M/HOAc, chloroform/methanol/acetic acid.

chromatography of a sample containing 17 amino acids (0.2 nmol each) [see Brown & Perham (1973)]. With the exception of bis(Dns-His)/bis(Dns-Tyr), all 17 amino acids were clearly separated. Rechromatography of this section with solvent 4 (Hartley, 1970) in the same direction as solvent 2 resolved these derivatives but compressed Dns-Leu, Dns-Ile, and Dns-Pro.

The elution of ^{3}H-labeled Dns amino acids from polyamide layers with subsequent quantitation by liquid scintillation has been described (Brown & Perham, 1973). Similar methods were examined for recovery of fluorescent dansyl derivatives. For these experiments, ~0.1-μL samples of the dansylated calibration standard containing 17 amino acids (50 pmol each) were spotted at several locations on a polyamide sheet and allowed to dry. Rectangular sections of ~25 mm^2 surrounding each spot were scraped from the plastic backing and placed in small test tubes, and samples were eluted with 0.5 mL of various solvents. After 30 min, 0.1 mL of the eluates were removed and diluted into methanol, and fluorescence was measured against appropriate blanks in a spectrofluorometer. The remaining eluate was evaporated under N_2, resuspended in a small volume of methanol, and chromatographed. The results (Table I) indicated that the maximal amount of fluorescent material was eluted with ethanol, methanol, or ethyl acetate/methanol/acetic acid (solvent 3). Although either methanol or ethanol elution resulted in similar fluorescence intensities, less time (~10 min) was required for elution with the former. The presence of Dns-OH in these samples had little influence on eluate fluorescence due to the altered excitation/emission spectra (332/464 nm) of this compound. Twofold variations in the size of polyamide sections removed had little effect on blank fluorescence. Chromatography of the concentrated eluates revealed all the appropriate derivatives.

The sensitivity and quantitative limits of the methanol-elution technique were examined by spotting increasing amounts of specific Dns amino acids on polyamide, followed by removal and elution into 2 mL of methanol. As in the above studies, fluorescence intensities were initially performed by transferring samples to cuvettes and examining in a spectrofluorometer. A significant increase in sensitivity was observed, however, by examining the elution tubes directly with a filter fluorometer. When quantitated in this manner, it was unnecessary to remove the sample of insoluble polyamide, as it remained below the lowest excitation aperture. When this procedure was used, the relative fluorescence intensities of eluted Dns-Asp and Dns-Arg were linear functions of concentration between 10 pmol and 1 nmol (Figure 2). Other

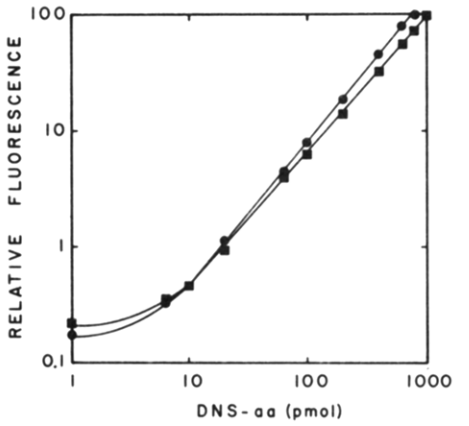

FIGURE 2: Fluorescence intensities of methanol-eluted Dns-aa as a function of concentration. Samples of Dns-Asp (■) and Dns-Arg (●) containing between 1 pmol and 1 nmol were spotted on polyamide. The sections containing the fluorescent spots were scraped off the sheet, and the derivatives were eluted into 2 mL of methanol. Relative fluorescence was determined by using a filter fluorometer as described in the text. The intensity obtained for 1 nmol of Dns-Asp was given a value of 100.

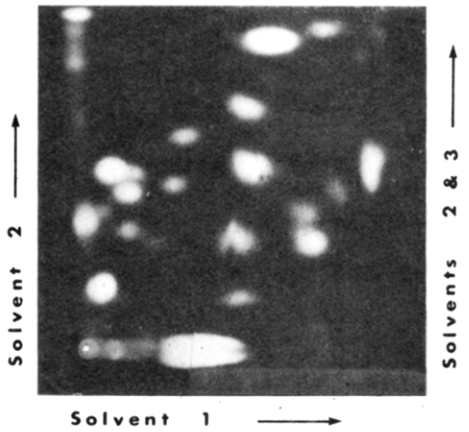

FIGURE 3: Two-dimensional TLC of the dansylated PC-III acid hydrolysate. Samples containing 100 μg of PC-III were hydrolyzed in 6 N HCl containing one drop of 5% phenol for 16 h at 110 °C, extracted 3 times with petroleum ether, and dried in vacuo over NaOH and P_2O_5 at 40 °C. Conditions of dansylation and chromatography were as described in Figure 1. Due to the time between chromatography and photography, the relative intensities of the Dns derivatives may not correlate with the data presented in Table II.

Dns-aa exhibited similar fluorescence intensities. These slight variations in the fluorescence intensity of different Dns-aa remained constant between runs and had little influence on quantitation.

Amino Acid Composition of PC-III. Previous investigations demonstrated that PC-III was unstable at reduced ionic strength or low pH (Carroll & Martinez, 1981a). These conditions were avoided where possible prior to chemical analysis so that the most active form of the bactericide was examined. This was accomplished by storing samples in 0.8–1.0 M potassium phosphate buffer, pH 7.4, at −20 °C until needed. Fractions were then desalted by chromatography on phosphocellulose in ammonium acetate and lyophilized. Greater than 80% of PC-III activity was recovered prior to lyophilization.

Thin-layer chromatography of dansylated PC-III acid hydrolysates generated patterns similar to the standards (Figure 3). Qualitative examination indicated the presence of a new spot (Dns-X) above and to the left of Dns-Ile (see Figure 1b) as well as the relative absence of Dns-Met and Dns-Phe. Like Dns-His/Dns-Tyr, Dns-X/Dns-Ile could be resolved by re-

Table II: Tentative Amino Acid Composition of PC-III[a]

amino acid	calcd[b] residues per mole: TLC[d]	paper electrophoresis[c]	assumed
Ala	1.11	0.97	1
Arg	1.07	1.07	1
Asp	0.52	1.04	(1)[h]
1/2-Cys	0.65		(1)
Glx	0.89	1.21	1
Gly	1.14	0.97	1
His	0.76	0.95	1
Ile	0.89	1.02	1
Leu	1.18	1.14	1
Lys	1.06	1.72	1
Me-Lys	0.93		1
Met	0.12		–
Phe	0.36	0.43	(–)
Pro	0.86	*e*	1
Ser	0.90[f]	0.81[f]	1
Thr	0.72[f]	0.87[f]	1
Trp	1.24[g]		1
Tyr	0.88	0.93	1
Val	0.72	0.65	1

[a] Following hydrolysis (6 N HCl, 110 °C, 16 h), samples were analyzed by dansylation and two-dimensional TLC as described or by high-voltage paper electrophoresis (pH 1.9) and quantiation with ninhydrin. Elution and quantitation procedures are described in the text. [b] Calculated assuming a molecular weight of ~1800 (Carroll & Martinez, 1981a). [c] Quantitated by elution of ninhydrin-aa and spectrophotometry. [d] Quantitated by elution of Dns-aa and fluorescence spectrophometry. [e] Not quantitated on paper with cadmium–ninhydrin. [f] Assumes 10% destruction during 16-h hydrolysis (Moore & Stein, 1963). [g] Examined in the native polypeptide fluorometrically (see text). [h] Values in parentheses showed the greatest variations.

chromatography in solvent 4. On the basis of intensity, it appeared that Dns-X either was a multiply dansylated residue or was present at a higher concentration than the other components.

Examination of several modified amino acids commonly found in proteins, both by TLC following dansylation or by high-voltage paper electrophoresis, indicated that N^{ϵ}-monomethyllysine was the only derivative to show the observed migration pattern. On paper electrophoresis at pH 1.9, this derivative had a mobility of 0.983 compared to that of lysine and could not be clearly resolved in mixed samples. When the serum-component amino acids were examined by paper electrophoresis (see below), a similar, poorly resolved lysine doublet was observed.

A tentative amino acid composition for PC-III is presented in Table II, along with values obtained by quantitating ninhydrin-stained amino acids following paper electrophoresis at pH 1.9. Although paper electrophoresis required considerably more material (~4 nmol of peptide) than did two-dimensional TLC (~20 pmol) for reproducibility, both procedures produced similar results. The dansyl two-dimensional TLC technique also established the presence of Pro (Dreyer & Bynum, 1967; Moore & Stein, 1963) and N^{ϵ}-methyl-Lys not clearly resolved on paper. Additonally, derivatives migrating in the positions of Dns-Cys and Dns-Asn (both destroyed by acid hydrolysis) were observed, but their concentrations varied between preparations. Although combining the values for Asn and Asp resulted in a calculated single Asn residue, only Asp is reported. Oxidation of samples with performic acid prior to analysis may help identify these residues.

When similar amounts of unknowns and standards were chromatographed simultaneously in either procedure, reproducibility between runs was consistantly better than 8%. These

Table III: Fatty Acid Content of PC-III[a]

fatty acid	nmol/(μg of protein)	ratio (fatty acid/18:2)
16:0	0.64	12.6
18:0	0.57	11.2
18:1	0.56	10.9
18:2	0.06	1.0

[a] Petroleum ether extracts from PC-III acid hydrolysates were methylated and then analyzed by gas–liquid chromatography. Concentrations were calculated by comparison with the positions and integrated peak areas of standard compounds.

data (and that described below) indicate that the peptide component of PC-III contained 17 amino acid residues, 24% basic and 35% nonpolar, with lysine the only multiple residue. On the basis of amino acid composition, the molecular weight of the peptide was estimated to be 2000.

The presence of tryptophan in protein hydrolysates could not be determined by using the above techniques due to its lability in acid. The absence of a clearly defined peak in the region of 275–295 nm also eliminated spectrophotometric analysis. Instead, advantage was taken of the intrinsic fluorescence of aromatic amino acids between 300 and 350 nm resulting from excitation at 270–280 nm (Teale & Weber, 1957). Fluorescence scans of the native peptide in water revealed a well-defined maximum at 346 nm, establishing the presence of Trp. When the published Trp/Tyr quantum yields for a series of natural and synthetic peptides were used (Cowgill, 1963; Weber & Young, 1964), a ratio of ca. 1.4:1 was calculated for PC-III, resulting in an estimated Trp content of 1 mol/mol of peptide. When an ϵ_m of 1.21×10^3 for Tyr and 5.49×10^3 for Trp at neutral pH are assumed, (Crammer & Neuberger, 1943) as well as a molecular weight of 2000 for PC-III (see above), an $E^{1\%}_{1cm}$ (280) of 33.5 was calculated for the purified sample. This value is in agreement with previous observations.

N-Terminal Analysis. Two-dimensional TLC of 4- and 18-h hydrolysates from dansylated PC-III preparations suggested that arginine was the only N-terminal residue present in these preparations (data not shown). Subsequent experimentation, however, indicated that Dns-Arg could not be separated from ϵ-Dns-Lys under the conditions described. The peptide, in which two lysine residues were found, thus contained an N-terminal Arg, or the N terminus was blocked such that only ϵ-amino groups were dansylated. Either result is consistant with previous electrophoretic data, suggesting the apparent homogeneity of PC-III (Carroll & Martinez, 1981a), although the presence of contaminating material cannot yet be excluded.

Lipid Content. The existence of free fatty acids in PC-III hydrolysates was also analyzed. Nearly equimolar amounts of 16:0, 18:0, and 18:1 fatty acids were found (Table III), with only traces of 18:2. A total of 1.82 nmol of fatty acids were found per μg of protein, suggesting a protein to fatty acid ratio (by weight) of ca. 3:1. Much lower ratios, i.e., larger amounts of lipid, were found associated with phosphocellulose column fractions prior to desalting.

Several attempts were made to analyze undegraded lipids present in active samples by TLC on silica gel. Although rhodamine or protoporphyrin stains were able to detect less than 10 μg of lipid after chromatography, spots were not visible in extracted samples. Rather than scale up to larger quantities, we chose to examine iodinated PC-III fractions before and after desalting on Sephadex G-10. Since unsaturated lipids also become iodinated (Shepherd et al., 1976), the amount of material required for analysis was greatly reduced and could

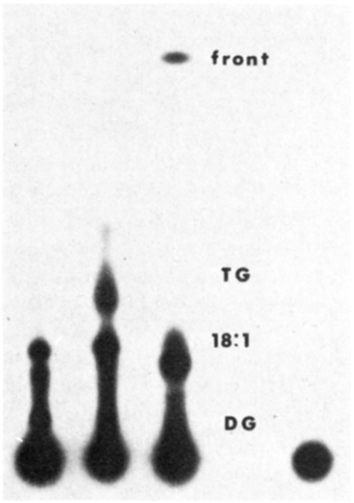

FIGURE 4: Chromatographic examination of neutral lipids present in PC-III. The bactericide was iodinated with ^{125}I by using Iodogen as described (Carroll & Martinez, 1981a) and then fractionated and chromatographed on silica gel by using the solvent system petroleum ether/diethyl ether/acetic acid (90:10:1). Positions were detected by autoradiography or, for the standard compounds, spraying with rhodamine 6G or protoporphyrin. (Lane 1) PC-III; (lane 2) petroleum ether extract of PC-III; (lane 3) butanol extract of PC-III; (lane 5) PC-III desalted on Sephadex G-10 in 50 mM phosphate buffer (pH 7.4) containing 0.5 M NaCl. The approximate positions of standard compounds are shown in lane 4: TG, triglycerides; 18:1, 18:1 free fatty acids (oleic acid); DG, diglycerides.

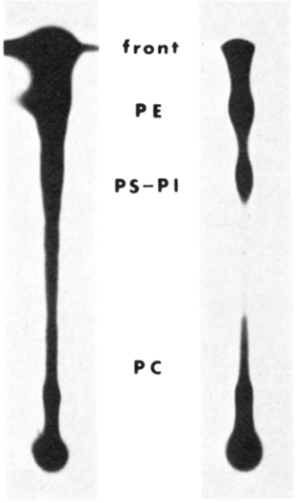

FIGURE 5: Chromatographic examination of polar lipids present in PC-III. The bactericide was fractionated as described and then chromatographed on silica gel G by using the solvent system chloroform/methanol/acetic acid/water (50:30:8:4). Conditions of detection and autoradiography were as described in Figure 4. (Lane 1) PC-III; (lane 2) PC-III desalted on Sephadex G-10. PE, phosphotidylethanolamine; PS, phosphotidylserine; PI, phosphotidylinositol; PC, phosphotidylcholine.

be detected by autoradiography. The positions of standard compounds were determined by spraying with the above lipid stains. Under these conditions, the iodinated preparation exhibited small amounts of neutral lipids (Figure 4, lane 1), most of which could be extracted with petroleum ether (lane 2) or butanol (lane 3). Desalting on Sephadex G-10 in 50 mM phosphate also removed most of this material (lane 5), which was composed primarily of free fatty acids and triglicerides. In contrast, much of the iodinated polar lipids (phospholipids) found in PC-III (Figure 5, lane 1) remained associated with the active polypeptide even after desalting (lane 2). When the lipids were extracted with butanol, 15% of the radioactivity associated with desalted fractions partitioned into the organic phase.

Carbohydrate Content. Small but consistent amounts of carbohydrate have been detected in the most highly purified preparations of the serum bactericide. Although absolute amounts varied, fractions desalted on Sephadex G-10, Bio-Rex 70, or phosphocellulose exhibited protein to carbohydrate ratios (by weight) between 2:1 and 4:1, with sliightly higher values found in the latter samples. That this material was present in the original biologically active PC-III was confirmed by analysis of individual column fractions. A small carbohydrate peak was found to coelute with the active peptide peak. Attempts to identify the carbohydrate moieties by mild acid hydrolysis and TLC on phosphate-buffered silica gel proved unsuccessful.

Discussion

An important step in delineating structure–function relationships within molecules of biological interest involves the chemical analysis of functional components. Such studies are often difficult to perform, however, due to insufficient material as well as the availability of sensitive instrumentation capable of such analyses. During our investigations into the nature and action of microbicidal agents present in normal mammalian sera, both areas of difficulty were encountered and prompted an examination of manual microanalytical techniques. The resultant procedures have been used to examine the composition and homogeneity of PC-III, an antibacterial fraction isolated from normal rabbit serum.

Our previous studies (Carroll & Martinez, 1981a) demonstrated that PC-III contains a low molecular weight, cationic polypeptide capable of reducing microbial viability in vitro and that the polypeptide component(s) may be responsible for biological activity. The compositional studies described here further support this contention and suggest that PC-III activity may reside in a single polypeptide chain. This conclusion follows both from the suggestive N-terminal analysis as well as the close agreement between the calculated molecular weight (based on amino acid composition) and that derived from mobility in peptide-resolving electrophoretic systems. Interestingly, the PC-III peptide itself appears somewhat unusual in that of the 17 amino acid residues present, lysine is the only multiple component. As one of these lysine residues may be monomethyl substituted on the ϵ-amino group, each residue thus represents a unique amino acid.

In addition to the peptide component, our data indicate that small amounts of lipid and carbohydrate are consistently found in the most highly purified preparations of PC-III. The composition of the active fraction (by weight) is ca. 50% protein, 25% lipid, and 25% carbohydrate. Whether these additional moieties are covalently associated with the peptide or necessary for activity remains unclear. However, since (i) their absolute amounts varied with the method of desalting and (ii) of the agents tested previously only proteases affected activity, much of this material may originate from carry-over of serum components. This appears especially true for the neutral lipids, most of which could be removed by extraction with petroleum ether or desalting on Sephadex G-10 (see Figure 4). Since lipid–protein interactions in serum lipoproteins are primarily hydrophobic (Morrisett et al., 1975), it seems likely that the material remaining associated with the peptide following desalting may do so through interactions with the six nonpolar amino acid residues. Column chromatography

in buffers containing detergents may allow complete separation and characterization of these components.

The above analyses were performed on fractions isolated from a single batch of pooled rabbit serum, and as such, it should be emphasized that additional studies need to be performed before the composition and diversity of PC-III is known. Analysis of PC-III in other serum pools as well as that in the sera of individual rabbits is in progress. Further characterization of the modified lysine residue should be possible by paper chromatography in phenol–cresol (DeLange et al., 1969). Moreover, substantiating data for both compositional and N-terminal analyses are currently being sought by high-performance liquid chromatographic techniques. Sequence studies have also been initiated, in hopes of providing information concerning the possible construction of synthetic antibacterial agents.

In view of the cationic nature of PC-III, the antibacterial activity of the isolated serum peptide may well reside in its content of 24% basic amino acids, i.e., lysine, arginine, and histidine. Other cationic compounds such as protamines and histones (Miller et al., 1942), polylysine (Watson & Bloom, 1952), or the cationic proteins from polymorphonuclear leukocytes (Lehrer et al., 1975; Odenberg & Olsson, 1975; Zeya & Spitznagel, 1966) or alveolar macrophages (Patterson-Delafield et al., 1980) are known to influence microbial viability. The action of PC-III as it relates to the above compounds will be dealt with in greater detail elsewhere (Carroll & Martinez, 1981b).

We have presented fluorometric methods for the microquantitation of amino acids and carbohydrates in the picomole range. Although not as sensitive as techniques employing isotopically labeled Dns-Cl, autoradiography, and densitometry (ca. 25 fmol vs. 10 pmol) (Burzgnshi, 1975), amino acid analysis as described offers significant savings in terms of time and especially of equipment required. In addition, it is anticipated that reproducibility could be enhanced by (i) using hydrolyzed amino acids as standards for dansylation and chromatography and (ii) dissolving the Dns-aa–polyamide sections directly in phenol. Preliminary experiments (data not shown) indicate that polyamide [poly(ε-caprolactam)] dissolves quite readily in commercial-grade phenol, but background fluorescence was quite high. The use of redistilled phenol should serve to minimize errors resulting from variations in the elution efficiencies of dansyl derivatives.

References

Beart, P. M., & Snodgrass, S. R. (1975) *J. Neurochem. 24*, 821–824.

Benson, J. R., & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 619–622.

Briel, G., & Newhoff, V. (1972) *Hoppe-Seyler's Z. Physiol. Chem. 353*, 540–553.

Brown, J. P., & Perham, R. N. (1973) *Eur. J. Biochem. 39*, 69–73.

Burzgnshi, S. R. (1975) *Anal. Biochem. 65*, 93–99.

Carroll, S. F., & Nelson, D. R. (1979) *Anal. Biochem. 98*, 190–197.

Carroll, S. F., & Martinez, R. J. (1981a) *Biochemistry* (preceding paper in this issue).

Carroll, S. F., & Martinez, R. J. (1981b) *Biochemistry* (following paper in this issue).

Cowgill, R. W. (1963) *Arch. Biochem. Biophys. 100*, 36–44.

Crammer, J. C., & Neuberger, A. (1943) *Biochem. J. 37*, 302–310.

Crowshaw, K., Jessup, S. J., & Ramwell, P. W. (1967) *Biochem. J. 103*, 79–85.

DeLange, R. J., Glazer, A. N., & Smith, E. L. (1969) *J. Biol. Chem. 244*, 1385–1388.

Dreyer, W. J., & Bynum, E. (1967) *Methods Enzymol. 11*, 32–39.

Glazer, A. N., DeLange, R. J., & Sigman, D. S. (1975) *Chemical Modification of Proteins*, pp 180–184, North-Holland Publishing Co., Amsterdam.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Hartley, B. S. (1970) *Biochem. J. 119*, 805–822.

Hill, D. W., Walters, F. H., Wilson, T. D., & Stuart, J. D. (1979) *Anal. Chem. 50*, 1338–1341.

Hirsch, J. G. (1960) *J. Exp. Med. 112*, 15–22.

Lehrer, R. I., Ladra, K. M., & Hake, R. B. (1975) *Infect. Immun. 11*, 1226–1234.

Malins, D. C., & Mangold, H. K. (1960) *J. Am. Oil Chem. Soc. 37*, 576–578.

Miller, B. F., Abrams, R., Dorfman, A., & Klein, M. (1942) *Science (Washington, D.C.) 96*, 428–430.

Moore, S., & Stein, W. H. (1963) *Methods Enzymol. 6*, 819–831.

Morrisett, J. D., Jackson, R. L., & Gotto, A. M., Jr. (1975) *Annu. Rev. Biochem. 44*, 183–207.

Odenberg, H., & Olsson, I. (1975) *J. Clin. Invest. 56*, 1118–1124.

Patterson-Delafield, J., Martinez, R. J., & Lehrer, R. I. (1980) *Infect. Immun. 30*, 180–192.

Privett, O. S., Dougherty, K. A., & Erdahl, W. L. (1973) in *Quantitative Thin Layer Chromatography* (Touchstone, J. C., Ed.) pp 57–93, Wiley, New York.

Roberts, R. R., Tew, J. G., & Donaldson, D. M. (1977) *Infect. Immun. 15*, 485–490.

Rogers, C. J., Chambers, C. W., & Clark, N. A. (1966) *Anal. Chem. 38*, 1851–1853.

Shepherd, J., Bedford, D. K., Morgan, H. G. (1976) *Clin. Chim. Acta 66*, 97–109.

Spivak, V. A., Shcherbukhin, V. V., Orlov, J. M., & Varshausky, JA. M. (1971) *Anal. Biochem. 39*, 271–281.

Stahl, E., & Kaltenbach, U. (1961) *J. Chromatogr. 5*, 351–355.

Sulya, L. L., & Smith, R. R. (1960) *Biochem. Biophys. Res. Commun. 2*, 59–62.

Teale, F. W. J., & Weber, G. (1957) *Biochem. J. 65*, 476–482.

Thomashow, M. F., & Rittenberg, S. C. (1978) *J. Bacteriol. 135*, 1015–1023.

Vroman, H. E., & Baker, G. G. (1965) *J. Chromatogr. 18*, 190–191.

Watson, D. W., & Bloom, W. L. (1952) *Proc. Soc. Exp. Biol. Med. 89*, 29–33.

Weber, G., & Young, L. B. (1964) *J. Biol. Chem. 239*, 1424–1431.

Woods, K. R., & Wang, K.-T. (1967) *Biochim. Biophys. Acta 133*, 369–370.

Zanetta, J. P., Vincendor, G., Mandel, P., & Gombos, G. (1970) *J. Chromatogr. 51*, 441–458.

Zeya, H. I., & Spitznagel, J. K. (1966) *J. Bacteriol. 91*, 755–762.