BBA 3997

STUDIES ON THE BIOSYNTHESIS OF PRODIGIOSIN IN *SERRATIA MARCESCENS*

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(Received July 30th, 1962)

SUMMARY

Prodigiosin, the blood-red pigment produced by *Serratia marcescens*, is a linear tripyrrole. Through a series of isotope incorporation experiments, it has been possible to show that this pigment, although a pyrrolic compound, is not closely related to the porphyrins biosynthetically. It has been demonstrated that δ-aminolevulinic acid, a specific precursor of porphyrins, is capable of penetrating the membrane of *Serratia marcescens* but is not used in the formation of prodigiosin.

The possibility that prodigiosin may be derived from four proline units has been studied and found to be untenable. The carboxyl carbon of proline is probably incorporated, as a single carbon unit, into the dipyrrolic precursor of prodigiosin produced by *Serratia marcescens* mutant 9-3-3. Incorporation experiments utilizing [^{14}C]proline (uniformly labeled), [1-^{14}C]proline, and [2-^{14}C]proline into both prodigiosin and its dipyrrolic precursor, indicate that C-2, and perhaps the entire ring, of proline is utilized mainly in the formation of the 2-methyl-3-amylpyrrole part of prodigiosin. Hydroxyproline is not utilized in the formation of ring II of prodigiosin.

On the basis of [2-^{14}C]glycine incorporation into both prodigiosin and its dipyrrolic precursor, it is apparent that the methyl carbon atom of glycine is incorporated with equal efficiency into both halves of the prodigiosin molecule. It is, however, probable that rings I and II are formed by a different mechanism than ring III.

INTRODUCTION

In 1950 HUBBARD AND RIMINGTON proposed that the tripyrrolic pigment prodigiosin is synthesized by *Serratia marcescens* via the same general metabolic pathway as the porphyrins[1]. This proposal stemmed from the observations that both carbon atoms of acetate and also the α-carbon and amino nitrogen atoms of glycine were incorporated into prodigiosin by *S. marcescens* as well as into porphyrins in other systems. It is now known that in the synthesis of porphyrins δ-aminolevulinic acid, the decarboxylated condensation product derived from glycine and succinyl-CoA, is the immediate precursor of the monopyrrole porphobilinogen. The tetrapyrrolic porphyrin nucleus is

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formed from four porphobilinogen units. MARKS AND BOGORAD[2] have recently re-investigated prodigiosin biosynthesis and have demonstrated that radio-carbon from δ-[5-^{14}C]aminolevulinic acid is not incorporated into the *S. marcescens* pigment but that radioactivity from [$^{14}C_5$]proline is introduced. Thus the biosynthesis of the pyrroles of prodigiosin now appears to be distinct from that of porphobilinogen and porphyrins.

Since the observations of MARKS AND BOGORAD on prodigiosin biosynthesis, ideas on the structure of prodigiosin have been revised. The branched tripyrrylmethene structure proposed by WREDE AND ROTHAUS[3] in 1934 has now been rejected in favor of a linear arrangement of the three pyrrole rings[4]. This recent structural information has been due in part to the existence of mutants of *S. marcescens* which accumulate prodigiosin precursors. One Serratia mutant, designated 9-3-3, has been shown to produce a dipyrrolic precursor of prodigiosin[5]. This dipyrrole is condensed enzymically with 2-methyl-3-amylpyrrole to produce prodigiosin in the living organism[6]. The structures of this dipyrrolic precursor and of prodigiosin are given in Fig. 1.

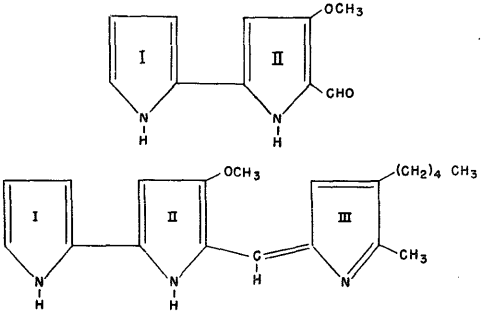

Fig. 1. (a) The dipyrrolic precursor of prodigiosin produced by *S. marcescens* mutant 9-3-3 (see ref. 4). (b) Prodigiosin[3].

The major portion of the present report is a further investigation of the utilization of proline, specifically of carbon atoms 1 and 2 of this amino acid, as well as of ^{14}C from glycine, and other selected compounds for the biosynthesis of prodigiosin by *S. marcescens*. The incorporation of ^{14}C from proline and glycine into the dipyrrolic precursor of prodigiosin produced by *S. marcescens* strain P-1 has been studied. Data are also included on the penetration of δ-aminolevulinic acid into *S. marcescens* and on the utilization of L- but not D-proline for prodigiosin formation.

EXPERIMENTAL

[2-^{14}C]Glycine, L-[$^{14}C_5$]proline, DL-[1-^{14}C]proline, DL-[2-^{14}C]glutamic acid, DL-[2-^{14}C]-ornithine, and DL-[2-^{14}C]hydroxyproline were purchased from the Volk Radiochemical Company. The compound δ-[4-^{14}C]aminolevulinic acid was purchased from the French Atomic Energy Research Institute, Saclay (France).

DL-[2-^{14}C]Proline was prepared from DL-[2-^{14}C]ornithine by the method of

HAMILTON[7]. The product was found to be identical with authentic [^{14}C]proline (Volk Radiochemical Company) when co-chromatographed in either a butanol–acetic acid–water (5:1:2) or a phenol–water (100:40, w/v) system using Whatman No. 1 paper.

A culture of *S. marcescens* was kindly supplied by Dr. J. MOULDER, Department of Microbiology, The University of Chicago.

In the experiments performed to study the utilization of [2-^{14}C]glycine and δ-[4-^{14}C]aminolevulinic acid in the synthesis of prodigiosin, the wild type cells were grown on the solid synthetic medium developed by WILLIAMS *et al.*[8]. When proline, hydroxyproline, ornithine, or glutamic acid labeled with ^{14}C was supplied as a possible substrate for prodigiosin biosynthesis, a mixture of amino acids in the same proportions as they occur in casein hydrolyzate was supplied *in lieu* of casein hydrolyzate.

After five days at 20° the cells were harvested from the agar in 0.9% saline. The cells were first disrupted by repeated freezing and thawing. Following this, 4 N NaOH was added to the broken-cell suspension until the color changed from red to orange. The prodigiosin was then extracted with petroleum ether (b.p. 30–60°). The emulsion formed was broken by the addition of small amounts of 95% ethanol. More pigment was recovered after the cells had been frozen and thawed than when this step was omitted. The pigment was next purified to constant specific activity in the manner outlined by MARKS AND BOGORAD[2].

We are grateful to Dr. U. V. SANTER of the Department of Microbiology, Yale University, for a culture of Rizki's P-1 mutant of *S. marcescens*[9]. This mutant is the same as the 9-3-3 mutant[6] insofar as both mutants produce the same dipyrrolic prodigiosin precursor. The P-1 mutant was grown in carboys of ten-liter capacity, each containing three liters of the synthetic medium developed by BUNTING[10]. Radioactive substrates were sterilized by filtration and added to the medium at the time of inoculation. Liquid cultures were aerated vigorously; this was required for dipyrrole synthesis by this mutant.

After five days of growth the dipyrrole was recovered from the culture medium by the chloroform extraction method of SANTER AND VOGEL[5] and was purified by recrystallization from absolute ethanol. The dipyrrole was assayed using the molar extinction co-efficient ($3.5 \cdot 10^4$ at 363 mμ in ethanol) determined by SANTER AND VOGEL. The radioactivity of an "infinitely thin" sample was determined in a windowless gas flow counter (39% efficient). The dipyrrole was repeatedly recrystallized until constant specific activity was achieved. All samples were counted to ± 5%.

The amount of pigment formed by Serratia cultures varied from time to time even under identical conditions (*e.g.* see Expts. 1 and 2 of Table I). The reasons for this behavior are not known to us. In the present studies in which comparisons were to be made of the utilization of different possible substrates, the labeled compounds were incorporated into media which were then inoculated with equal aliquots from a single culture. The newly inoculated cultures in each experiment were maintained under identical conditions for the same period of time until harvest.

RESULTS

The incorporation of δ-aminolevulinic acid into prodigiosin

The observation of MARKS AND BOGORAD[2] that δ-aminolevulinic acid is not utilized during the synthesis of prodigiosin was confirmed. 50 mg of δ-[4-^{14}C]aminolevulinic

acid (specific activity $3.01 \cdot 10^5$ counts/min/mg carbon), containing approximately seven times the radioactivity used by MARKS AND BOGORAD[2], was supplied to a culture of wild-type cells. The prodigiosin which was recovered had a specific activity of $2.50 \cdot 10^3$ counts/min/mg carbon. Thus, the utilization of this compound for prodigiosin synthesis was approx. 0.1 %.

In an attempt to determine whether the failure of *S. marcescens* to employ δ-aminolevulinic acid for prodigiosin formation might simply result from the impermeability of the cells to the acid, the utilization of carbon atom four of δ-aminolevulinic acid for the synthesis of compounds other than prodigiosin was investigated. *S. marcescens* was grown on a medium containing δ-[4-^{14}C]aminolevulinic acid. The cells were washed with saline five times prior to pigment extraction. Following extraction of the pigment, the petroleum ether-insoluble residue, including broken cells, was concentrated to approx. 50 ml total volume by vacuum distillation. The cell envelopes, etc. were removed from the concentrated solution by centrifugation at 1000 × *g* for 30 min. Concentrated hydrochloric acid was added to the supernatant solution until it was 6 N with respect to the acid. It was then heated for 1 h at 100°, filtered, and neutralized with NaOH. Desalting was accomplished by means of the ion-exchange technique of PIEZ *et al.*[11]. The desalted hydrolyzate was evaporated to dryness and chromatographed on Whatman 3MM paper in a butanol–acetic acid–water (5:1:2) system. Six radioactive spots were observed. These materials were eluted, and each appeared homogeneous on rechromatography. One of the compounds behaved as did δ-aminolevulinic acid in both phenol and butanol chromatographic systems using Whatman No. 1 paper. Attempts to identify the other five compounds through a study of their behavior in various chromatographic systems and their ultraviolet absorption spectra yielded no conclusive results, although three of these spots developed a blue color with ninhydrin.

The incorporation of radiocarbon from [^{14}C]proline into prodigiosin and its dipyrrolic precursor

[$^{14}C_5$]*Proline*: The results of studies on the incorporation into prodigiosin of L-[$^{14}C_5$]proline, [1-^{14}C]proline and [2-^{14}C]proline are summarized in Table I. The

TABLE I

THE INCORPORATION OF [$^{14}C_5$]PROLINE, [1-^{14}C]PROLINE AND [2-^{14}C]PROLINE INTO PRODIGIOSIN

Experiment No.	*Substrate*	*(mg)*	*Total activity (counts/min)*	*Specific activity of substrate (counts/min/mg carbon)*	*Specific activity of prodigiosin (counts/min/mg carbon)*	*Utilization (%)*
1a	L-[$^{14}C_5$]Proline	44	$4.40 \cdot 10^6$	$2.34 \cdot 10^5$	$1.58 \cdot 10^4$	6.8
b	L-[1-^{14}C]Proline	44	$2.30 \cdot 10^6$			
	D-[1-^{14}C]Proline	1	$2.30 \cdot 10^6$	$1.00 \cdot 10^5$	$2.40 \cdot 10^4$	24
c	L-[2-^{14}C]Proline	50	$1.00 \cdot 10^6$			
	D-[2-^{14}C]Proline	1	$1.00 \cdot 10^6$	$3.89 \cdot 10^5$	$9.57 \cdot 10^3$	24
2a	L-[1-^{14}C]Proline	44	$2.30 \cdot 10^6$			
	D-[1-^{14}C]Proline	1	$2.30 \cdot 10^6$	$1.00 \cdot 10^5$	$6.61 \cdot 10^3$	7
b	L-[2-^{14}C]Proline	50	$2.35 \cdot 10^6$			
	D-[2-^{14}C]Proline	1	$2.35 \cdot 10^6$	$9.01 \cdot 10^4$	$6.07 \cdot 10^3$	7
c	[1-^{14}C]Proline	25	$2.30 \cdot 10^6$			
	[2-^{14}C]Proline	25	$2.35 \cdot 10^6$	$1.78 \cdot 10^5$	$1.08 \cdot 10^4$	6

data from investigations on the utilization of these same three substrates in the formation of the dipyrrolic precursor of prodigiosin are given in Table II.

The data presented in Table I indicate that [$^{14}C_5$]proline is incorporated into prodigiosin with about 6.8% efficiency*. For dipyrrole synthesis, however, L-[$^{14}C_5$]proline is used with only 3.1% efficiency (Table II).

TABLE II

THE INCORPORATION OF [$^{14}C_5$]PROLINE, [1-^{14}C]PROLINE, AND [2-^{14}C]PROLINE INTO THE DIPYRROLIC PRECURSOR OF PRODIGIOSIN

Experiment No.	*Substrate*	*mg*	*Total activity (counts/min)*	*Specific activity of substrate (counts/min/mg carbon)*	*Specific activity of dipyrrole (counts/min/mg carbon)*	*Utilization (%)*
3a	L-[$^{14}C_5$]Proline	1305	$3.02 \cdot 10^7$	$4.44 \cdot 10^4$	$1.39 \cdot 10^3$	3.1
b	L-[1-^{14}C]Proline	1305	$1.55 \cdot 10^7$			
	D-[1-^{14}C]Proline	2	$1.55 \cdot 10^7$	$2.27 \cdot 10^4$	$8.17 \cdot 10^3$	36
c	L-[2-^{14}C]Proline	1305	$1.20 \cdot 10^7$			
	D-[2-^{14}C]Proline	2	$1.20 \cdot 10^7$	$1.77 \cdot 10^4$	$1.00 \cdot 10^2$	0.3

It was of interest to determine whether the portion of the dipyrrole derived at least in part from proline was incorporated into prodigiosin. No mutant capable of cross-feeding with the P-1 mutant to produce prodigiosin was available; consequently, the incorporation of ^{14}C-labeled dipyrrole produced by *S. marcescens* mutant P-1 into prodigiosin by wild-type cultures was studied.

The P-1 mutant of Serratia was grown in liquid peptone–glycerol medium[5] containing [^{14}C]proline. The dipyrrole which was recovered had a specific activity of $1.58 \cdot 10^3$ counts/min/μmole. It was sterilized by filtration and then added, in a small amount of ethanol, to unsolidified agar prepared with WILLIAM's synthetic medium[2] in a 5-l diptheria toxin bottle. The vessel was then inoculated with wild-type cells of *S. marcescens* and harvested after five days, as described above. The prodigiosin recovered from this culture had a specific activity of $4.90 \cdot 10^2$ counts/min/μmole. It is thus apparent that the portion of the dipyrrole molecule derived from proline is incorporated into prodigiosin by wild-type cells. The dilution of the isotope observed in the above experiment is most probably due to endogenous substrates in the wild-type organism.

[1-^{14}C] and [2-^{14}C]proline: The incorporation of carbon atoms 1 and 2 from proline into both prodigiosin and its dipyrrolic precursor was studied in an attempt to determine which portions of the proline molecule were utilized in prodigiosin biosynthesis.

It is evident from the data presented in Table I that radio-carbon from [1-^{14}C]-proline is incorporated into prodigiosin with less dilution than isotope from uniformly labeled proline. This difference might be accounted for in one of two ways: First, the carboxyl carbon atom of proline might be used in prodigiosin biosynthesis more directly than the remainder of the amino acid. Or, second, since the carboxyl-labeled

* "Efficiency of utilization," expressed as per cent, is the ratio of the specific activity of the product (*e.g.* prodigiosin or its dipyrrolic precursor) to the specific activity of the compound supplied to the culture (*i.e.* the substrate) in any particular experiment times 100. This value is thus also the reciprocal of the dilution of the radioactivity of the substrate.

proline used in the incorporation experiments was a racemic mixture, D- as well as L-proline might be used in the synthesis of prodigiosin.

To test the latter possibility, each of two cultures was grown on a medium containing 41 mg of L-proline (specific activity $2.11 \cdot 10^5$ counts/min/mg carbon). One of the cultures contained, in addition to the L-proline, 40 mg of D-proline. It was found that the prodigiosin formed had approximately the same specific activity in both experiments. D-Proline, therefore, does not appear to be utilized in prodigiosin synthesis. The results of these experiments are summarized in Table III. In view of this observation, the percentage utilization, in cases where racemic mixtures are supplied as substrates, is expressed in terms of the amount of the L-form alone. It is still unclear why radio-carbon from [$^{14}C_5$]proline is incorporated into prodigiosin with greater dilution than that from [1-^{14}C]proline.

TABLE III

THE NON-UTILIZATION OF D-PROLINE FOR PRODIGIOSIN SYNTHESES

Experiment No.	*Substrate*	*mg*	*Total activity (counts/min)*	*Specific activity of substrate (counts/min/mg carbon)*	*Specific activity of prodigiosin (counts/min/mg carbon)*	*Utilization (%)*
4a	L-[^{14}C]Proline	40	$4.40 \cdot 10^6$	$2.11 \cdot 10^5$	$7.72 \cdot 10^3$	3.8
b	L-[^{14}C]Proline + D-proline	40	$4.40 \cdot 10^6$	$2.11 \cdot 10^5$	$8.05 \cdot 10^3$	3.8

The data presented in Table I indicate that the carboxyl and the number two carbon atoms of proline are utilized with approximately equal efficiency for prodigiosin biosynthesis. However, carbon atom two of proline is not used appreciably in the formation of the dipyrrole, whereas the carboxyl carbon atom is so used (Table II). Furthermore, when [1-^{14}C]proline and [2-^{14}C]proline are added to the same culture the incorporation observed is approximately equal to the sum of the isotope incorporated into prodigiosin by two cultures, each of which is supplied with one of the isotopically labeled prolines.

The incorporation of [2-^{14}C]glycine into prodigiosin and its dipyrrolic precursor

The utilization of glycine carbon atom 2 for the synthesis of prodigiosin and its dipyrrolic precursor was investigated. 52 mg of [2-^{14}C]glycine (specific activity $2.70 \cdot 10^5$ counts/min/mg carbon) were supplied to the wild-type cells as described above. The prodigiosin recovered had a specific activity of $5.65 \cdot 10^3$ counts/min/mg carbon. Thus, the methyl carbon of glycine is utilized with 2.1% efficiency. For dipyrrole synthesis [2-^{14}C]glycine is used with 2.6% efficiency. 775 mg of [2-^{14}C]glycine, specific activity $5.64 \cdot 10^4$ counts/min/mg carbon, were used; the purified dipyrrole had a specific activity of $1.39 \cdot 10^3$ counts/min/mg carbon.

The incorporation of glutamic acid, ornithine and hydroxyproline into prodigiosin

In order to test further the utilization of proline and related metabolites, as well as the proposal[2] that hydroxyproline might give rise to the methoxyl-bearing ring (II), the degree of incorporation into prodigiosin of [2-^{14}C]hydroxyproline, as well as two

precursors of proline, [2-^{14}C]ornithine and [2-^{14}C]glutamic acid, was studied. The results of these experiments are summarized in Table IV. Ornithine was incorporated into prodigiosin with approx. 8% efficiency, but glutamic acid and hydroxyproline were not incorporated.

TABLE IV

THE INCORPORATION OF [2-^{14}C]GLUTAMIC ACID, [2-^{14}C]ORNITHINE AND [2-^{14}C]HYDROXYPROLINE INTO PRODIGIOSIN

Experiment No.	*Substrate*	*mg*	*Total activity (counts/min)*	*Specific activity of substrate (counts/min/mg carbon)*	*Specific activity of prodigiosin (counts/min/mg carbon)*	*Utilization (%)*
5a	L-[2-^{14}C]Glutamic acid	110	$1.15 \cdot 10^6$	$2.46 \cdot 10^3$	0	0
	D-[2-^{14}C]Glutamic acid	1	$1.15 \cdot 10^6$			
b	L-[2-^{14}C]Ornithine	50	$2.37 \cdot 10^6$	$1.04 \cdot 10^5$	$8.37 \cdot 10^3$	8
	D-[2-^{14}C]Ornithine	1	$2.37 \cdot 10^6$			
c	L-[2-^{14}C]Hydroxyproline	42	$2.27 \cdot 10^6$	$1.68 \cdot 10^5$	0	0
	D-[2-^{14}C]Hydroxyproline	1	$2.27 \cdot 10^6$			

DISCUSSION

The occurrence of five radioactive compounds different from δ-aminolevulinic acid in hydrolyzates of *S. marcescens* supplied δ-[4-^{14}C]aminolevulinic acid indicates that this compound can enter the cells and be metabolized; thus its failure to serve as an effective substrate for prodigiosin cannot be attributed to impermeability. This conclusion is supported by the observation that when δ-[^{14}C]aminolevulinic acid with seven-fold more radioactivity than used by MARKS AND BOGORAD[2] was supplied to a culture of *S. marcescens*, slight incorporation of isotope into prodigiosin was detected. Consequently, the present data support the contention[2] that although prodigiosin is a pyrrolic compound it is not synthesized from δ-aminolevulinic acid as are the porphyrins.

Studies with mutants of *S. marcescens* reveal that prodigiosin is formed in two discrete steps: (a) the formation of a dipyrrole (Fig. 1a) and (b) the condensation of the dipyrrole with independently synthesized 2-methyl-3-amylpyrrole; the latter constitutes ring III of prodigiosin. The finding reported here that radioactivity from [2-^{14}C]proline enters the 2-methyl-3-amylpyrrole moiety of prodigiosin, but not rings I and II, suggests that the entire heterocyclic ring of proline may be utilized for its formation. The components of the dipyrrolic precursor appear to arise differently; this is indicated by the utilization of the carboxyl carbon atom but not of the carbon atom 2 of proline for its formation.

The pattern of proline carbon atom utilization for the synthesis of the dipyrrole suggests the possibility, which is not conclusively demonstrated in the present experiments, that the carboxyl carbon atom is used only after it has been removed, as a single carbon unit, from proline. Thus, the proline carboxyl carbon atom may constitute a further source of single carbon units in metabolism of *S. marcescens* and other organisms.

The failure of *S. marcescens* to utilize [2-^{14}C]hydroxyproline for prodigiosin formation when taken together with data on the incorporation of specific carbon

atoms of proline demonstrates (a) that proline but not hydroxyproline is used for the synthesis of ring III of prodigiosin, and (b) that, although ring II of prodigiosin contains a methoxyl side chain, the hydroxyproline ring is no more effective a substrate for this part of the pigment molecule than is the ring of proline— neither is used.

In view of the observations that radioactivity from ornithine and proline can be incorporated into prodigiosin, it was surprising that glutamate was not utilized for pigment formation by *S. marcescens*. It is possible that Serratia cannot form glutamic semialdehyde from glutamate or that the dilution and/or diversion of glutamate was so great as to make its incorporation, after conversion to proline, undetectable.

The earlier observations[1,2] that the methyl carbon of glycine is utilized in prodigiosin synthesis have been confirmed. This observation has been extended with the finding that the methyl carbon of glycine is utilized with approximately equal efficiency in the formation of the two portions of prodigiosin studied here. It is of interest to note that HUBBARD AND RIMINGTON[1] reported that the methyl carbon and the amino nitrogen of glycine are utilized equally in prodigiosin synthesis; however, the present studies with proline suggest the possibility that the α-carbon of glycine may be incorporated into the pyrrole rings of prodigiosin by more than one metabolic path.

ACKNOWLEDGEMENTS

This work was made possible by grants from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service (A-1010) and from the National Science Foundation. It was also supported in part by the Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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