Biosynthesis of Prodigiosin, a Secondary Metabolite of *Serratia marcescens*

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Prodigiosenes (prodigiosin and prodigiosin-like pigments) are known to be synthesized by only one genus of *Eubacteriales* and by two genera of *Actinomycetales*. Biosynthesis by *Serratia marcescens* occurs over a relatively narrow range of temperatures, although the bacteria grow over a broad range. When cultures of *S. marcescens* were incubated at 27 C in 1.0% casein hydrolysate, viable count and protein attained maximal values within 24 to 48 h, whereas prodigiosin did not reach a maximum until 96 h. The greatest amount of pigment was synthesized when cultures were in the senescent phase of growth. Suspensions of nonproliferating bacteria incubated at 27 C in only L-alanine also synthesized prodigiosin, although at a slower rate than growing cultures. Kinetics of growth for the wild-type, red *S. marcescens* and a white mutant were identical when incubated at 27 C, but the wild type produced abundant pigment. These results plus other data obtained from the literature suggest that prodigiosin is a secondary metabolite. The importance of this proposal to understanding the function of prodigiosin in *S. marcescens* is discussed.

Numerous microorganisms synthesize small-molecular-weight compounds that have no demonstrable function in the cells. Maximal production generally occurs after cellular multiplication has ceased, and, because the substances are not required for the primary metabolism of cellular growth and multiplication, they are called secondary metabolites (3). This term defines the compounds, but their classification according to chemical nature is a Sisyphean task. Although a specific secondary metabolite usually is produced by a single or a few species, bacteria and fungi synthesize an enormous variety, ranging from complex antimetabolites and antimicrobial agents, such as actinomycin and erythromycin, to simple aliphatic acids, such as itaconic (3). Thus, secondary metabolites are identified not by their chemistry but by the metabolic and taxonomic peculiarities of their formation.

In addition to the above characteristics, secondary metabolites can be recognized by the effect of physicochemical factors that are involved in their production, such as temperature, oxygen, and concentrations of phosphate and metallic ions (9, 33). Data presented in this paper show that the production of prodigiosin by *Serratia marcescens* has many of these characteristics and establish that this pigment should be considered a secondary metabolite.

**MATERIALS AND METHODS**

**Organisms and growth media.** The Nima strain of *S. marcescens* and a white mutant obtained from it by treatment with ultraviolet light were used. When grown aerobically at room temperature, strain Nima produced prodigiosin. The white mutant did not form prodigiosin even when grown in the presence of various other mutants that synthesize intermediates along the pathway for biosynthesis of the pigment (22, 37). Therefore, it was concluded that this white mutant not only was incapable of synthesizing prodigiosin but also could not produce any of the known intermediates in the pathway. Except for pigmentation, both Nima and the white mutant gave identical biochemical reactions that were typical of *S. marcescens*.

Inocula for both strains were prepared from stock cultures carried on tryptic soy agar (Difco) by inoculating bacteria into liquid minimal medium in test tubes. These cultures were grown at 27 C for 24 h without shaking, and then 0.1-ml portions of the cultures were inoculated into test tubes containing 10 ml of minimal medium. After growth without shaking at 27 C for 24 h, the bacteria were harvested by centrifugation and then washed three times with 0.86% saline buffered at pH 7.2 with 0.01 M phosphate buffer. The washed bacteria were slightly pink. For the experiments, 0.1 ml of suspension containing 10^8 washed bacteria/ml of buffered saline was inoculated into 10-ml portions of medium contained in 50-ml Erlenmeyer flasks. The flasks were closed with cotton plugs and were incubated without shaking for the desired period of time in a water bath that
maintained the proper temperature within ±0.5 C, as continuously monitored during the experiments by a recording thermometer.

Growth media. Liquid minimal medium (6) contained (wt/vol): 1% glycerol, 0.5% ammonium citrate, 0.05% magnesium sulfate, 1.0% dipotassium phosphate, 0.5% sodium chloride, and 0.005% ferric ammonium citrate. In some experiments, this medium was supplemented by the addition of 1.0% (wt/vol) casein hydrolysate (N-Z Case peptone, Sheffield Chemical, Norwich, N.Y.). Complete medium was prepared by adding (wt/vol): 0.2% casein hydrolysate and 0.1% yeast extract (Difco). Media were adjusted to pH 7.2 before autoclaving.

Preparation of cell suspensions in alanine. Nonpigmented cells of Nima were obtained by growing cultures without shaking for 72 h at 38 C in minimal medium. The nonpigmented cells were harvested by centrifugation, washed three times in buffered saline, resuspended to one-half the original volume of the culture in 0.85% saline, and then supplemented with 10 mg of l-alanine/ml, a concentration that will not permit multiplication of strain Nima when added to minimal medium (36). Amounts of 10 ml of this suspension were incubated at 27 C without shaking in 50-ml Erlenmeyer flasks. Pigment formation was determined during incubation for 7 days.

Analytical procedures. Protein was determined by the method of Lowry et al. (19) with bovine serum albumin as a standard. Prodigiosin was measured by a modification of the method of Hubbard and Rimington (17) in which the bacteria were digested by boiling with 1 N NaOH for 1 h in a water bath. Pigment was extracted from the digest with absolute ethanol, followed by a second extraction of the ethanolic solution with petroleum ether. The latter extract was dried in a boiling-water bath; the residue was dissolved in acidified ethanol (10 ml of ethanol plus 1 ml of 1 N HCl), and the solution then was read in a Beckman spectrophotometer, model DU, at 553 nm. Concentrations of pigment were calculated by use of the specific absorbancy for prodigiosin of 51.5 × 10^3 liter per g per cm (34).

Viable counts were determined by standard plate count procedures with the use of a complete medium (13) solidified with agar. After incubation at 27 C for 48 h, viable cells were counted. In counting, no distinction was made between surface or subsurface (pigmented or nonpigmented) colonies.

RESULTS

Generic distribution of prodigiosenes. The parent nucleus of prodigiosin and prodigiosin-like pigments was named prodigiosene (Fig. 1) by Hearn et al. (15). As shown in Table 1, pigments isolated and chemically identified as to structure all contain the prodigiosene nucleus and are closely related to one another. Only microorganisms of the class Schizomycetes are known to produce prodigiosenes. These species of bacteria occur in two orders, Eubacteriales and Actinomycetales.

Among the Eubacteriales, synthesis of prodigiosene is associated only with the genus _Serratia_, although Lewis and Corpe (18) reported production of pigment similar to prodigiosin by two unidentified marine bacteria. Two of the pigments, 2-methyl-3-amyl-6-hydroxyprodigiosene and 2-(2-pyryl)-4,6-dimethoxyprodigiosene, are synthesized by mutants of _S. marcescens_ and may not be produced by wild-type strains.

Prodigiosenes are synthesized by members of two families of _Actinomycetales_, _Actinomycetaceae_ and _Streptomycetaceae_. _Nocardia madurae_, _N. pelletieri_, and _Streptomyces longisporusruber_ each synthesize two pigments, one of which has a cyclic side chain attached to the prodigiosene nucleus. Ability to produce these cyclic compounds may be characteristic of species in the _Actinomycetaceae_. Pigments of lower molecular weight are produced by _S. marcescens_, whereas _N. madurae_, _N. pelletieri_, and _S. longisporusruber_ produce pigments of higher molecular weights.

Effect of incubation temperature upon biosynthesis of prodigiosin. As can be seen in Fig. 2, the Nima strain of _S. marcescens_ showed little variation in total growth, as measured by protein, over temperatures ranging from 16 to 32 C. Kinetic studies on these cultures showed that growth was slower at the lower temperatures, but after incubation for 5 days the total amount of protein was about the same for all temperatures between 16 and 32 C. On the other hand, biosynthesis of prodigiosin occurred over a narrower range, with maximal production being between 24 and 28 C.

Kinetics of prodigiosin biosynthesis. When incubated at 27 C, cultures of _S. marcescens_ produced only minimal amounts of prodigiosin during the logarithmic phase of growth. Maximal production of pigment occurred during the stationary phase, and the amount of pigment in the bacteria reached a

![Fig. 1. Chemical structure of prodigiosene (15), the parent nucleus of prodigiosin and prodigiosin-like pigments. Three carbon atoms in the bipyrrrole portion of the molecule and one in the monopyrrole are not numbered because substitutions on them would destroy the basic linear tripyrrole structure of prodigiosene.](image-url)
peak sometime after 5 days of incubation. These characteristics of production were enhanced in cultures growing in 1.0% (wt/vol) casein hydrolysate, as shown in Fig. 3. In this medium, little increase in viable count occurred after incubation for 24 h; protein synthesis began to level at about 48 h, but maximal biosynthesis of prodigiosin occurred between 48 and 96 h, after multiplication and growth had almost ceased. Prodigiosin is characteristically produced by senescent cells in the stationary phase of growth.

Production of prodigiosin by nonproliferating cells. Recently, my associates and I described a method by which prodigiosin biosynthesis could be induced in nonproliferating cells by addition of single amino acids (24, 35, 36). The most effective amino acids for induction were alanine, histidine, and proline, but aspartic acid, glutamic acid, hydroxyproline, ornithine, and serine also induced some pigmentation. Kinetics for synthesis by stationary cultures in the presence of L-alanine are shown in Fig. 4. Production showed a lag similar to that for growing cells (Fig. 3), but the maximal amount of pigment was produced later. Stationary cultures of nonproliferating cells produced less pigment than stationary growing cultures. During the time of pigment production, there was no increase in viable count or protein, establishing the fact that the cultures were nonproliferating.

Kinetics of growth in pigmented and nonpigmented cultures. Figure 5 compares growth of the wild-type strain Nima to that of a white mutant. The curves for increases in viable count and protein are almost superimposable. Prodigiosin biosynthesis by the wild-type strain followed a pattern similar to that shown in Fig. 3, although the maximal amount of pigment was less because this culture was grown in a medium containing only 0.1% casein hydrolysate. No difference in growth was evident between the pigmented and nonpigmented strains.

**DISCUSSION**

The data presented establish that prodigiosin and secondary metabolites have several characteristics in common. The pigment is a small-molecular-weight substance that is produced by only a few species of microorganisms. Growth of bacteria and biosynthesis of the pigment occur in two distinct phases. Bu'Lock
et al. (5) called these two phases the trophophase (nourishment phase), for the period of cellular growth, and the idiophase (individual phase), for the period when specific secondary metabolites peculiar to individual bacteria are produced. The two phases are clearly separated during production of prodigiosin by *S. marcescens* (Fig. 3). Biosynthesis of prodigiosin occurs over a narrower range of temperature than growth, and nonproliferating cells produce pigment. Since the kinetics for growth of pigmented and nonpigmented strains of *S. marcescens* are identical, prodigiosin has no metabolic function that is reflected in cellular multiplication.

In his review, Weinberg (33) cited several other characteristics that may distinguish secondary metabolites. For example, their synthesis by nonproliferating cells can be inhibited by glucose, by phosphate, and by chloramphenicol. These same substances inhibit biosynthesis of prodigiosin by nonproliferating cells of *S. marcescens* (1, 2, 36). Secondary metabolites may be located in the cell envelope of microorganisms, as is true of prodigiosin (23), although prodigiosin is not released into the medium, as is characteristic of many secondary metabolites. Biosynthesis of prodigiosin, in contrast to biosynthesis of cellular materials for growth, has a narrower tolerance for iron (29) and a narrower range of aeration (16). Nonproliferating cells synthesize the pig-

![Fig. 3. Kinetics of growth, as measured by viable count and protein, and prodigiosin formation in stationary cultures of Serratia marcescens incubated at 27 C in medium containing 1.0% (wt/vol) casein hydrolysate.](image)

![Fig. 4. Kinetics of prodigiosin formation in suspensions of nonproliferating Serratia marcescens incubated as stationary cultures at 27 C in 10 mg of L-alanine/ml. Determinations of protein and viable count are also shown.](image)
ment under alkaline conditions (36). Again, these conditions are characteristic for production of other secondary metabolites (33). In addition, prodigiosin is one member of a closely related family of molecular species, the prodigiosenes (Table 1). A characteristic of other secondary metabolites is their existence as members of closely related chemical families (33).

Secondary metabolites may arise by different biosynthetic pathways than primary metabolites (3, 4, 33). Several investigations indicated that the pyrrole groups of prodigiosin arise from amino acids (26, 27, 28, 35) and acetate (8), rather than through the succinate-glycine cycle, as is characteristic of the pyrrole groups of porphyrins, compounds that are involved in primary metabolism.

Both the present experiments and those of others provide evidence that prodigiosin is a secondary metabolite. The significance of this proposal lies not so much in characterizing prodigiosin as a secondary metabolite as in the implication the suggestion has regarding function of the pigment. Failure to establish a function for prodigiosin in *S. marcescens* has perplexed investigators (7, 21, 27), and Stavri and Marx (27) argued that if the pigment were of no use to the bacterium the ability to synthesize prodigiosin would have been lost. But, as Weinberg (33) pointed out, one hypothesis for the function of secondary metabolites (38) suggested that the process of formation is more important than the specific product. Formation of the substances by microbes no longer capable of dividing removes from the cellular milieu unused primary metabolites that might accumulate and become lethal for the cell. Secondary metabolism converts these potentially toxic metabolites into innocuous end products. Thus, secondary metabolism is of value because removal of the primary metabolites may prolong survival of the microorganisms. However, prodigiosin itself cannot be the compound that traps possible toxic materials, because a nonpigmented mutant maintains the same viable count over a period of incubation for 7 days as does the pigmented wild type (Fig. 5). Perhaps an early, colorless precursor not yet identified is the key compound. Investigations of various white mutants may reveal this substance.

Synthesis of secondary metabolites such as prodigiosin that have no demonstrable function in the bacteria also offers a paradox in which useful cellular macromolecules (genes and pro-
PRODIGIOSIN, A SECONDARY METABOLITE

Vol. 25, 1973

Fig. 6. Scheme of prodigiosin biosynthesis by Serratia marcescens. Two immediate precursors have been identified in mutants, MAP (2-methyl-3-amylpyrrole) and MBC (4-methoxy-2,2'-bipyrrrole-5-carboxyldihyde). Other classes of at least nine different mutants have been isolated in pathways leading to both MAP and MBC, but the nature of the intermediates has not been determined (22, 37).

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LITERATURE CITED

1. Angeles, L. T., R. P. Williams, and C. L. Gott. 1965. Effect of variation in medium on chloroquine inhibition of pigment production by Serratia marcescens. Acta Med. Philipp. 11(Ser. III):131-136.
2. Blizzard, J. L., and G. E. Peterson. 1963. Selective inhibition of proline induced pigmentation in washed cells of Serratia marcescens. J. Bacteriol. 86:1136-1140.
3. Bu'Lock, J. D. 1961. Intermediary metabolism and antibiotic synthesis. Advan. Appl. Microbiol. 3:293-342.
4. Bu'Lock, J. D. 1967. Essays in biochemistry and microbiological developments. John Wiley & Sons, Inc., New York.
5. Bu'Lock, J. D., D. Hamilton, M. A. Hulme, A. J. Powell, H. M. Smalley, D. Sheppard, and G. N. Smith. 1965. Metabolic development and secondary biosynthesis in Penicillium urticae. Can. J. Microbiol. 11:765-778.
6. Bunting, M. I. 1940. A description of some color variants produced by Serratia marcescens strain 274. J. Bacteriol. 40:57-68.
7. Carrara, G. 1963. I pigmenti batterici. Riv. Ist. Sieroteraf. Ital. 38:306-325.
8. Cushey, R. J., D. R. Anderson, S. R. Lipesky, R. J. Sykes, and H. H. Wasserman. 1971. Carbon-13 Fourier transform NMR spectroscopy. II. Pattern of biosynthetic incorporation of 1-13C and 2-14C acetate into prodigiosin. J. Amer. Chem. Soc. 93:6284-6286.
9. Demain, A. L. 1968. Regulatory mechanisms and the industrial production of microbial metabolites. Lloydia 31:395-418.
10. Gerber, N. N. 1969. Prodigiosin-like pigments from...
Actinomadura (Nocardia) pelletieri and Actinomadura madurae. Appl. Microbiol. 18:1-3.

11. Gerber, N. N. 1970. A novel, cyclic, tripodyle pigment from Actinomadura (Nocardia) madurae. Tetrahedron Lett., p. 809-812.

12. Gerber, N. 1971. Prodigiosin-like pigments from Actinomadura (Nocardia) pelletieri. J. Antibiot. (Tokyo) 24:636-640.

13. Goldschmidt, M. C., and R. P. Williams. 1968. Thiamine-induced formation of the monopyroyle moiety of prodigiosin. J. Bacteriol. 96:609-616.

14. Harashima, K., N. Tsuchida, T. Tanaka, and J. Nagatsu. 1967. Prodigiosin-25 C. Isolation and chemical structure. Agr. Biol. Chem. 31:481-489.

15. Hearn, W. R., M. K. Elson, R. H. Williams, and J. Medina-Castro. 1970. Prodigiosene [5-(2-pyrrrole)-2,2'-dipyrrylmethene] and some substituted prodigiosenes. J. Org. Chem. 35:142-146.

16. Heisenmann, B. A. J. Howard, and H. J. Palocz. 1970. Influence of dissolved oxygen levels on production of L-asparaginase and prodigiosin by Serratia marcescens. Appl. Microbiol. 19:800-804.

17. Hubbard, R., and C. Rimington. 1950. The biosynthesis of prodigiosin, the tripodylemethene pigment from Bacillus prodigiosus (Serratia marcescens). Biochem. J. 46:220-225.

18. Lewis, S. M., and W. A. Corpe. 1964. Prodigiosin-producing bacteria from marine sources. Appl. Microbiol. 12:13-16.

19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265-275.

20. McQuilten, K. 1965. The physical organization of nucleic acid and protein synthesis, p. 135-158. In M. R. Pollock and M. H. Richmond (ed.), Function and structure in microorganisms. Cambridge Univ. Press, London.

21. Marchal, J. G. 1958. Les bacteries chromogenses. J. Pharmaceutiques Francaises. Tech. Pharm. 5:(page numbers not provided).

22. Morrison, D. A. 1966. Prodigiosin synthesis in mutants of Serratia marcescens. J. Bacteriol. 91:1599-1604.

23. Purkayastha, M., and R. P. Williams. 1963. Association of pigment with the cell envelope of Serratia marcescens (Chromobacterium prodigiosum). Nature (London) 187:349-351.

24. Qadri, S. M. H., and R. P. Williams. 1972. Induction of prodigiosin after shift-down in temperature of nonproliferating cells of Serratia marcescens. Appl. Microbiol. 23:704-709.

25. Rapport, H., and K. G. Holden. 1962. The synthesis of prodigiosin. J. Amer. Chem. Soc. 84:635-642.

26. Shrimpton, D. C., G. S. Marks, and L. Bogorad. 1963. Studies on the biosynthesis of prodigiosin in Serratia marcescens. Biochim. Biophys. Acta 71:408-415.

27. Stavri, D., and A. Marz. 1961. Recherches sur le mechanism de synthèse de la prodigiosine par le Serratia marcescens. Arch. Rioum. Pathol. Exp. Microbiol. 20:287-294.

28. Tanaka, W. K., L. B. deMedina, and W. R. Hearn. 1972. Labelling patterns in prodigiosin biosynthesis. Biochem. Biophys. Res. Commun. 46:731-737.

29. Waring, W. S., and C. H. Werkman. 1943. Iron requirement of heterotrophic bacteria. Arch. Biochem. 1:425-433.

30. Wasserman, H. H., D. J. Friedland, and D. A. Morrison. 1968. A novel dipyrrolyldipyrrmethene prodigiosin analog from Serratia marcescens. Tetrahedron Lett. p. 641-644.

31. Wasserman, H. H., G. C. Rodgers, Jr., and D. D. Keith. 1966. The structure and synthesis of undecyldiprogiosin. A prodigiosin analogue from Streptomyces. Chem. Commun. p. 825.

32. Wasserman, H. H., G. C. Rodgers, and D. D. Keith. 1969. Metacyclodiprogiosin, a tripodyle pigment from Streptomyces longisporus ruber. J. Amer. Chem. Soc. 91:1263-1264.

33. Weinberg, E. D. 1970. Biosynthesis of secondary metabolites: roles of trace metals. Advan. Microbial Physiol. 4:1-44.

34. Williams, R. P., C. L. Gott, and J. A. Green. 1961. Studies on pigmentation of Serratia marcescens. V. Accumulation of pigment fractions with respect to length of incubation time. J. Bacteriol. 81:376-379.

35. Williams, R. P., C. L. Gott, and S. M. H. Qadri. 1971. Induction of pigmentation in nonproliferating cells of Serratia marcescens by addition of single amino acids. J. Bacteriol. 106:444-448.

36. Williams R. P., C. L. Gott, S. M. H. Qadri, and R. H. Scott. 1971. Influence of temperature of incubation and type of growth medium on pigmentation in Serratia marcescens. J. Bacteriol. 106:438-443.

37. Williams, R. P., and W. R. Hearn. 1967. Prodigiosin, p. 410-432. In D. Gottlieb and P. D. Shaw (ed.), Antibiotics, vol. 2. Springer-Verlag, Berlin.

38. Woodruff, H. B. 1966. The physiology of antibiotic production: the role of the producing organism, p. 22-46. In B. A. Newton and P. E. Reynolds (ed.), Biochemical studies of antimicrobial drugs. Cambridge Univ. Press, London.