Prodigiosin. I. Antibiotic Action on *Coccidioides immitis in Vitro*.* (17533)

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Prodigiosin is a thermostable, water insoluble, red pigment produced by *Serratia marcescens* under certain conditions. The dye has stimulated interest because of its ability as a contaminant to discolor water and food. This characteristic is better known than its inhibitory action against microbial agents.

The antibiotic properties of prodigiosin recorded in the literature indicate a possible wide spectral range of activity against bacteria and protozoa. No evidence concerning its fungistatic action could be found. Hettche(1) first reported the bacteriostatic activity of prodigiosin for staphylococci and *Bacillus anthracis in vitro*. The growth of *B. anthracis* was inhibited in a dilution of 1:200,000 after 3 hours' exposure and a dilution of 1:25,000 appeared effective against staphylococci after one hour's exposure. He was unable to demonstrate any activity against the gram-negative organisms, *Escherichia coli* and *Salmonella paratyphi*. Fischl(2) demonstrated that prodigiosin would protect mice against nagana. A single subcutaneous injection of 10 mg of prodigiosin in sweet almond oil caused the disappearance of *Trypanosoma brucei* from the peripheral blood.

Eisler and Jacobsohn(3) demonstrated that sterile boullion extracts of *S. marcescens* inhibited the growth of *Corynebacterium diphtheriae* and of *Neisseria gonorrhoeae*. Their results, however, were not directly related to dye content. Singh(4) reported toxic effects of the pigment of *S. marcescens* on soil amoeba. The concentration of the pigment which prevented amoebae from consuming an edible substrate of bacteria was not reported.

The naturally occurring dye was first isolated by Kraft,(5) who called it "prodigiosine." Wrede, Hettche, and Rothhaas(6) presented a series of articles on the chemical properties of prodiogiosin and identified the structure as a tripyrrole methane.

Materials and methods. Abundant pigment production was obtained from growth of *S. marcescens* on thick mannite agar plates (15.0 cm) adjusted to pH 5.0.† Surface inoculation with a single strain of *S. marcescens* (Stanford Z-4 strain) was followed by incubation at 22°C for from 5 to 7 days. The surface growth was harvested by scraping and contained large amounts of both intracellular and extracellular red pigment which was extracted by a modification of Wrede's method (Lack and Botts).(7) Yields of from 1.0 to

* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Hettche, H. O., *Arch. Hyg.*, 1932, v107, 348.
2. Fischl, V., *Z. f. Immunitats.*, 1935, v85, 77.
3. Eisler, M., and Jacobsohn, I., *Z. f. Hyg. Infekt.*, 1936, v117, 76.
4. Singh, B. N., *Nature*, 1942, v149, 168.
5. Kraft, E., Thesis Wurzburg, 1902 (from Wrede, F., and Hettche, O., *Berichte d. Deutschen Chemischen Gesellschaft*, v929, v22, II Abblg. B, 2678).
6. Wrede, F., and Hettche, O., *Berichte d. Deutschen chem. Gesellsch.*, 1929, v22, II Abblg. B, 2678; Wrede, F., *Z. f. Hyg. d. Infect—Krankl*, 1930, v111, 531; *ibid., Z. f. physiol. chem.*, 1932, v210, 125; Wrede, F., and Rothhaas, A., *Z. f. physiol. chem.*, 1933, v215, 67; *ibid., Z. f. physiol. chem.*, 1933, v219, 267; *ibid., Z. f. physiol. chem.*, 1933, v222, 203; *ibid., Z. f. physiol. chem.*, 1934, v226, 95.

†Mannite agar formula:

Mannite	20 g
Neopeptone	10 g
Meat extract	3 g
$MgSO_4$	1.25 g
Brewer's yeast	1 g
Agar	20 g
Q.S. ad H_2O	1000 cc

Adjusted to PH 5.

7. Lack, A., and Botts, E., in press.

TABLE I.
Fungistatic and Fungicidal Activity of Prodigiosin Base and Certain Salts on *Coccidioides Immitis in vitro.*

Derivative	Fungistatic conc., ×1000	Fungicidal conc., ×1000
Base, purified	1:500	1:100
Hydrochloride	1:500	1:100
Perchlorate	1:500	1:100
Glutamic acid (water soluble)	1:250	1:83
Tartrate	1:100	1:50
Sulfonate	1:10	0
Sulfonamide	1:1	0

2.0 mg of prodigiosin were obtained from approximately 10 sq cm of wet surface growth. Repeated purifications yielded a prodigiosin from which several new compounds were made.‡ Each compound was tested *in vitro* for its fungistatic and fungicidal action against *Coccidioides immitis* (Strain S.F.S. 46). Inasmuch as prodigiosin base is insoluble in water, tests were made to determine the most satisfactory solvent. Propylene glycol and ethanol were found to be suitable vehicles for *in vitro* and *in vivo* tests. Propylene glycol is sufficiently miscible in water to permit uniform dilutions of the derivatives to be tested in liquid synthetic culture medium.

Serial dilutions of prodigiosin in propylene glycol or ethanol from 1:10,000 to 1:10,000,000 were made in a synthetic medium.§ Controls with equivalent amounts of propylene glycol or ethanol in this medium were run in parallel. Each tube was inoculated with a 0.1 ml of a known concentration of chlamydospores which was diluted to give a range of from 2.0 to 3.0 $\times$ 10^4 spores. After incubation of 24 and 48 hours at 34°C 0.1 ml was transferred from each tube to 9.9 ml of the liquid synthetic medium as a test for viability. The subcultures were incubated at 34°C and inspected for growth after from 10 to 14 days on a basis of no growth (0) to abundant growth (++), indicating first signs of spore germination to rich mycelial development.

Results. The prodigiosin first prepared was fungistatic in a dilution of 1:250,000. Subsequent purifications increased the activity to 1:500,000 (Table I). Fungicidal activity as determined by the subcultures showed no growth at 1:100,000 dilutions. Propylene glycol controls revealed no inhibition of *C. immitis* except in the first tube of the series containing 10% propylene glycol. The hydrochloride and perchlorate salts of prodigiosin base inhibited growth in a dilution of 1:500,000. The tartrate maintained moderate effectiveness; the sulfonate and sulfonamide derivatives showed an almost complete loss of activity. A water soluble preparation was made by fusion of glutamic acid with prodigiosin base. This glutamic acid compound demonstrated effective fungistatic activity at a 1:250,000 dilution.

A comparison was made of the fungistatic and fungicidal effectiveness of prodigiosin purified bases when dissolved in propylene glycol or ethanol. Inhibition of growth of *C. immitis* was obtained in a dilution of 1:500,000 in both series. Although definite inhibition occurred at a dilution of 1:1,000,000, after several weeks' incubation germination appeared first in the propylene glycol and then the alcohol series. The propylene glycol and alcohol controls showed abundant growth in all except the initial tubes containing equivalent amounts of diluent. The tubes were subcultured at 48 hours to test the fungicidal activity. Slightly better results were obtained

‡ The preliminary investigation of the chemical constitution and general properties of prodigiosin were performed with the assistance of Dr. Frederick Proescher and Mr. V. Sycheff, formerly of the Santa Clara County Hospital laboratory. Subsequent extension of similar chemical research has been with the aid of Dr. Elbert Botts in the laboratory of the Birmingham Veterans Administration Hospital, Van Nuys, Calif.

§ Synthetic media:

	g
NH_4Cl	10
Na Acetate	10
KH_2PO_4	2
K_2HPO_4	6

H_2O q.s. ad 1000 cc pH 6.12.

in the propylene glycol series. Complete loss of viability of all chlamydospores after 48 hours' exposure was established at a prodigiosin dilution of 1:100,000.

Summary. Prodigiosin was fungistatic for *Coccidioides immitis* in dilutions through 1:500,000. It was fungicidal after 48 hours' exposure in dilutions through 1:100,000. Prodigiosin hydrochloride, perchlorate and a water soluble glutamic acid derivative demonstrated similar *in vitro* effectiveness.

Received October 14, 1949. P.S.E.B.M., 1949, v72.

Pregnancy in Alloxan Diabetic Rats. (17534)

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Because of the well recognized frequency of complications in the course of pregnancy in clinical diabetes, this study was undertaken to determine the course of pregnancy in rats suffering from uncontrolled chronic diabetes. Since August 1946, when these studies were begun, 4 reports(1-4) have appeared dealing with this subject each with a slightly different approach to the problem and each with somewhat varying results.

Methods. Twenty-seven 70-90 day old Wistar strain female rats were fasted overnight and given a single subcutaneous dose of alloxan varying from 140-175 mg per kg. Where a single dose failed to produce a persistent diabetic state, repeated injections of increasing doses were employed up to 205 mg per kg. In some rats it was necessary to administer as many as nine doses of alloxan in order to achieve permanent diabetes. In 2 rats permanent diabetes did not appear despite repeated injections of alloxan. During the entire period covered by these studies the animals were kept in individual metabolism cages and the following data were obtained daily, 7 days a week, for periods up to 448 days: (a) total fluid intake, (b) total food intake, (c) total urine volume, (d) total quantitative glycosuria, (e) qualitative acetonuria, (f) vaginal smears for determination of oestrus. The animals were given free access to a constant mixture of crushed Purina Lab Chow (Protein 25%, Carbohydrate 50%, Fat 6.3%) reinforced with the following: reduced iron 0.2 g; sodium chloride (iodine-free) 10 g; sodium chloride (iodized) 2.7 g; riboflavin 5 mg, vitamin B_6 5 mg, vitamin D 3000 U.S.P. units, vitamin E 100 mg, choline 15 mg, per kilo of chow in order to bring the ration up to an adequate level for breeding as recommended by the Ralston Purina Company.

Because of the frequent occurrence of a transient diabetic state after alloxan injection, matings were performed when possible only after the establishment of permanent diabetes by placing one young adult male of the same strain into the metabolism cage with each female on the day of oestrus. Animals were considered definitely diabetic only when they fulfilled both of the following criteria: (1) persistent glycosuria greater than 0.5 g per 24 hours as measured by Benedict's Picrate method, and (2) repeated non-fasting venous blood sugars in excess of 200 mg% obtained from tail blood or cardiac puncture and measured by the technique of Lauber and Mattice.(5) Fasting blood sugars were not employed in this study, because it was thought that repeated overnight fasts might introduce an irregular influence on the nutritional or

1. Davis, M. E., Fugo, N. W., and Lawrence, K. G., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 638.

2. Miller, H. C., *Endocrinology*, 1947, v40, 251.

3. Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, v25, 131.

4. Sinden, J. A., and Longwell, B. B., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 607.

5. Lauber, F. V., and Mattice, M. R., *J. Lab. and Clin. Med.*, 1944, v29, 113.