trodes (Fig. 3). Although the amplitude of the recorded potential was reduced somewhat using the wick electrodes, the wave forms are essentially the same. The mass of the cotton wick electrodes is extremely low and is a very flexible system, permitting the tip of the tuft to follow the movements of the heart. The wave form of the ECG of a control preparation indicates no change even after 1.5 hours of continuous recording.

Using the system described, it is possible to denote the difference between a control recording of an isolated cardiac preparation and that following experimental procedures, illustrating that the cotton wicks retain their position and that no injury is produced from their use.

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Microbiological Assay of Aspartocin in Pharmaceutical Preparations Containing Chlortetracycline

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An agar diffusion procedure, using *Staphylococcus epidermidis* ATCC No. 12,228 and 0.1 *M* calcium chloride solution as diluent for aspartocin standard and samples, was evaluated. Satisfactory standard curves for aspartocin were obtained with concentrations of compound ranging from 0.5 mcg./ml. to 50 mcg./ml.; chlortetracycline (CTC) did not interfere in the assay of aspartocin-CTC mixtures.

THE FERMENTATION, isolation, and antimicrobial properties of aspartocin have been previously reported (1–4). It is active primarily against Gram-positive bacteria; and although somewhat similar to amphomycin (5), aspartocin may be separated from this compound and identified by paper chromatography or electrophoresis (4).

For microbiological assay, an agar-plate method using *B. subtilis* and alkaline conditions was proposed (4); however, the tetracyclines are highly active against this test organism, and aspartocin-tetracycline mixtures would be difficult to assay under these conditions. With this in mind, the application of a tetracycline-resistant test organism was investigated.

Staphylococcus epidermidis ATCC #12,228 is routinely employed in this laboratory for neomycin assays, particularly with mixtures containing streptomycin and tetracycline, where resistance to these antibiotics (6) would be essential. Since the aspartocin antibacterial spectrum indicates that low concentrations inhibit staphylococci and streptococci *in vitro* (2), *Staphylococcus epidermidis* ATCC #12,228 was evaluated for use in aspartocin microbiological assays.

EXPERIMENTAL

The method outlined is a modification of FDA cylinder-plate procedures generally used in our laboratory for antibiotic assays; a more detailed description of antibiotic cylinder-plate methods appears in Grove and Randall (7).

Test Organism.—*Staphylococcus epidermidis* ATCC #12,228 (available from the American Type Culture Collection) is subcultured every 2 weeks on nutrient agar slants, incubated overnight at 30–35° and stored at 4°.

For inoculum, suspend the growth from a stock slant in sterile 0.9% saline solution and inoculate a Roux flask containing 200 ml. sterile Difco Penassay seed agar; incubate overnight at 30–35°. Harvest this growth with approximately 50 ml. sterile saline solution and store at 4°; this concentrated suspension may be used at least 5 to 6 days. Before the actual assay, determine what dilution of the concentrate will give 80% light transmission using a Lumetron model #400 colorimeter and 650 mμ filter. Use trial plates to determine the optimum concentration of diluted suspension for the assay (usually 0.5 to 1%).

Assay Medium.—Difco Penassay seed agar (antibiotic medium #1) is autoclaved 15 minutes at 121°, cooled, and adjusted to pH 8.0 with 4% NaOH solution.

Diluent.—The diluent was calcium chloride 0.1 *M* in distilled water for aspartocin standard and unknowns; anhydrous $CaCl_2$ (reagent grade) 11.1 Gm. dissolved in distilled water to make 1,000 ml. of solution (pH 5.8); it was autoclaved for 15 minutes at 121°.

Aspartocin Standard Curve.—Sodium aspartocin (Lot #31460-165A) with an assigned potency of 1,000 mcg./mg.[1] was used for the standard.

Weigh 50 to 100 mg. of the standard and dissolve in sufficient distilled water to obtain 1,000 mcg./ml. stock solution. Dilute further in calcium chloride solution to obtain 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, and 0.39 mcg./ml.; the reference point solution is at 10 mcg./ml. The stock solution is held refrigerated and may be used at least 7 days.

Preparation of Test Plates.—The assay agar is cooled to about 45° and inoculated with the test organism. A 6-ml. volume of inoculated agar is distributed evenly into each flat-bottomed Petri dish (Pyrex #3162) equipped with an unglazed ceramic cover, and allowed to solidify on a perfectly level surface.

The agar is allowed to harden 15 to 20 minutes at room temperature before placing six stainless steel Penicylinders on each plate. These cylinder plates

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[1] Obtained from the Biochemical Research Section, Lederle Laboratories Division, American Cyanamid Co.

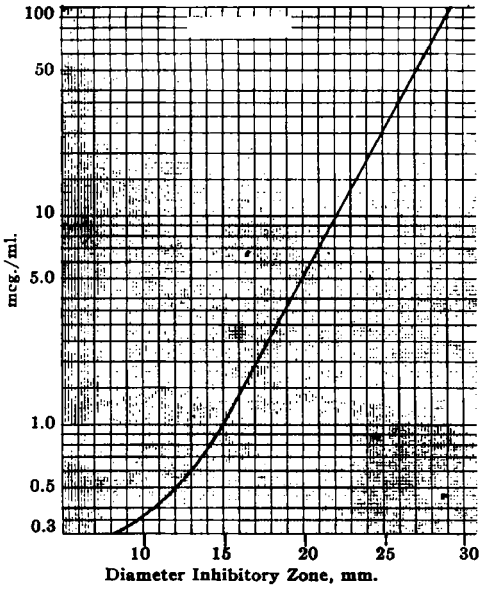

Fig. 1.—Cylinder plate standard curve for sodium aspartocin (Lot #3146C-165A) in 0.1 *M* $CaCl_2$ *vs. S. epidermidis* #12,228.

are held about 10 minutes before filling the cylinders with solutions.

CONDUCTING THE ASSAY

Aspartocin Standard Curve And Unknowns.—Three plates are set for each point on the standard curve. On each plate, fill three alternate cylinders with the 10 mcg./ml. reference solution and the other three with the standard curve solution. Incubate all plates 16–18 hours at 30–32° and measure the diameter of the inhibitory zones. (A Quebec colony counter equipped with an etched glass millimeter scale is used in this laboratory.) Average all of the reference point readings, and use this value to adjust the averages of reference point and standard solution for the three plates set for each point on the standard curve. For example, if all the readings of the reference solution average 20 mm. and, on a particular set of three plates the reference solution averages 19.8 mm., the correction is plus 0.2 mm. for the other solution's response on that same set of three plates.

Plot all points, including the reference point, on 3 cycle semilog paper, using the logarithmic scale for the potency (mcg./ml.). Draw the best straight line to fit the points (see Fig. 1).

The test sample is similarly assayed on three plates after diluting to an estimated 10 mcg./ml. in $CaCl_2$ solution. The sample zone size is adjusted to fit the curve as determined by the response of the reference point. This corrected zone size is then converted directly to mcg./ml. from the standard curve, and the value multiplied by the dilution factor for sample potency.

In the dose-response curve comparisons, the same basic procedure was employed with variations in diluent and culture media; *B. subtilis* or *B. cereus* var. *mycoides* spore suspensions were used for inoculum in specific trials, as indicated (Table I).

Aspartocin–CTC complexes or dry-powder mixtures, such as tablets or capsules, may be extracted in a Waring Blendor using 0.01 *N* HCl to obtain

TABLE I.—SODIUM ASPARTOCIN DOSE-RESPONSE CURVES

Organism	Test Conditions: Agar Medium	Dilutions[a] Prepared In	Average mm. Zone Size Per mcg./ml. Aspartocin Solution At: 50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.195	Diluent Control
S. epidermidis ATCC #12,228	Difco Penassay seed agar pH 8.0	0.1 *M* K_2HPO_4, pH 8.7	23.6	20.6	17.2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
		1% ammonium carbonate, pH 9.0	25.5	23.3	16.6	10.0	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
		0.1% ammonium carbonate, pH 8.8	24.2	22.4	18.0	13.8	Neg.	Neg.	Neg.	Neg.	...	Neg.
		0.1 *M* $CaCl_2$, pH 5.8	27.3	24.8	22.3	20.8	18.5	16.6	14.2	10.5	...	Neg.
		Distilled H_2O, pH 5.9	22.7	19.6	16.9	11.3	Neg.	Neg.	Neg.	Neg.	...	Neg.
		0.1% $Ca(OH)_2$, pH 12.0	20.6	18.9	16.5	14.1	12.5	11.2	10.4	10.2	10.4	Neg.
		0.9% NaCl, pH 5.1	22.2	19.7	17.7	13.3	9.0	Neg.	Neg.	Neg.	Neg.	Neg.
		0.1 *M* KH_2PO_4, pH 5.0	20.5	17.1	11.9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	Difco nutrient agar pH 7.0 plus $CaCl_2$ after sterilization to obtain 0.1 *M*	0.1 *M* $CaCl_2$, pH 5.8	25.3	23.3	20.6	18.6	16.6	14.3	11.3	9.5	...	Neg.
		Distilled H_2O, pH 5.9	25.4	24.5	22.5	19.9	17.3	15.0	12.0	10.1	...	Neg.
B. cereus var. *mycoides* ATCC #11,778	Difco Penassay base pH 8.0 plus $CaCl_2$ after sterilization to obtain 0.1 *M*	0.1 *M* K_2HPO_4, pH 8.4	19.0	18.0	16.8	15.1	13.9	12.7	12.0	11.0	...	Neg.
		0.1 *M* sodium bicarbonate, pH 7.9	25.6	24.5	22.9	21.3	20.2	18.7	17.2	15.4	...	Neg.
		0.1 *M* sodium citrate, pH 7.2	24.6	23.7	21.8	20.8	19.6	18.0	17.2	15.9	...	Neg.
B. subtilis ATCC #6,633	Difco nutrient agar pH 8.0	0.1 *M* K_2HPO_4, pH 8.7	29.3	28.2	26.0	23.3	20.8	17.6	12.0	Neg.	Neg.	Neg.

[a] Diluent pH value may represent that obtained as prepared, or after adjustment with the appropriate base or acid.

TABLE II.—COMPARISON STANDARD CURVES USING 0.1% AMMONIUM CARBONATE DILUENT AND *B. Subtilis* (4, 9)

Preparation	Average mm. Zone Size Per mcg./ml. Aspartocin Solution At— 100	50	25	12.5	6.25
Sodium aspartocin Lot #50A	27.3	25.0	23.8	21.7	19.7
Calcium aspartocin Lot #85A	27.0	24.8	22.8	20.2	18.1

TABLE III.—SODIUM ASPARTOCIN ACTIVITY IN THE CTC MICROBIOASSAY TEST SYSTEM (10)

Test Conditions— Test Organism	Agar	Curve Diluent	Average mm. Zone Size Per mcg./ml. Aspartocin At— 50	25	12.5	6.25	3.12	1.56	Control
B. cereus var. *mycoides* ATCC #11,778	Difco Penassay base adjusted to pH 5.7	0.1 *M* KH_2PO_4 pH 4.7	21.8	19.3	16.8	14.3	11.5	Neg.	Neg.

TABLE IV.—MICROBIOLOGICAL ASSAY ASPARTOCIN–CTC MIXTURES[a]

Preparation Number	Theoretical Content, % Aspartocin	CTC	Activity Assay Value, % Aspartocin	CTC	Colorimetric CTC (11)
#582-43	13.5	76	12.5	68.5	...
#582-118-1	27	65	28.8	62.0	61.1
#582-118-2	27	65	24.9	61.5	59.0

[a] These products prepared by Mr. F. H. Smith of our Laboratories.

an estimated 500–1,000 mcg. aspartocin/ml.; assay dilutions are prepared in 0.1 *M* $CaCl_2$ diluent. Portions of this same extract may be tested for CTC content (Table IV).

RESULTS AND DISCUSSION

Satisfactory standard curves for aspartocin were obtained with *S. epidermidis* ATCC #12,228 and 0.1 *M* calcium chloride solution as diluent for the reference material and samples under test.

Several assay systems were compared, and the inhibitory-zone responses for graded doses of aspartocin appear in Table I.

The influence of diluent, pH, and concentration of salts in agar-diffusion streptomycin assays is well known (8). In this respect the potentiation of aspartocin activity (2) was of particular interest. When 0.1 *M* calcium chloride solution was used as a diluent, the aspartocin standard curve showed good sensitivity (Fig. 1). The addition of 0.1 *M* calcium chloride in the assay agar also appeared satisfactory; however, this made the culture medium hazy and responses at low concentrations might be difficult to measure. Calcium as calcium hydroxide and chloride as sodium chloride did not appear to enhance aspartocin activity in the agar diffusion assay. Similarly, the responses obtained with sodium and calcium aspartocin salts were not significantly different (Table II) when ammonium carbonate was used as the diluent.

The responses using the *B. subtilis* procedure, as proposed (4), appear in Table II; however, aspartocin dilutions were prepared in 0.1% ammonium carbonate in distilled water instead of 1% ammonium carbonate, and responses were less erratic (9). Although suitable standard curves may be obtained with *B. subtilis* and *B. cereus* var. *mycoides*, the tetracycline sensitivity of these organisms limits their usefulness in the assay of aspartocin–tetracycline mixtures.

In the determination of CTC in aspartocin–CTC mixtures, the response of aspartocin in the usual CTC cylinder-plate assay (10) indicated relatively low activity; aspartocin alone at 3 mcg./ml. showed a small zone of inhibition (Table III). This response may be insignificant in the presence of an equivalent weight of CTC. However, if a mixture is predominantly aspartocin with low CTC content present, a mixed standard curve (aspartocin plus CTC in an appropriate ratio) may be more useful for the assay of CTC content.

Experimental aspartocin–CTC mixtures were evaluated by the *S. epidermidis*–0.1 *M* $CaCl_2$ test system; the results of these assays (Table IV) indicated no interference in the aspartocin or CTC determinations. Chlortetracycline microbiological values were confirmed by colorimetric assay (11), and for aspartocin by nitrogen determinations.

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