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Research Article

CHEMICAL STUDIES ON POLYMYXIN B [1]

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Polymyxin is a generic term for chemotherapeutic antibiotics elaborated in fermentations of various media by strains of *Bacillus polymyxa* (1). The known polymyxin polypeptides (A, B, C, D, and E) have closely similar chemical and biological properties, and have in common L-α, γ-diaminobutyric acid, L-threonine and an unidentified C_9 optically-active fatty acid. In addition to these acids, polymyxin A (aerosporin) contains D-leucine (2). Polymyxin A, first described by Ainsworth, Brown and Brownlee, was isolated by Brownlee and Bushby, and Catch and Friedmann (3) from broth cultures by adsorption onto a suitable carbon and elution with aqueous acetone containing sulfuric acid. Further purification was obtained by converting the antibiotic to the helianthate and subsequently to the hydrochloride. Polymyxin D, which is composed of the constituents of polymyxin A plus serine, was described by Benedict and Langlykke (4) and Stansly *et al.* (5). It was purified by adsorption onto carbon after clarification of the fermentation liquors, elution with acid-methanol and precipitation with acetone. Further purification by Shepherd *et al.* (6) involved butanol extraction, fractional precipitation of the picrate and conversion to the hydrochloride. Hydrolyzates of polymyxin B (7), another basic polypeptide produced by strain CN 1419 of *B. polymyxa* (Wellcome Foundation Culture Numbers), show the presence of D-leucine and phenylalanine as well as the three constituents common to all the polymyxins.

During the course of studies on polymyxin B designed to eliminate toxic impurities and prepare high potency material, a procedure was developed involving adsorption of the active principle on cotton sodium succinate, elution with dilute sulfuric acid, precipitation of the antibiotic with 1-(4-chloro-o-sulfophenyl)-5-hydroxy-3-methyl-4-(p-(p-tolylsulfonoxy)-phenylazo)-pyrazole (Polar Yellow), liberation of the antibiotic, precipitation as its free base and conversion to the acid salt. The antibiotic was subsequently crystallized as its naphthalene β-sulfonate by a procedure described by Wilkinson (8).

The homogeneity of material from each purification stage was studied by partition chromatography on paper and by chemical and biological means. The paper partition chromatographic studies were carried out by a method similar to that described by Goodall and Levi (9) for penicillins. After developing and drying the paper strips, they were placed on the surface of nutrient agar previously seeded with *Escherichia coli* instead of *Bacillus subtilis*. In this way, the positions of the zones of inhibition established the presence of another active substance in certain polymyxin B fractions.

Crude material obtained from the cotton sodium succinate column was found to produce a histamine-like lowering of the blood pressure when injected into anesthetized cats. The histamine-like substance was removed from the polymyxin B by purification through its Polar Yellow salt. Fractions of moderately high potency (*ca.* 6500 units/mg.), obtained by a dual purification through the Polar Yellow salt, showed two spots on the chromatogram indicating the presence of an active minor component associated with the polymyxin B. When the acid hydrolyzates of each of these active materials were chromatographed, however, identical amino acid spectra were obtained.

In addition to the recognized components of polymyxin B, an unknown substance was routinely found high in the solvent band of the paper chromatogram of hydrolyzates of this material. Consideration of the R_F differences between glycine and alanine, alanine and valine, valine and leucine, and leucine and the unknown patch, 0.2, 0.15, 0.15, 0.1, respectively, led to the suggestion that the substance might be a C_7-amino acid. Consequently, the three leucine homologs RCH_2-

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$CHNH_2COOH$ were synthesized in which the R groups were $-CH_2CH_2CH_2CH_3$, $-CH_2(CH_3)$-$CHCH_3$ and $-CH(CH_3)CH_2CH_3$. Chromatography of these known C_7-amino acids showed them to resemble the unknown patch, but none was identical with it. In a like manner, a similar sample of polymyxin hydrochloride separated into nine fractions by alumina chromatography showed that the hydrolyzates of all of these fractions were indistinguishable from the products of pure polymyxin B. The minor component produced by the strain was eliminated by adding alkali and precipitating the free polymyxin B base. After separating the insoluble antibiotic, the presence of the extraneous substance could be demonstrated by chromatographing the filtrate on paper. Materials of high biological activity purified through the free base followed by crystallization of the naphthalene β-sulfonate salt (8) showed but a single active component by partition chromatography.

METHODS

Paper chromatography. An apparatus for the partition chromatography method using paper was designed after suggestions of Consden *et al.* (10) and Goodall and Levi (9). The method of paper chromatography using ascending flow, described by Williams and Kirby (11), was adapted to the work discussed here by using the butanol-15% acetic acid-water solvent system and developing in one dimension only. Both intact materials and hydrolyzates were similarly analyzed on paper (12).

Hydrolysis of all samples was carried out by a standard procedure. The sample was dissolved in 5 N HCl (5 to 10% W/V) and heated for 2.5 hours in a sealed tube placed in a steam bath. This solution was evaporated to dryness in an open dish on a steam bath, and the residue was dissolved in water so that the stock solution prepared for chromatography corresponded to a concentration of 100 μg./μl. of intact material.

Solutions to be chromatographed were placed on spots along a line 2 inches from the edge of a sheet of Whatman No. 1 paper in amounts containing 10 to 100 μg. of intact or 500 to 1,000 μg. of hydrolyzed polymyxin B. After drying, the sheet was suspended in the chamber, inoculated edge down, for at least one hour before starting the development, so that the paper could come to equilibrium with the atmosphere of the chamber. The lower edge of the paper was then placed in a trough containing butanol-acetic-acid-water mixture and the chromatogram was developed by an ascending solvent flow for about 15 hours. The developed sheet was dried in an oven at 60° and then sprayed with a 0.25% solution of ninhydrin in n-butanol. Redrying for five minutes brought out the amino acid-ninhydrin color. Comparison of the R_F values of the amino acids obtained from hydrolyzates of polymyxin B fractions was made with known acids and with known acids added to the test material.

Biological assay. In these studies, the method of biological assay was essentially that developed by Stansly and Schlosser (13) and modified by Benedict and Stodola (14) using *Brucella bronchiseptica,* strain NRRL B-140. A polymyxin hydrochloride, containing 6,650 units/mg., supplied by the Wellcome Research Laboratories (England), was used as a standard.

The paper chromatograms of intact materials were further examined for antibiotic activities by drying at 50°, after development, and pressing on agar plates (13" × 17") seeded with *E. coli.* After refrigerating at 0° for two hours, the paper was removed from the surface. of the agar and the agar plate was incubated at 37° for 15 hours. Active substances in certain samples were located by the zones of inhibition of the organism.

EXPERIMENTAL

Clarification of fermentation broth. Eight liters of a freshly harvested broth was adjusted to pH 2 with dilute sulfuric acid and heated at 90° C. for 30 minutes so as to coalesce the cells and thin out the mucilage. About 60 gm. of Super-Cel was added and the mixture was filtered. The clarified filtrate showed no appreciable loss of activity.

Adsorption of polymyxin on cotton sodium succinate. Cotton acid succinate (15) was prepared by dissolving 25 gm. of fused sodium acetate and 200 gm. of succinic anhydride in 1,500 ml. of glacial acetic acid and submerging 50 gm. of cotton in this solution at 100° for 48 hours. The partially esterified cotton was filtered, washed with water, with dilute hydrochloric acid and finally thoroughly with water. The cotton acid succinate was found to be a poor cation exchange medium for polymyxin. Therefore, the cotton acid succinate was converted to cotton sodium succinate by suspending the former in water and titrating with dilute sodium hydroxide to a phenolphthalein endpoint. After filtering and washing with water, the product was dried *in vacuo* at 50° for 24 hours.

Eight liters of the clarified polymyxin B broth (2,000 units/ml.) was poured into a column containing 50 gm. of cotton sodium succinate. About 4% of the original activity in the polymyxin B broth was found in the effluent. The column was washed with 2,000 ml. of water (less than 10 units/ml. in wash) and the antibiotic (93%) was eluted from the cotton with 500 ml. of 0.25 N sulfuric acid. Previous experiments had shown that

the polymyxin B eluted from the cotton sodium succinate column contained a histamine-like substance which was detected by the lowering of the blood pressure in cats.

Isolation of polymyxin B using Polar Yellow. The histamine-like substance could be removed from the polymyxin B eluate by purification through its Polar Yellow salt. Hence, the acid eluate was neutralized to pH 3.5 with sodium hydroxide and treated with 18 gm. of Polar Yellow. After stirring the precipitate for 15 minutes, the mixture was filtered, washed well with water and dried *in vacuo* at 50° for 48 hours. The dried mixture was suspended in 1 liter of a mixture of 85% acetone-15% methanol and treated with 25 ml. of a solution of triethylamine sulfate (0.45 gm./ml.) in methanol in order to convert the polymyxin B into the methanol insoluble sulfate. After it was shaken for one hour, the mixture was filtered, the precipitate was washed with acetone-methanol mixture and finally with acetone. After it was dried, the cake was triturated in 500 ml. of water, filtered, and washed with water. The colorless filtrate was vacuum freeze-dried; a yield of about 75% of the active substance was obtained. The product had a potency of 5,200 units/mg. when assayed against *B. bronchiseptica* and had 2.3% ash.

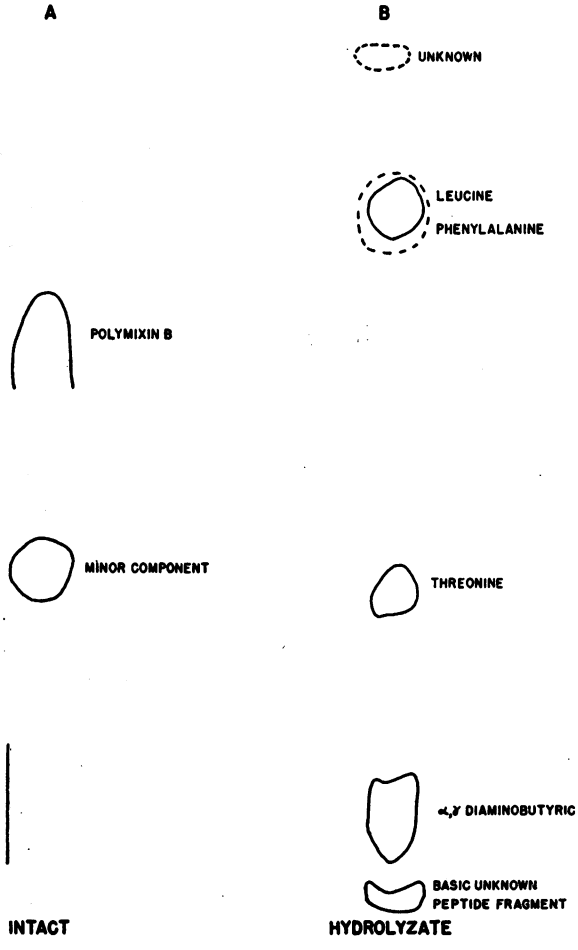

FIG. 1

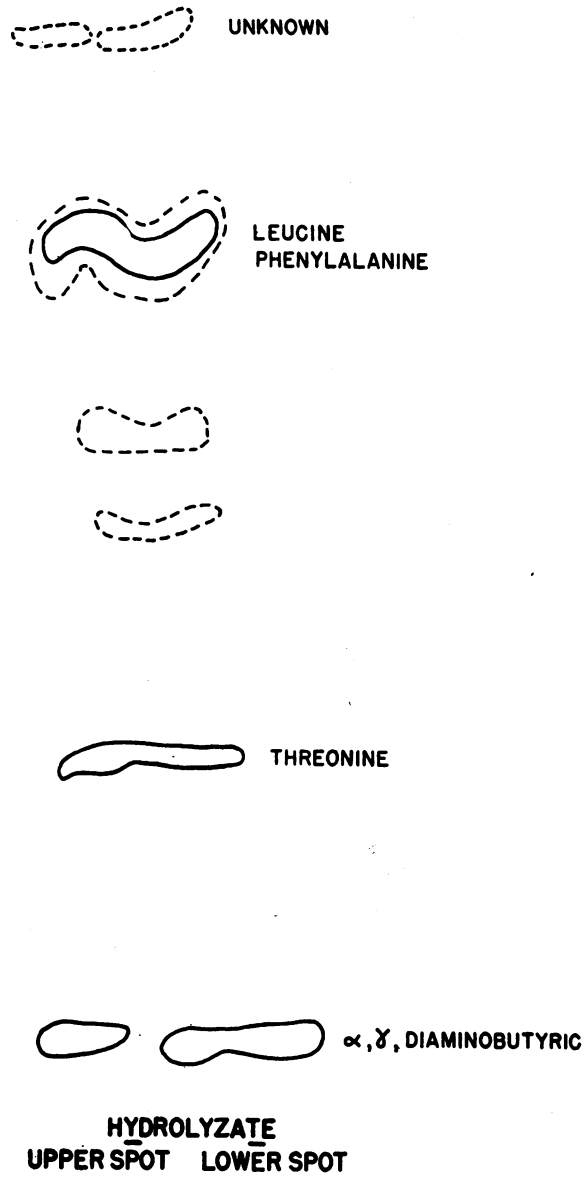

FIG. 2

Partition chromatograms of this intact material

showed a spot corresponding to the R_F value of polymyxin B and in addition the presence of a biologically active lower second patch (Figure 1A). However, the hydrolyzate of the intact material showed only the presence of the expected components (Figure 1B).

In the course of investigating the contaminant, a comparatively large amount of the material was put onto chromatographing paper as a swath instead of individual spots. After the usual 15-hour development, a very small portion of this swath was ninhydrin-treated to locate the spots. The remainder of the paper was cut horizontally so that two strips were obtained, each holding one component. The material held on each paper was eluted and hydrolyzed separately. It was found, on chromatographing the hydrolyzates, that both spots gave identical amino acid spectra (Figure 2).

Attempts to isolate enough of the active extraneous material for use in determining its contribution to the toxicity in animals were made by studying the chromatography of the substances on alumina.

Brockmann alumina was treated with 50% sulfuric acid to pH 6.0, backwashed with distilled water until the washings were sulfate-free and dried 24 hours at 100° C. The dried alumina was transferred to the column as a slurry in methanol. After the column was packed, a solution of polymyxin B hydrochloride (60,000 units/ml.) in anhydrous methanol was carefully poured into the column. The chromatogram was developed with absolute methanol, 95%-, 90%-, 80%-, 50%-methanol, water, and finally with 0.1 N and 0.5 N sulfuric acid. After the third fraction, each solution leaving the column was collected until it showed less than 500 units/ml., whereupon the chromatogram was developed with the succeeding more dilute methanol. Twelve fractions were collected; the methanol was evaporated from each and the residue was vacuum freeze-dried. The results of the column are summarized in Table I.

Portions of the first nine alcohol-developed fractions were redissolved and chromatographed intact on paper. The purest fraction (No. 1) appeared to be composed only of polymyxin B while the lowest potency fraction (No. 4) seemed to be made up mainly of the minor component produced by this strain. After going through a minimum, fractions 6, 7, and 8 appeared to be composed

TABLE I

Fractions of polymyxin B from alumina column

Fraction number	Developing solution	Potency *units/mg.*
1	100% methanol	6750
2	100% methanol	5350
3	100% methanol	4150
4	95% methanol	3530
5	90% methanol	4750
6	80% methanol	5750
7	80% methanol	5750
8	50% methanol	6050
9	50% methanol	3700
10	water	2040
11	0.1 N sulfuric acid	—
12	0.5 N sulfuric acid	40

mainly of polymyxin B. Further examinations showed that the hydrolyzates of all of these fractions yielded identical amino acid spectra, indicating that the two active substances are qualitatively related (Figure 1A). This evidence is based upon the relative solubilities of the active components in two solvents, and in turn, on their relative speeds on two chromatograms. There was found to be some important difference between the active substances because the lower component appears to be only weakly active biologically. The most effective technique for eliminating the second component involves the precipitation of polymyxin B as its free base. This was achieved by the use of amines, ammonia, sodium hydroxide, etc.

The purification of polymyxin B by precipitation of its free base. Thirteen grams of polymyxin B, obtained from the Polar Yellow purification, were dissolved in 200 ml. of water. To the cooled solution (0° C.) was added an aqueous solution of triethylamine until no further precipitation occurred (pH 11.0). The polymyxin-B-free base was filtered over Super-Cel, washed with ice water and finally with ether until it was free of triethylamine. The base was suspended in water and dissolved by the addition of dilute sulfuric acid (pH 2.5), and warming to 50°. It was neutralized (pH 5.5) with barium hydroxide and filtered from the barium sulfate. The clear filtrate was vacuum freeze-dried: yield 85%; potency 7,200 units/mg.; ash 0.5%. The optical rotation increased from $-61°$ to $-76°$; and on chromatographing it on paper only one spot was observed corresponding to the R_F value for polymyxin B. The filtrate from the triethylamine precipitation was also vacuum freeze-dried after neutralization to pH 5.5; only about 10% of the activity was

found in this portion. Paper chromatography showed this intact material to be composed mainly of the lower active component.

Crystalline polymyxin B naphthalene β-sulfonate. This crystalline salt was prepared (8) by suspending 12 gm. of polymyxin-B-free base in 200 ml. of water, and adding a solution of naphthalene β-sulfonic acid with constant stirring to pH 5. The sulfonate was precipitated as a heavy oil. After standing, the supernatant liquid was decanted. The oil was washed three times with 150 ml. portions of water and dissolved in the minimum amount of ethanol. Additional alcohol was added until the solution appeared turbid whereupon it was allowed to stand at 5° for 18 hours. The sulfonate precipitate was filtered and washed with small portions of ethanol. It was then suspended in about 250 ml. of ethanol at 50° C. and treated with water until the sulfonate completely dissolved. The solution was stirred with 1 gm. of Darco G–60 at 50° for one-half hour and filtered. Ethanol was then added to the decolorized solution until cloudiness appeared, and the solution was allowed to cool slowly. The white, crystalline sulfonate crystallizes as very fine needles. The sulfonate was recrystallized by redissolving the crystals and repeating the procedure described above. Analysis of polymyxin B naphthalene β-sulfonate (dried *in vacuo* at 100° C. at 0.1 mm. Hg): C, 49.11%; H, 6.81%; S, 6.31%; N, 13.41%; $[\alpha]^{25}_D = -63.3°$ (c, 1% in 75% ethanol in water); m.p. 235–8° C. with decomposition.

The crystalline polymyxin B naphthalene β-sulfonate was converted to the hydrochloride by suspending the sulfonate in anhydrous methanol and adding concentrated hydrochloric acid until complete solution took place. Polymyxin hydrochloride was precipitated by pouring the acidified methanol solution into six volumes of acetone since polymyxin B naphthalene β-sulfonate is completely soluble in this mixture. The polymyxin B hydrochloride, after drying, showed $[\alpha]^{25}_D = -75.7°$ (c, 1% in 75% ethanol in water).

Partition chromatography on paper of the naphthalene β-sulfonate and the sulfate produced by metathesis reaction consistently showed a single component. Acid hydrolyzates of this component showed the presence of threonine, leucine, phenylalanine, α,γ-diaminobutyric acid, and a C_9 optically active fatty acid.

Acid hydrolysis of crude and crystalline materials yielded an optically active acid previously isolated and described by Catch, Jones and Wilkinson (2). After heating in concentrated hydrochloric acid, the fatty acid component was isolated by extraction with ether and distillation of the solvent. The yield of fatty acid was approximately 5% by weight of intact polymyxin of 5,000 units/mg. The crude acid was distilled *in vacuo* under nitrogen, and a clear colorless product was obtained with the following properties: b.p. 117–120° C. at 7 mm.; n 1.4307; $[\alpha]^{25}_D = +32°$ (c, 1% in methanol); sp. gr. 0.93; mol. wt. (sodium hydroxide titration) 157.3. *Anal.* Calcd. for $C_9H_{18}O_2$: C, 68.4; H, 11.4. Found: C, 68.2; H, 11.4. Amide: m.p. 96° C. p-Bromphenacyl ester: m.p. 60° C.; $[\alpha]^{25}_D = -25°$ (c, 1% in methanol). This optically active isomer of pelargonic acid has been recently shown to be *d*-6-methyloctan-1-oic acid by Wilkinson (8).

SUMMARY

A method for the recovery, purification and crystallization of polymyxin B is described in which the fermentation broth is clarified, adsorbed on cotton sodium succinate, eluted, precipitated as the polymyxin B Polar Yellow salt, converted to the free base and crystallized as the naphthalene β-sulfonate salt. Purification through the Polar Yellow salt removes a histamine-like contaminant, but does not remove a second minor active component the presence of which was demonstrated by partition chromatography on paper. The extraneous active substance is soluble in excess of base and can be separated from the insoluble basic polymyxin B. Paper chromatograms of the crystalline polymyxin B naphthalene β-sulfonate show a single component. The composition of the hydrolyzates of the polymyxin B and the minor active constituents are qualitatively related since they show the same acid spectra: Threonine, leucine, phenylalanine, α,γ-diaminobutyric acid, and a C_9 optically active fatty acid.

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