*STUDIES ON THE BIOSYNTHESIS OF PRODIGIOSIN IN SERRATIA MARCESCENS**

By Gerald S. Marks and Lawrence Bogorad

DEPARTMENT OF BOTANY, UNIVERSITY OF CHICAGO

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Prodigiosin, the red pigment of *Serratia marcescens*, has been characterized as a tripyrrylmethene[1] (Fig. 1) although this structure has never been confirmed by synthesis. Its biosynthesis is of considerable interest both for its own sake and in relation to porphyrin biosynthesis where a tripyrrylmethane has been postulated as an intermediate by Turner[2] and, more recently, by Bogorad and Granick[3] and by Shemin.[4] In 1949 Hubbard and Rimington[5] investigated the biosynthesis of prodigiosin and showed that the nitrogen and the methylene carbon atoms of glycine are utilized in its biosynthesis whereas the carboxyl carbon atom is not; they also found that both carbon atoms of acetate are incorporated into the pigment. Since similar observations had been made in studies of heme biosynthesis Hubbard and Rimington concluded that prodigiosin biosynthesis should be given due consideration in the elaboration of any final scheme of porphyrin biogenesis.

During the last decade many of the details of porphyrin biosynthesis have been elucidated. It is now known that succinyl-Co A condenses with glycine to give

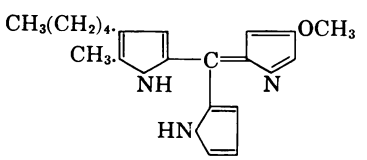

FIG. 1.—Prodigiosin.

5-aminolevulinic acid with the loss of CO_2 from the carboxyl group of glycine.[6] Two molecules of 5-aminolevulinic acid condense to form porphobilinogen which is then converted to uroporphyrinogen III. It was of interest to ascertain whether prodigiosin is indeed derived from 5-aminolevulinic acid and porphobilinogen as suggested by the data of Hubbard and Rimington. Another possibility which we felt merited consideration was that prodigiosin is derived from proline. This idea was put forward by Kost[7] on the basis of a study of the effects of amino acids on growth and pigment production of *Serratia marcescens*. The aim of the present investigation was to decide between these two alternatives.

Experimental.—5-Aminolevulinic acid hydrochloride-5-C^{14}, prepared from glycine-2-C^{14} by an unpublished method, was provided by Drs. F. Sparatore and D. Mauzerall of the Rockefeller Institute. Glycine-2-C^{14} was purchased from the Volk Radiochemical Company and L-proline-C^{14} from the Nuclear Chicago Corporation. We are grateful to Dr. James Moulder of the Department of Microbiology, University of Chicago, for a culture of *Serratia marcescens*.

In the experiments with 5-aminolevulinic acid-5-C^{14} and with glycine-2-C^{14} *Serratia marcescens* was grown on the medium of Williams *et al.*[8] For the experiments with L-proline-C^{14} the same medium was used except that a synthetic amino acid mixture was substituted for the enzymatic casein hydrolysate. This mixture

corresponded in composition to casein[9] except that hydroxyglutamic acid was omitted. The medium (250 ml. in a 5 l. diphtheria toxin bottle) was autoclaved and a solution of the C^{14} compound to be tested was sterilized by filtration and added just prior to the solidification of the agar. The inoculated flask was kept at room temperature in the dark and harvested after 5 days.

Prodigiosin was extracted from the bacteria by the alkaline extraction procedure described by Hubbard and Rimington[5] and then purified essentially as described by these authors. After extracting the pigment from petroleum ether (b.p. 30–60°C) with 85 per cent ethanol containing 1 per cent acetic acid, the ethanolic solution was diluted with an equal volume of water. The aqueous-ethanolic solution was then treated with aqueous sodium hydroxide until the color changed to orange, whereupon the pigment was extracted with petroleum ether. This procedure was repeated several times until the specific activity of the prodigiosin remained constant. In cases where the prodigiosin was found to be radioactive its specific activity was determined again after paper chromatography by a modification of the method of Williams *et al.*[8] Descending paper chromatography was used rather than circular chromatography between glass plates. A small amount of blue pigment, suggested previously to be a dimer of prodigiosin,[10] remained at the origin but the bulk of the pigment which was pink in color moved close to the solvent front. This was eluted with chloroform and in each case its specific activity was found to be unchanged. The concentration of prodigiosin was determined spectrophotometrically in chloroform containing 1 per cent acetic acid using the molar extinction coefficient given by Hubbard and Rimington.[5] The pigment was then plated as an infinitely thin layer and its radioactivity measured in a windowless gas flow counter (efficiency 39%). The sample and background were counted long enough to bring the standard deviation for the corrected count down to ±5 per cent.

Results and Discussion.—The results of these experiments are summarized in Table 1.

TABLE 1

UTILIZATION OF C^{14}-LABELED COMPOUNDS BY *Serratia marcescens*

C^{14}-Labeled Compound	Amount, mg	Total Activity, Counts/Min	Specific Activity of Labeled Compound, Counts/Min/mg of C	Specific Activity of Prodigiosin, Counts/Min/mg of C
Glycine-2-C^{14}	52.5*	2.17×10^6	129,500	1,550
5-Aminolevulinic acid hydrochloride-5-C^{14}	2.62	0.9×10^6	956,000	0†
L-Proline-C^{14}	40	2.2×10^6	105,200	3,560

* 50 mg of glycine-2-C^{14} was added and it was calculated from the composition of casein[9] that 2.5 mg of glycine was present in the medium.

† The sample (36.7 μ grams) and background were counted for 60 minutes each. The net count rate was −0.5 cpm and the S.D. ±1 cpm. Similar results were obtained with additional samples.

In confirmation of the results of Hubbard and Rimington[5] glycine-2-C^{14} was incorporated into prodigiosin. 5-Aminolevulinic acid-5-C^{14}, however, was not incorporated. Thus it appears that prodigiosin is not derived biosynthetically from the same pyrrolic precursor as porphyrins, viz., porphobilinogen. The possibility does exist that the 5-aminolevulinic acid was unable to pass through the cell membrane thus accounting for the lack of incorporation. However, experiments dem-

onstrating radioactivity in other cellular components derived from 5-aminolevulinic acid, such as porphyrins, purines, serine or methionine,[4] would exclude such a possibility. Observations made so far indicate that 5-aminolevulinic acid is able to penetrate the cell membrane: (a) Cells washed ten times with saline remained highly radioactive. (b) After extraction of the washed cells with 5 per cent trichloroacetic acid and then with chloroform-methanol, the residue was extracted with 10 per cent trichloroacetic acid at 90°C; the supernatant containing nucleic acids[11] and the residue containing crude protein were both found to be radioactive.

Results obtained with L-proline-C^{14} are in accord with the observation that 5-aminolevulinic acid is not a precursor of prodigiosin. L-Proline is approximately $2^1/_2$ times as efficient a precursor of prodigiosin as glycine whereas in heme synthesis, in the rat, according to Shemin and Rittenberg,[12] glycine is a much more efficient precursor than proline. All the evidence therefore favors L-proline, or a closely related derivative, rather than 5-aminolevulinic acid, as an intermediate in prodigiosin biosynthesis. It is possible that (a) glycine and proline are utilized for the biosynthesis of different parts of the prodigiosin molecule or (b) that the

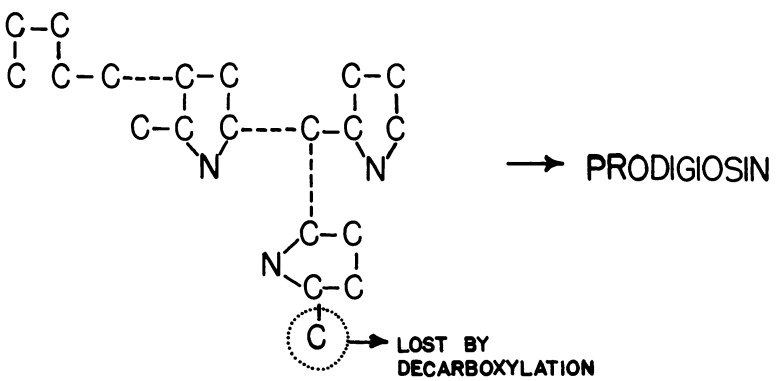

FIG. 2.—Hypothetical scheme for biosynthesis of prodigiosin from 5-carbon units.

methylene carbon and the nitrogen of glycine are incorporated into prodigiosin via proline or a closely related derivative. Experiments are now in progress to decide between these two possibilities. In any event, it is clear that the methylene carbon and the nitrogen of glycine are not incorporated into prodigiosin via 5-aminolevulinic acid. Furthermore, the pyrrolic precursor of prodigiosin found in a *Serratia marcescens* mutant,[13] having the formula $C_{10}H_{10}N_2O_2$, may also be derived from proline or a closely related derivative. Two hypotheses of prodigiosin biosynthesis,[14, 15] both of which are based on the assumption that prodigiosin and porphyrin biosynthesis are closely linked, appear to require reconsideration in the light of the present observations. It would seem rather that prodigiosin biosynthesis is closely linked with the metabolism of 5-carbon units, such as proline, ornithine, and glutamic acid, and that *Serratia marcescens* is an excellent organism for profitably studying the interrelationships of these acids.

As a working hypothesis we would like to suggest that the pyrrolic precursor from the *Serratia marcescens* mutant as well as prodigiosin are built up of 5-carbon units and that the amyl group in prodigiosin is derived from a similar deaminated 5-carbon unit. The proposed scheme as depicted in Figure 2 is based on the as-

sumption that the structure of prodigiosin is correctly represented in Figure 1. Furthermore, inspection of the prodigiosin molecule suggests that at least the methoxyl-bearing ring may be derived directly from hydroxy-proline.

Summary.—Glycine-2-C^{14} was incorporated into prodigiosin, the red pigment of *Serratia marcescens*, in confirmation of the results of Hubbard and Rimington. 5-Aminolevulinic acid-5-C^{14} was, however, not incorporated showing that the 3 pyrrole rings of prodigiosin must be derived from a precursor other than porphobilinogen, the pyrrolic precursor of porphyrins. Experiments with L-proline-C^{14} indicated that proline or a closely related metabolic derivative is a precursor of prodigiosin.

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*THE ROLE OF TRANSCARBOXYLATION IN PROPIONIC ACID FERMENTATION**

BY ROBERT W. SWICK† AND HARLAND G. WOOD

DEPARTMENT OF BIOCHEMISTRY, WESTERN RESERVE UNIVERSITY, SCHOOL OF MEDICINE, AND DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH, ARGONNE NATIONAL LABORATORY

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The evidence that propionibacteria form propionate by the direct decarboxylation of succinyl CoA[1] has been reviewed by Wood *et al.*[2] It has generally been considered that carbohydrates, e.g., glucose, glycerol, etc. are catabolized to pyruvate which is then converted to oxalacetate by the fixation of carbon dioxide. Oxalacetate is reduced to succinate, which in turn is esterified with CoA and decarboxylated to propionyl CoA and CO_2. Therefore the reduction of one mole of pyruvate to propionate would be expected to involve the fixation and release of one mole of CO_2.