Purification of Antibacterial Compounds from Calf Liver[1] (37181)

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During attempts to extract polymyxin antibiotics from tissues it was found that normal liver contained an antimicrobial substance (1). Acid extracts of untreated rabbit liver, muscle, kidney, brain, heart, and lung were found to produce zones of inhibition in the polymyxin B cup-plate assay utilizing *Bordetella bronchiseptica* as the test organism. Substitution of *E. coli* or *Klebsiella* as test organisms still revealed zones of inhibition by extracts. The rabbits were known not to have received antibiotics in their food and homogenates of feed pellets did not exhibit antibacterial activity. This report deals with an attempt to characterize the antibacterial substances utilizing normal calf liver as the major source of material since it was as active as rabbit tissues and readily available. Evidence will be presented that the active substance in tissues is a fraction rich in glutamic acid.

Methods. Twenty-five grams of fresh calf livers were homogenized for 2 min in 95 ml of 0.2 *N* H_2SO_4 using a Sorvall Omni-Mixer. The homogenate was stirred at 6° for 1 hr. Two hundred milliliters of chloroform–methanol (2:1) were added. The mixture was homogenized and centrifuged in Sorvall RC-2 at 10,000*g* for 20 min. The upper aqueous layer was recovered and briefly subjected to flash evaporation to remove the methanol. After adjusting the pH to 6.5 with NaOH, the solution was lyophilized. The yield of the solid was 2.0 g and it was stored at −20°.

A portion of the lyophilized powder, 230 mg, was dissolved in 3 ml of H_2O and applied to a column of Sephadex G-10 (2.5 × 38 cm). The column was eluted with water and 3.7 ml fractions were collected. The absorbances at 260 and 280 nm of the effluent fractions were measured.

DEAE-Sephadex A-25 was equilibrated with 0.05 *M* NaCl containing 0.001 *M* sodium acetate, pH 4.9, and packed in a column (2 × 53 cm). Elution from this column was carried out with increasing concentration of NaCl. The mixing chamber contained 350 ml of 0.05 *M* NaCl in 0.001 *M* sodium acetate, pH 4.9, and the reservoir contained 350 ml of 1.0 *M* NaCl in 0.001 *M* sodium acetate, pH 4.5. The flow rate was 60 ml/hr and 3.7 ml fractions were collected. The absorbance at 260 nm of the effluent fractions was measured. The contents of the tubes comprising the peaks were pooled, lyophilized and desalted on Sephadex G-10, 2.5 × 38 cm, prior to microbiological assay.

Bacterial assays were performed by the cup-plate method with *B. bronchiseptica, Ps. aeruginosa, S. aureus* 6538P, *E. coli, Klebsiella,* and *Proteus* as the test organisms. Only *B. bronchiseptica* was used throughout the purification of the antibacterial compounds. Prior to assay all the organisms were subcultured in tryptic soy broth (Difco) and grown overnight at 37°. This suspension was diluted 1:500-fold with the bacterial medium and 10-ml portions of this solution were used for each petri dish. For *B. bronchiseptica* the medium was 2% agarose (Sea Kem, Marine Colloids) in Medium 199 adjusted to pH 8.0, and for the other bacteria it was Difco Antibiotic Medium No. 1, pH 6.6. After pipetting 0.3 ml of the antibacterial test solution in the steel cylinder placed on top of the agar, the plates were incubated for 18 hr at 37°, and the diameter of the clear zones was measured.

The lyophilized fractions were weighed out and dissolved in distilled, deionized water to give the following concentrations; lyophilized

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liver extract, 215 mg/ml; fraction A, 25 mg/ml; fraction B 53 mg/ml; fraction D II, 6.0 mg/ml. The pH was adjusted to the desired value with dilute H_2SO_4 or with dilute NaOH, and twofold serial dilutions were carried out. The initial concentrations of the amino acids and glutamate derivatives were 40–70 μmole/ml. The zone size of the serially diluted samples ranged from 10.0–24 mm (including the 8-mm diameter of the cup). At each pH value tested the zone size plotted against the log scale of milligrams of material, gave a straight line. In each set of *B. bronchiseptica* assays polymyxin B, 100 γ/ml, was included as a positive control. Throughout the purification procedure there was good reproducibility using different batches of calf's liver. With a given solution of an antibacterial compound the zone sizes were reproducible from assay to assay within one tube dilution.

With *B. bronchiseptica* there were no zones of inhibition with the following control compounds: NaCl up to 1.6 *M*; 0.1 *M* sodium acetate, pH 3.5–5.0; Medium 199, pH 2.3–6.4; Na_2SO_4 up to 0.3 *M*.

Results. The initial gel filtration on Sephadex G-10 separated the lyophilized liver extract into 2 major fractions, A and B, both of which had antibacterial activity against *B. bronchiseptica* (Fig. 1). Fraction A, the larger molecular weight material, was eluted in tubes 19–24 (void volume) and it comprised 4% of the original starting material on a weight basis. It exhibited antibacterial activity in the entire range tested, pH 3.5–8, but was more active at the lower pH values. The minimum inhibitory concentration (m.i.c.) of fraction A in the *B. bronchiseptica* assay was 12.5 mg/ml, using a 0.3 ml aliquot (Table I).

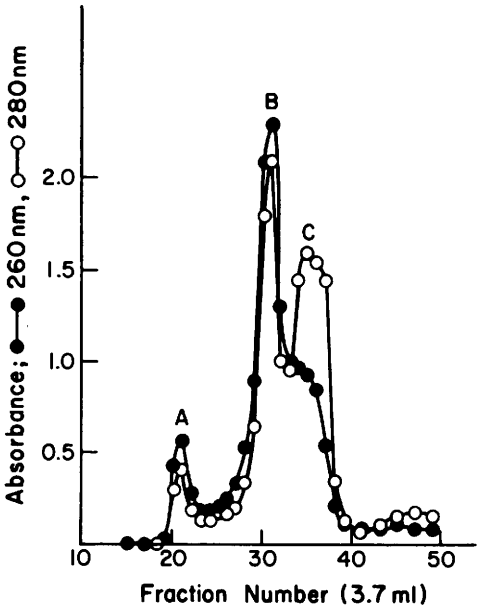

FIG. 1. Fractionation of the lyophilized calf liver extract, 230 mg, on Sephadex G-10, 2.5 × 38 cm. The column was eluted with water; 3.7-ml fractions were collected.

TABLE I. Minimum Inhibitory Concentration Values Against *Bordetella bronchiseptica*

Liver fraction or compound	mg/ml[a]
Lyophilized liver extract	26.8
A fraction from Sephadex G-10	12.5
B fraction from Sephadex G-10	13.2
DII fraction from DEAE-Sephadex A-25	0.375
L-Glutamate	1.5
L-Aspartate	2.0

[a] 0.3 ml of each solution was used in the assay. The pH of the solution was adjusted to 3.6; the pH of the bacterial medium was 8.0.

Fraction B was eluted in tubes 25–32 and it represented 78% of the original material on a weight basis. This material inhibited bacteria only at low pH values and was devoid of activity above pH 5.5. The m.i.c. against *B. bronchiseptica* at pH 3.6 was 13.2 mg/ml (Table I). Survey of other microorganisms revealed that fraction B was also active against *E. coli* and *Klebsiella* and at pH 3.6 gave m.i.c. values of 22.6 and 45.2 mg/ml, respectively. It was not active against *S. aureus, Pseudomonas,* or *Proteus.* Since fraction B contained most of the antibacterial activity it was purified further. Fraction C (Fig. 1) did not have antibacterial activity.

Fraction B was resolved into 3 components, DI, DII, and DIII, by chromatography on DEAE-Sephadex A-25 using a concentration gradient of NaCl (Fig. 2). After desalting each of the peaks, they were assayed at pH 3.6 using *B. bronchiseptica.* Of the total antibacterial activity in fraction B, 70–80% was associated with the DII peak. This peak was consistently eluted at 0.24 *M* NaCl and rep-

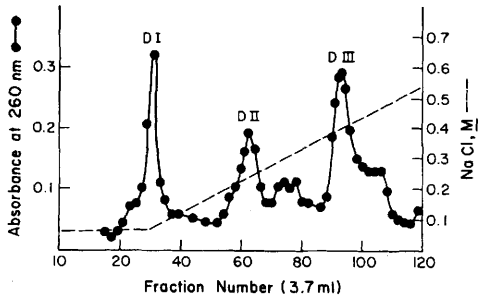

FIG. 2. DEAE-Sephadex A-25 chromatography of fraction B (375 mg). Column size: 2 × 53 cm. Flow rate: 60 ml/hr; 3.7 ml fractions.

resented 0.4% of the lyophilized liver extract by weight. Its m.i.c. at pH 3.6 was 0.375 mg/ml as compared with 26.8 mg/ml for the lyophilized liver extract (Table I).

The material contained in DII was ninhydrin (2) and Lowry (3) positive indicating the presence of peptide bonds and free amino groups. The anthrone test for reducing sugars, the orcinol test for pentoses, and phosphate determination were negative. Hydrolysis of the material in 6 *N* HCl for 20 hr at 100° yielded a mixture of several amino acids. As determined by the Beckman-Spinco Analyzer, glutamic acid comprised 81 mole% of the total amino acids, while aspartic acid, cysteic acid, and glycine accounted for 8.2, 5.0, and 3.8 mole%, respectively. Cysteine, cysteic acid, and glycine as well as other neutral amino acids did not inhibit *B. bronchiseptica* at pH 3.6 or above at concentrations up to 14 mg/ml. Glutamic and aspartic acid were strong inhibitors (Table I) and their derivatives, such as glutamine, glutathione, *N*-carbamyl glutamate, *N*-carbamyl aspartate, diethyl glutamate, and *p*-aminobenzoyl glutamate were also inhibitory in the *B. bronchiseptica* assay at concentrations in the same range as those of the free amino acids. With all of these compounds the extent of inhibition decreased with increasing pH and was not observed above pH 5.5 at the levels tested. Glutamate alone under these conditions did not inhibit *S. aureus, E. coli, Pseudomonas, Proteus,* and *Klebsiella.*

Analyses of fraction A indicated that this material in addition to peptide material also contained reducing sugars. Of the amino acid constituents, glutamic acid comprised 12% of the mixture per mole basis, aspartic acid, 6.5%; lysine, 13%; proline, 12%; and glycine, 11%. Upon mild heating or prolonged storage at —20°, fraction A tended to decompose into smaller molecular weight fragments. The total sample still had antibacterial activity toward *B. bronchiseptica,* but in contrast to the wide pH range of the fresh material, these preparations were active only at the lower values.

Discussion. Compounds such as spermine (4), and basic proteins (5–9) are known to inhibit the growth of bacteria. Gram-positive organisms including *B. anthrax, S. aureus,* and *M. tuberculosis* are particularly susceptible to inhibition by these compounds although gram-negative bacteria are also affected. Basic protein fractions from polymorphonuclear leukocytes (10) and an α-keto aldehyde from HeLa cells (11) have been reported to be active against both gram-positive and negative organisms. The test organism used in this study, *B. bronchiseptica,* a gram-negative bacterium, was not inhibited by spermine, spermidine, or by basic proteins such as protamine or pig brain histone. This indicates a marked difference in the nature of the material described here and the basic protein derived from calf thymus described by Hirsch and Dubos (12) and Skarnes and Watson (13).

The most active fraction of calf liver prepared in the present study was found to contain a high proportion of glutamic acid. Glutamic acid itself and many of its derivatives also inhibited the growth of *B. bronchiseptica.* Since the derivatives were inhibitory at levels comparable with those of free glutamate and they showed the same pH dependence, it is likely that they are hydrolyzed to the parent compound. These data indicate that the antibacterial activity of the DII fraction to a large extent may be accounted for by its high content of glutamate, most of which appears to be peptide bound although some of it may be free. The small amounts of glutamate and glutamine (150 and 100 mg/liter, respectively) contained in Medium 199, which was used routinely in the *B. bronchiseptica* assay, were below the levels re-

quired for inhibition and control experiments over the whole pH range were negative.

Organic acids such as propionic acid, benzoic acids (14), and aliphatic fatty acids are known to possess antimicrobial properties (15, 16). This type of anion-induced inhibition is augmented as the pH of the medium is lowered (14, 16). A similar pH dependence was found in the present study and is probably related to the anionic character of the γ-carboxyl group of glutamate, the pK of which is 4.25.

To date no systematic studies of the antibacterial properties of aspartate and glutamate have appeared. The antibacterial activity of glutamate against *B. bronchiseptica* is of particular interest because this compound is ubiquitous in animal tissues both in the free and in the bound form. For example, the glutamic acid content of cat liver and kidney is 66 and 137 mg/100 g wet weight, respectively (17) and many animal proteins are rich in glutamate (18). The possibility should be considered that glutamate-containing compounds may play a role *in vivo* in limiting infectious processes in tissue undergoing necrosis at acid pH.

Summary. An antimicrobial substance has been purified from normal calf's liver. Activity is directed primarily against gram-negative bacteria, particularly *Bordetella bronchiseptica,* the test organism used in these studies. Peptides rich in glutamic acid appear to be responsible and probably account for the fact that activity is augmented as the pH is lowered.

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