[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY OF THE UNIVERSITY OF SANTA CLARA, SAN JOSE STATE COLLEGE, AND THE JOHNS HOPKINS UNIVERSITY]

Products from *Serratia marcescens*[1,2]

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Received July 21, 1958

Procedures for the isolation of pure prodigiosin from *Serratia marcescens* are described. Prodigiosin occurs in the bacterial strain studied mainly, if not entirely, as an acid derivative. Palmitic acid and an amide, $C_{24}H_{42-44}O_7N_2$ are isolable from the bacterium.

The specta of ethyl and isopropyl alcohol solutions of the perchlorate are influenced due to the interaction of the perchlorate with the solvent, which functions as a Lewis base.

Prodigiosin, obtainable from the red pigment produced by the bacterium *Serratia marcescens*, is reportedly[3] 2,2′2″-(4-*n*-amyl-4′-methoxy-5-methyl)tripyrrylmethene. The proposed structure is unique for a naturally occurring polypyrryl type compound and is of further interest in view of the suggested role of tripyrrylmethanes in the biosynthesis of porphyrins.[4] However, the product used in the original structural study was of questionable purity.[5] Furthermore, assuming the purity to be adequate for the structural study, the proposed formula is not definitely established and merits reinvestigation.

Morgan and Tanner[6] reported the isolation of purified prodigiosin through a procedure involving chromatography on alumina. We have isolated the compound from the reaction of prodigiosin perchlorate with sodium hydroxide *via* solvent extraction and chromatography on powdered sugar. A far more convenient process than column chromatography is to simply stir a petroleum ether solution of the crude compound with magnesium oxide. The dark color is removed from the solution and pure prodigiosin can be obtained through crystallization. The infrared and ultraviolet-visible absorption spectra for the purified compound are shown in Figures 1 and 2,[7] respectively.

The value for ϵ_{max} at 466 mμ is 4.3×10^4. The properties reported for pure prodigiosin by Morgan and Tanner are, for the most part, identical with those for our product. However, distinct absorption bands shown by our product at 7.14, 8.14, and 11.37 μ are not described by them. It was considered that possibly prodigiosin was changed during the Morgan and Tanner procedure. Starting with our product we repeated their process. From melting point data the product and starting compound appeared to be identical, and the infrared spectra were found in fact to be the same, rather than different. Accordingly, the earlier report is apparently incomplete. It is of interest that although the infrared spectrum for prodigiosin is consistent with certain features[8] of the provisional formula, opposing opinions[10-12] have been registered concerning the tripyrrylmethene linkage. A study aimed at the elucidation of the structure of prodigiosin is in progress by us.

Molecular weights of 520 and 540 were found for prodigiosin in benzene at 30° using the method of Signer.[13,14] From these measurements, calculation for

$$(C_{20}H_{25}ON_3)_2 \rightleftharpoons 2C_{20}H_{25}ON_3$$

given an equilibrium constant of $1.3\text{–}1.4 \times 10^{-3}$.

(1) Presented in part before the Division of Organic Chemistry at the 132nd National Meeting of the American Chemical Society, New York, N. Y., September 10, 1957.

(2) This investigation was supported in part by a research grant, E-541, from the National Microbiological Institute, Public Health Service, to San Jose State College and in part by a research grant, E-1335, from the National Institute of Allergy and Infectious Diseases, Public Health Service, to the University of Santa Clara.

(3) F. Wrede and A. Rothhaas, *Z. physiol. Chem.*, **226**, 95 (1934).

(4) W. J. Turner, *J. Lab. Clin. Med.*, **26**, 323 (1940).

(5) C. M. Weiss, *J. Cellular Comp. Physiol.*, **34**, 467 (1949).

(6) E. N. Morgan and E. M. Tanner, *J. Chem. Soc.*, 3305 (1955).

(7) The weak absorption near 540 mμ arises from a trace of acid in the solution. In three solutions examined there was no apparent relationship between intensity and concentration.

(8) The broad absorption starting at 2.78 and extending to 3.32 μ is in the region for pyrrole NH and CH stretchings; the 3.45, 3.53, 6.84–6.90 and 7.30–7.37 μ bands can be associated with CH stretching and bending modes of the substituents appearing in the suggested formula; the band at 13.73 μ can be interpreted as arising from rocking vibrations of the methylene groups in the amyl substituent; the 6.16 and 6.44 μ bands are in the region appropriate to C=N stretching and NH bending modes, respectively; the 8.69–8.98 μ band complex falls in the region where C—O stretching vibrations of a methoxyl group attached to a pyrrole ring might appear.[9]

(9) Assignments in this article are based upon private communication with Professor Nelson Fuson (Fisk University), to whom we are indebted in this connection and otherwise, and comparisons with the data contained in L. J. Bellamy's *The Infrared Spectra of Complex Molecules*, Methuen and Co., Ltd., London, 1954.

(10) R. Hubbard and C. Rimington, *Biochem. J.*, **46**, 220 (1950).

(11) A. Treibs and K. Hintermeier, *Ann.*, **605**, 35 (1957).

(12) A. Treibs and R. Galler, *Angew. Chem.*, **70**, 57 (1958).

(13) A. Steyermark, *Quantitative Organic Microanalysis*, The Balkiston Co., New York, 1951, p. 292.

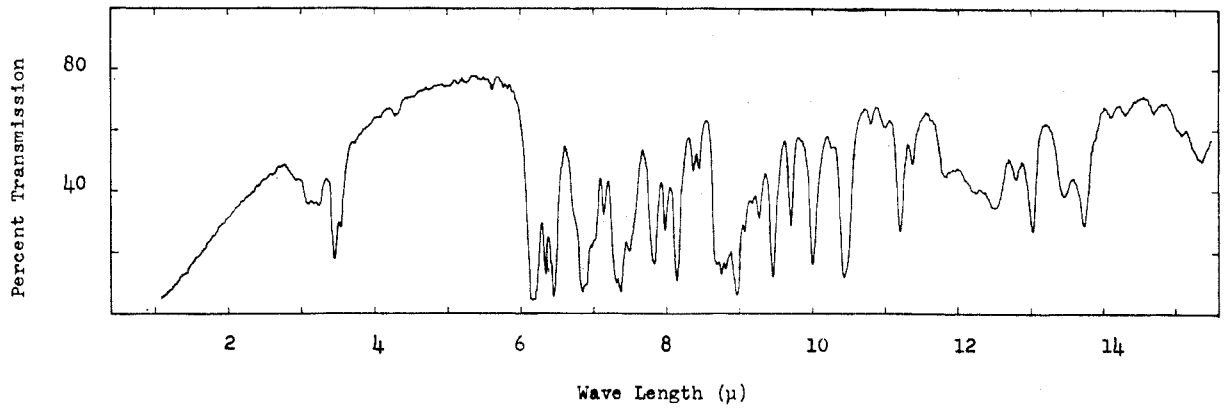

Fig. 1. Infrared spectrum for prodigiosin (KBr).

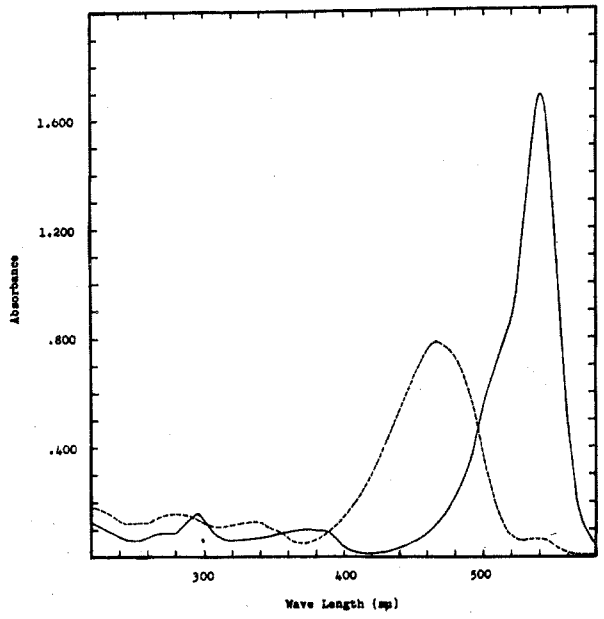

Fig. 2. ---Prodigiosin, 0.580 mg./ml. isopropyl alcohol solution. —Prodigiosin perchlorate in acidified isopropyl alcohol. Solution 13 of Table I.

Wrede's isolation[16] of the zinc-prodigiosin complex from growth of the bacterium on a solid medium containing a zinc salt indicates that prodigiosin is truly a bacterial product. Our attempts to isolate the free base directly from the bacterium through solvent extraction and chromatography were unsuccessful. Instead, a red grease having a green sheen was obtained. The ultraviolet-visible absorption spectrum for this fraction is like that for prodigiosin perchlorate (Fig. 2) with λ_{max} at 534 mμ in 95% ethyl alcohol and upon treatment with perchloric acid, prodigiosin perchlorate was formed. Furthermore, while the isolation of prodigiosin in the preceding fashion was not fruitful, when the bacterial growth is treated with sodium hydroxide in ethyl alcohol, the mixture immediately changes color from a red to an orange, corresponding to the change from an acid derivative of prodigiosin to the free base, and prodigiosin was isolated from the mixture. A magenta colored oil which was separated from the bacterial pigment gave similiar results. It is concluded that prodigiosin exists principally, if not entirely, as an acid derivative in the strain of *Serratia marcescens* studied by us.[17]

Prodigiosin hydrochloride[18] was isolated in a small amount, along with prodigiosin, from the mixture derived from the reaction of prodigiosin perchlorate with alkali. Its presence is attributed to an artifact.[20] In addition, palmitic acid and a compound, $C_{24}H_{42-44}O_7N_2$, were isolated from the bacterium. The infrared spectrum for the latter in a mineral oil mull contains a strong carbonyl band at 5.81 μ. In addition, bands at 3.02, 6.04 (shoulder at 6.11) and 6.46 μ are consistent with a secondary amide group.

Prodigiosin perchlorate exhibits an absorption spectrum in 95% ethyl alcohol which is dependent upon its concentration. The decrease in the molecular extinction coefficient at 536–538 mμ with dilution, as shown in the accompanying table, is accompanied by a rise in absorption in the neighborhood of the maximum for the free base. This sug-

(14) From Wrede and Hettche's crypscopic measurements[15] in benzene, we calculate molecular weights for their product of 603 and 630. As in the present study, the higher value was obtained for the more concentrated solution. A benzene solution of prodigiosin at a concentration of 6 g./l., which is more concentrated than either of the solutions studied by us, does not show a Tyndall effect. Hence, our results, which are not questionable from the standpoint of purity, must be due to association.[15]

(15) F. Wrede and O. Hettche, *Ber.*, **22**, 2678 (1929).

(16) F. Wrede, *Z. physiol. Chem.*, **210**, 125 (1932).

(17) A narrow orange zone that occured at the leading edge of the principal pink zone, from which the red grease was obtained, may possibly have arisen from some free prodigiosin, but this was not investigated.

(18) At the time our original manuscript was submitted this compound had not been identified and the similarity in its properties with that which Efimenko, Kuznetsova, and Yakimov[19] claimed to be the hydrochloride was noted. Since then the nature of the compound has been established.[20]

(19) O. M. Efimenko, G. A. Kuznetsova, and P. A. Yakimov, *Biokhimiya*, **21**, 416 (1956), kindly translated by Dr. W. Graf.

(20) A. J. Castro, J. F. Deck, M. T. Hugo, L. R. Williams, and M. R. Zingg, *J. Org. Chem.*, **23**, 1232 (1958).

gested a proton exchange reaction involving the perchlorate and the alcohol, which functions as a Lewis base. In agreement with this, as shown in the table, in the presence of added perchloric acid, all of the solute is kept in the protonated form and the dilution effect is eliminated. Similar results were obtained with isopropyl alcohol as the solvent.

EXPERIMENTAL[21]

Bacterium growth. The organism, a substrain of the Stanford Z-4 strain, exhibited the characteristics described[22] for *Serratia marcescens*. Growth at room temperature on Lack's solid medium[23] showed good red pigmentation having a green metallic sheen. A modification of the Lack medium, wherein the quantity of beef extract was reduced to 1.5 g./l. and an additional 1.5 g./l. of Difco autolyzed yeast was added, also give satisfactory results. For large scale production the bacterium was grown on the medium contained in paper covered, 8 × 10 in. enamel Cesco photography trays. Alternately, the paper covers were removed and the trays stored in pairs in an upright and inverted fashion, so that the upper tray served as a cover for the lower one. Sterile apparatus and media were used. The bacterial growth was scraped from the medium 3–7 days after inoculation and stored in a refrigerator. Eighteen kg. of bacterial mud was obtained from 627 l. of medium.

Prodigiosin perchlorate. The perchlorate was prepared essentially by the method of Wrede and Hettche.[15] The yield of crude product averaged 1.8 g./kg. of bacterial growth. After further recrystallization, the purple solid when examined microscopically appears as clusters of red blades, m.p. 226.0–228.0° (dec.). Some difficulty was experienced in determining the decomposition point, for it is somewhat hard to judge. A decomposition point as high as 234.0–236.0° was recorded for one sample.

Anal. Calcd. for $C_{20}H_{26}O_5N_3Cl$: C, 56.67; H, 6.18; N, 9.91; Cl, 8.37. Found: C, 56.69; H, 6.31; N, 9.89; Cl, 7.89.

In the purification of this compound it was found necessary to remove the oily film that forms on the surface of the hot solution of the crude perchlorate. Furthermore, the perchlorate was found occasionally to be accompanied by a brown filamentous solid, which is apparent under a microscope. This can be removed through recrystallization.

Prodigiosin. (*a*) *Purification through chromatography on powdered sugar.* Two g. of prodigiosin perchlorate, m.p. 226–228° (dec.), in 350 ml. of 95% ethanol was treated with somewhat more 10% aqueous sodium hydroxide than that necessary to change the color of the mixture from deep red to orange-brown, and the mixture was extracted with ligroin (d, 0.67–0.69). The ligroin extract was washed with water, filtered, and applied to a column of California and Hawaiian confectioners powdered sugar, 9.5 cm. (diam.) × 65 cm. After development with ligroin several different colored zones were apparent preceding the principal zone which was pink in color and occupied the major portion of the column. An orange eluate was collected. The principal pink zone was removed, sucked dry on a Büchner funnel and the orange filtrate was combined with the orange eluate from the column. The resulting solution was concentrated and applied to a second column of powdered sugar. Evaporation of the orange eluate which was collected yielded an orange oil having a distinct green sheen. The oil crystallized and after two recrystallizations from ligroin, prodigiosin was obtained as red crystals showing a little green reflex, m.p. 151.5–152.9° (dec.).

TABLE I

ABSORPTION MAXIMUM FOR PRODIGIOSIN PERCHLORATE

No.	Solvent	Perchlorate Conc. mg./100 ml.	λ_{max} mμ	ϵ_{max}
1	95% EtOH	0.64	536–538	6.5×10^4[a]
2		0.400	536–538	5.6[a]
3		0.107	536–538	2.6[a]
4	Acidified 95% EtOH	0.604	537	11.6[b,d]
5		0.400	538	11.4[b,d]
6		0.400	538	11.8[b,d]
7		0.200	537	11.4[b,d]
			Avg.	11.6 × 10^4
8		0.63	536	11.1[c,e]
9		0.400	538	11.4[c,d]
10		0.106	538	11.2[c,e]
			Avg.	11.2 × 10^4
11	i-PrOH	0.624	542	10.2
12		0.497	542	9.7
13	Acidified i-PrOH	0.624	540	11.5[c,f]

[a] Average of two measurements. [b] Perchlorate dissolved in solvent containing perchloric acid. [c] Perchlorate dissolved in solvent followed by addition of perchloric acid. [d] 1.00 ml. of 0.0829*M* $HClO_4$ per 100 ml. solution. [e] 0.99 ml. of *ca.* 1*M* $HClO_4$ per 100 ml. solution. [f] 1.00 ml. of *ca.* 1*M* $HClO_4$ per 100 ml. solution.

The perchlorate was prepared by addition of 5% aqueous perchloric acid to a hot 95% ethanol solution of prodigiosin, boiling the mixture for a short time and allowing it to stand. Red crystals deposited which after one recrystallization from a mixture of 95% ethyl alcohol and 5% aqueous perchloric acid melted at 235.0–236.0° (dec.). The product gave a positive Beilstein test.

Anal. Calcd. for $C_{20}H_{26}O_5N_3Cl$: N, 9.91. Found: N, 9.56.

The ultraviolet-visible absorption spectrum for this compound is the same as that for authentic prodigiosin perchlorate.

The dye which remained absorbed after the main pink zone from the first chromatogram had been sucked dry was eluted with chloroform and the solvent evaporated. The portion of the residue that was insoluble in ligroin was successively crystallized from 95% ethyl alcohol and a mixture of benzene and iso-octane. This yielded 0.0333 g. of prodigiosin hydrochloride,[20] a magenta colored solid, m.p. 146.9–151.2° (dec.). Recrystallization from a mixture of benzene and iso-octane gave a product which melts with decomposition at 150.0–150.5°.

(*b*) *Magnesium oxide purification.* A 0.532 g. sample of prodigiosin perchlorate, m.p. 226.8–229.1° (dec.), in 95% ethyl alcohol was decomposed with sodium hydroxide as before. The resulting water washed, sodium sulfate dried petroleum ether extract of crude prodigiosin was concentrated and stirred with magnesium oxide. The discolored magnesium oxide was removed by filtration, rinsed with solvent, and the combined petroleum ether filtrates were concentrated. The bright red solution that remained was allowed to evaporate in the dark yielding 0.2169 g. of red crystals, m.p. 152.0–153.0° (dec.). From the mother liquor after further treatment with magnesium oxide, a second crop of crystals weighing 0.0672 g. (combined yield 70%), m.p.

(21) Melting points were taken with a Fisher-Johns apparatus, and are uncorrected. Ultraviolet-visible absorption spectra were measured with a Beckman Model DU Spectrophotometer or a Cary Model 11M. The latter was made available to us through the courtesy of Professor William Mansfield Clark, to whom we are indebted. Infrared spectra were determined with a Model 21 Perkin-Elmer Spectrophotometer using a sodium chloride prism.

(22) E. S. Breed, E. G. D. Murray, and A. P. Hitchens, *Bergey's Manual of Determinative Bacteriology*, The Williams and Wilkens Co., Baltimore, 6th ed., 1948, p. 479.

(23) A. Lack, *Proc. Soc. Exptl. Biol. Med.*, **72**, 656 (1950).

150.1–152.0° (dec.), was obtained. A mixture melting point of prodigiosin purified in this fashion with that from a powdered sugar chromatogram showed no depression.

A sample of crude prodigiosin obtained from the Parke, Davis Co. was purified similarly and after crystallization from iso-octane melted at 151.0–152.5°. The infrared spectra for this and the preceding product are the same as that from powdered sugar.

Anal. Calcd. for $C_{19}H_{22}N_3(OCH_3)$: C, 74.27; H, 7.79; N, 12.99; OCH_3, 9.60. Found: C, 74.36, 74.42; H, 7.59, 7.89; N, 12.65; OCH_3, 9.55.

(*c*) *Adsorption on alumina.* Prodigiosin, m.p. 150.2–152.1° (dec.) was treated essentially according to the procedure described by Morgan and Tanner[6] for the isolation of the pure compound. Aluminum Co. of America activated alumina, grade F-20, was used as an adsorbent. The product, m.p. 152.3–153.7° (dec.), was dark red and exhibited considerable green reflux. A mixture melting point with authentic prodigiosin showed no depression.

(*d*) *Isolation from the alkali digestion product from Serratia marcescens.* Following substantially the Lack and Botts[24] modification of Wrede and Hettche's procedure,[15] 1181 g. of bacterial mud was digested overnight with approximately an equal volume of 10% sodium hydroxide. A volume of 95% ethanol equal to that of the alkali was added and the mixture was extracted repeatedly with ligroin. The combined, water washed ligroin extracts were concentrated, and upon standing a brown gelatinous mass precipitated. This was moved by filtration, dried and stored. When examined about 34 months later, crystals of prodigiosin were apparent in the solid. The mixture was extracted with cyclohexane in a Soxhlet apparatus. The extract was evaporated and prodigiosin, m.p. 151.0–152.5° (dec.) was obtained from the residue *via* the magnesium oxide procedure. The infrared spectrum for this product agrees with that for prodigiosin obtained from the perchlorate.

(*e*) *Isolation from alkali digestion of magenta oil from Serratia marcescens.* Ten g. of the magenta oil, isolated from *Serratia marcescens*, as described below, was dissolved in 25 ml. of methanol. To this, 1.55 g. of sodium hydroxide dissolved in a little water and 15 ml. of methanol was added, followed by a 10 ml. methanol rinse. The mixture was stirred for an hour and shaken with an ether–petroleum ether–cyclohexane mixture. Sodium chloride was added to break the emulsion that formed and the hydrocarbon-ether solution was separated. The aqueous layer and the dark solid remaining were extracted with ether. The water washed, combined, sodium sulfate dried extracts were applied to a column of alumina (Woelm, non-alkaline, activity grade I) and the main scarlet band, after elution with ethyl alcohol and subsequent treatment with magnesium oxide as in the preceding, gave a dark semicrystalline mass. Upon the application of this in petroleum ether to a second column of alumina and development with ethyl alcohol the principal zones were orange and red with the former above the latter. As these moved down the column the region occupied by the latter decreased. The two were eluted with alcohol and the product from the eluate after treatment with magnesium oxide as before yielded 0.0407 g. of prodigiosin, m.p. 150.3–152.0° (dec.). The infrared spectrum for this product was the same as that for the product from the powdered sugar chromatogram.

Molecular weight of prodigiosin. The Signer apparatus, mounted in a constant temperature water bath held at 30°, was rotated slowly so that the solutions alternately filled the burets and the corresponding bulbs. Volumes were read periodically until constant. Equilibrium was reached with the following benzene solutions: 2.194 mg. of prodigiosin per 0.7512 ml. and 1.309 mg. of fluorenone per 1.2937 ml., 3.519 mg. of prodigiosin per 0.8080 ml. and 3.294 mg. of 2,2′-(3,3′,5,5′-tetramethyl-4,4′-dicarbethoxy)dipyrrylmethene per 1.1848 ml. From the first experiment, the molecular weight for prodigiosin is 520 and the equilibrium constant is 1.4×10^{-3}; from the second, 540 and 1.3×10^{-3}.

Solvent extract of Serratia marcescens. Repeated extraction of 4.9 kg. of the bacterial growth with cold 95% ethyl alcohol followed by evaporation of the extracts at reduced pressure yielded 186 g. of a viscous red liquid. To a solution of this in 800 ml. of 95% ethyl alcohol, an equal volume of ligroin was added followed by 300 ml. of water. This caused the separation of a dark ligroin layer, a dark aqueous alcohol layer, and a dark solid. The aqueous alcohol layer and dark solid were extracted together repeatedly with ligroin, the dark insoluble solid was removed, and the extraction of the aqueous alcohol layer continued until successive extracts showed only a small, if any, difference in color. The combined, water washed ligroin extracts were evaporated at diminished pressure and the oily residue, 96.3 g., was stored in a refrigerator. After three days, 7.8 g. of a dark red oily solid had precipitated. Upon further standing in the cold an additional 2.8 g. of a red solid precipitated and a magenta oil remained. The latter was noted to contain a little water.

Crude fractions of palmitic acid were isolated from the red oily solid through a series of operations involving successive crystallizations, using petroleum ether as the most common solvent, chromatography of the residual oil and subsequent crystallization of the red oil which was isolated from the chromatogram. In the chromatography step, powdered sugar was used as an adsorbent and a 2:1 mixture[25] of petroleum ether and ethyl ether was used to develop the column and elute the red fraction. Crude portions of the acid were also isolated from the magenta oil following chromatography of one portion on powdered sugar and another on celite (Analytical Filter Aid), in a manner like that of the foregoing case, followed by successive crystallizations of the resulting red oil obtained from these, and repetition of these steps. The crude fractions of palmitic acid were combined, decolorized with charcoal in methanol, and crystallized from aqueous methanol. The purified acid was obtained as shiny white crystals, m.p. 62.3–63.7°. The second red solid, wt. 2.8 g., that had precipitated from the ligroin soluble portion of the bacterial extract was similarly decolorized and crystallized yielding an additional quantity of the acid, m.p. 63.1–63.9°.

Anal. Calcd. for $C_{16}H_{32}O_2$: C, 74.94; H, 12.58; Neut. Eq., 256. Found: C, 74.94; H, 12.52; Neut. Eq., 258.

A mixture melting point with an authentic sample of palmitic acid showed no depression and the two samples show identical infrared absorption spectra.

The red aqueous alcohol solution obtained in the initial solvent fractionation of the bacterial extract was concentrated at reduced pressure and a red tar, 53.6 g., mixed with a brown aqueous solution remained. Through a process of crystallization from aqueous ethyl alcohol, mechanical separation, decoloration with charcoal in ethyl alcohol, and recrystallization from aqueous ethanol 0.53 g. of white needles, m.p. 154.3–156.0°, were isolated from 10 g. of the red tar.

Anal. Calcd. for $C_{24}H_{42}O_7N_2$: C, 61.25; H, 9.00; N, 5.95. Calcd. for $C_{24}H_{44}O_7N_2$: C, 60.99; H, 9.38; N, 5.93. Found: C, 61.00, 61.25; H, 9.08, 9.13; N, 5.39, 6.11.

Mol. wt. Calcd. for $C_{24}H_{42}O_7N_2$: 471. Calcd. for $C_{24}H_{44}O_7N_2$:473. Found[26] (Rast; camphor): 460, 477.

In another experiment, the ethyl alcohol extract of the bacterium was transferred to cyclohexane and the cyclohexane solution was applied to a column of powdered sugar. The main pink zones from this and a similar column after elution with chloroform or benzene were combined and after evaporation a red oil remained. A ligroin solution of the oil

(24) A. Lack and E. D. Botts, private communication.

(25) R. P. Williams, J. A. Green, and D. A. Rappoport, *J. Bacteriol.*, **71**, 115 (1956).

(26) G. Weiler and F. B. Strauss, 164, Banbury Road, Oxford, England.

applied to a column of powdered sugar gave a principal dark pink colored zone. The center portion of the dark pink zone was eluted with chloroform and a red grease having a green metallic sheen remained after removing the solvent. Qualitative analysis of the product like this obtained from another experiment showed that it contained nitrogen; sulfur and halogen were absent. Treatment of the red grease in 95% ethyl alcohol with perchloric acid in the described fashion gave prodigiosin perchlorate, m.p. 224.5–226.0° (dec.).

Anal. Calcd. for $C_{20}H_{26}O_5N_3Cl$: Cl, 8.37. Found: 8.40.

The ultraviolet-visible absorption spectrum for the perchlorate is the same as that for authentic prodigiosin perchlorate.

Acknowledgment. We are grateful to Mrs. J. C. Kurtz, Mr. M. Bilitch, Dr. A. E. Blood, Mr. D. K. Fisher, Mr. J. Walter, and Dr. George C. Kleinspehn for assistance with certain phases of this investigation and to Mr. Martin Black of the Parke, Davis Co. for a supply of prodigiosin.

SANTA CLARA, CALIF.

[CONTRIBUTION FROM THE WELLCOME RESEARCH LABORATORIES]

Unsymmetrically Substituted Piperazines. XII.[1] Benzhydrylpiperazines and Related Compounds with Spasmolytic and Anti-Fibrillatory Action[2]

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Received October 17, 1958

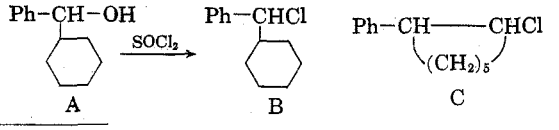
In a study of compounds showing activity against artificial fibrillation, a number of *o*-substituted benzhydrylpiperazines and related benzhydrylamines have been prepared.

In the course of work on piperazines having spasmolytic action, it was found that monoquaternary salts of *ortho*-substituted benzhydrylpiperazines[3] and of partially reduced benzhydrylpiperazines[4] had strong atropine-like action. A number of the ditertiary bases intermediate to these quaternary salts were also submitted to pharmacological screening. Some of these, especially those having *o*-alkyl substitution in the benzhydryl moiety, showed considerable activity in suppressing artificial fibrillation in experimental animals.[5]

In following up this lead we prepared the series of piperazine derivatives, data on which are presented in Table I. Since, *a priori*, it could not be assumed that the observed activity was dependent on a piperazine portion or indeed on any single feature of this type of substance, there were also prepared for comparison a number of benzhydrylamines, substituted ethylenediamines, *etc.* Many of these have already been reported from other sources but some appear to be new compounds. Their physical properties are shown in Table II and in the experimental part. The physiological studies showed that in fact the anti-fibrillatory action was not a function of the piperazine moiety although favorably influenced thereby. The critical requirements seem to be those of an antihistaminic. Activity is augmented, however, by *ortho*-substitution in the benzhydryl moiety.

The quaternary salts, data on which are presented in Table III, were largely prepared as spasmolytics. Compounds XXX, XXXIII, and XXXV have anti-cholinergic activities on isolated tissue of the same general order as atropine. Compounds XXVII–XXIX were tested for anthelmintic activity in mice against *Syphacia obvelata*;[6] of these the most active was XXVIII.

The quaternary salts XXX–XXXVI were prepared by quaternization of hexahydrobenzhydryl-*N'*-methylpiperazine[7] or of Compound III with the appropriate alkyl iodide, usually in acetone. The preparation of these ditertiary bases was initially rather troublesome, resulting in poor yields and leading to a search for an alternate route of synthesis.[4,8] Because of these poor yields and the apparent impurity of the bases as initially obtained, it was suspected that hexahydrobenzhydryl chloride might undergo a rearrangement either in formation from the carbinol, on refluxing with thionyl chloride, or in reaction with *N'*-alkyl piperazine in the sense:



(1) Paper XI in this series, M. Harfenist and E. Magnien, *J. Am. Chem. Soc.*, **80**, 6257 (1958).

(2) The work reported here is part of a joint research carried out in cooperation with the Pharmacology Department of these laboratories.

(3) R. Baltzly, W. S. Ide, and E. Lorz, *J. Am. Chem. Soc.*, **77**, 4809 (1955).

(4) P. B. Russell and R. Baltzly, *J. Am. Chem. Soc.*, **77**, 629 (1955).

(5) C. H. Ellis, *Ann. N. Y. Acad. Sci.*, **64**, 552 (1956); C. H. Ellis and L. N. Sivertsen, *Arch. intern. pharmacodynamie*, **116**, 17 (1958).

(6) H. W. Brown, K. L. Hussey, K. F. Chan, M. Harfenist, R. V. Fanelli, and E. Magnien, in preparation.

(7) R. Baltzly, S. Dubreuil, W. S. Ide, and E. Lorz, *J. Org. Chem.*, **14**, 775 (1949).

(8) R. Baltzly, E. Lorz, P. B. Russell, and F. M Smith, *J. Am. Chem. Soc.*, **77**, 624 (1955).