LABELING PATTERNS IN PRODIGIOSIN BIOSYNTHESIS

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Summary: Biosynthesis of prodigiosin in Serratia marcescens was studied in two systems. In surface culture of mutant strains, isotope from proline-1-^{14}C was incorporated via the non-volatile bipyrrole intermediate only. Pigment of a liquid-cultured wild-type strain was oxidized to maleimides. Isotope from proline-1-^{14}C or methionine-methyl-^{14}C appeared in methoxymaleimide only; unsubstituted maleimide was labeled by proline-5-^{14}C but not by glycine-2-^{14}C. Proline thus seems to enter prodigiosin intact, forming the unsubstituted pyrrole ring and the adjacent alpha-carbon of the methoxypyrrole ring.

Final step in the biosynthesis of prodigiosin (2-methyl-3-amyl-6--methoxy-prodigiosene (1), red pigment of the microorganism Serratia marcescens), is condensation of 4-methoxy-2,2'-bipyrrole-5-carboxaldehyde (MBC) with 2-methyl-3-amylpyrrole (MAP) (2). Strain WF produces MAP, but not MBC; strain 933 produces MBC, but not MAP. We grew surface cultures in a dual-chamber apparatus that allowed volatile MAP to pass from strain WF to strain 933, and isolated and counted the syntrophic prodigiosin produced by strain 933. We also grew wild-type strain Nima in Harned's medium (3) containing 1% Difco Bactoneopeptone and transferred the poorly pigmented cells to fresh medium in which Difco Bactopeptone replaced Bactoneopeptone. In 12 hr of vigorous aeration the cells produced abundant prodigiosin, which was purified and oxidized to maleimides and CO_2. Maleimide, methoxymaleimide, and amylmaleimide were separated by thin later chromatography (TLC) and scanned for radioactivity.

METHODS

System I. The surface culture apparatus consisted of a glass baking dish (diam. 215 mm, depth 75 mm), with a 125 x 65 mm crystallizing dish in

the center. Each chamber contained 200 ml of Harned's 2% agar medium (3). An inverted baking dish cover was sealed on with tape during autoclaving and incubation. Inocula were 1 ml of 18-hr tube cultures in Williams' medium (4), with S. marcescens strain 933 in the inner chamber and strain WF in the outer chamber. Radioactive compound was added to the appropriate chamber at the time of inoculation. After 4 days incubation in the dark at 27-28°, the cells were washed from the surface with 30 ml of 0.9% NaCl. The pigmented 933 cells were treated with NaOH and acetone, and the pigment was extracted into hexane. Prodigiosin was purified (5) and chromatographed as the free base in hexane on diatomaceous earth. Electronic and ir spectra were identical to those of wild-type prodigiosin (6).

System II. S. marcescens strain Nima was grown in a rotary incubator shaker at 28° for 54 hr in a 2-liter Erlenmeyer flask containing 1 liter of Harned's medium (3) made with Bactoneopeptone. The centrifuged cells were resuspended in 1 liter of Harned's medium made with Bactopeptone. Radioactive amino acid and a small amount of Dow Corning Antifoam C emulsion were added. The culture was now vigorously aerated for 12 hr by bubbling air through a gas dispersion tube. Pigment was extracted from the centrifuged cells by NaOH-acetone-hexane. The broth also was extracted with hexane. The combined solution of the free base was dried (Na_2SO_4) and evaporated under reduced pressure. The residue was dissolved in dichloromethane (75 ml) and washed with 1 N NaOH, water, and 1 N HCl. The dried CH_2Cl_2 solution was concentrated under reduced pressure to 5 ml. Addition of 15 ml of hexane and concentration of the CH_2Cl_2-hexane solution under reduced pressure gave 10-20 mg of crystalline prodigiosin for oxidation (7). A solution of 10 mg of pigment in 1 ml of acetone and 25 ml of 15% (v/v) H_2SO_4 was placed in a 100-ml round-bottom flask equipped for gas flow. Chromium trioxide (1 g in 5 ml water) was added dropwise for 5 minutes. CO_2-free N_2 gas was swept through the reaction vessel into a saturated solution of $Ba(OH)_2$ to trap CO_2 produced. The reaction was stirred for 1 hr at room temp. Neutral maleimides were extracted (8) and submitted to TLC (see legend of Fig. 1 for

details). Mass spectra of maleimides from TLC plates were obtained with a Cary Atlas CH4 mass spectrometer (7,8).

RESULTS

System I. See Table I. Reading across, columns e and f show how a labeled compound was utilized for prodigiosin via MBC compared with utilization for "cell protein" in strain 933; columns i and j show utilization for prodigiosin via MAP and for "cell protein" in strain WF. Reading down columns e and i, utilization of the two prolines for prodigiosin via each intermediate can be compared. Note that the carboxyl-labeled proline was in the racemic form. Comparisons between other substrates are not valid because the true specific activity of the compound in the medium was not known.

System II. See Figure 1. The added compounds had higher specific activities (approx. 10^8 c/min/mg) and the prodigiosin isolated was more highly radioactive (3.6-90 x 10^6 c/min/mg) than in System I. Recoveries of CO_2 from the CrO_3 oxidation were erratic; we noted higher radioactivity in the $BaCO_3$ when methoxymaleimide was labeled.

DISCUSSION

Qadri and Williams (9) showed that, in nonproliferating cells of S. marcescens, isotope from methionine-methyl-^{14}C was incorporated into prodigiosin with about 50 times the efficiency of isotope from methionine--carboxyl-^{14}C. In our oxidation experiments, methoxymaleimide alone was labeled by methionine-methyl-^{14}C, presumably via methylation of the hydroxy analog (10) of MBC. However, labeling of C_5 or the methyl group on C_2 of the prodigiosene nucleus is not excluded by our experiments.

Isotope from L-proline-U-^{14}C has been shown to be incorporated efficiently into prodigiosin (11) and also into MBC (12). Shrimpton et al. (12) concluded that, although prodigiosin incorporated isotope from both proline--1-^{14}C and proline-2-^{14}C, MBC utilizes only the carboxyl carbon of proline. Data from our System I show that the proline carboxyl carbon is utilized only via MBC, and data from our System II show that methoxymaleimide alone

TABLE I. UTILIZATION OF ISOTOPE FROM C-LABELED COMPOUNDS IN SYSTEM I

Compound Added		Addition to MBC-producing Strain 933				Addition to MAP-producing Strain WF			
a.	b.	c.	d.	e.	f.	g.	h.	i.	j.
Labeled compound (amount added)	Sp. act. compound	Sp. act. prodigiosin	Sp. act. cell protein	Utilization for prodigiosin	Utilization for cell protein	Sp. act. prodigiosin	Sp. act. cell protein	Utilization for prodigiosin	Utilization for cell protein
mg	c/min/mg	c/min/mg	c/min/mg	(100c/b)	(100d/b)	c/min/mg	c/min/mg	(100g/b)	(100h/b)
L-Pro-U-^{14}C (44)	394,000	68,700	11,300	17.4%	2.9%	7,570	17,700	1.9%	4.5%
DL-Pro-1-^{14}C (44)	394,000	27,200	6,600	6.9%	1.7%	0	8,550	0%	2.2%
Gly-2-^{14}C (*)	131,000	74,000	57,800	56.5%	44.1%	3,550	95,900	2.7%	73.2%
D-GlcN-1-^{14}C (+)	337,000	8,900	19,400	2.6%	5.8%	6,600	15,200	2.0%	4.5%

*1.6 mg of glycine-2-^{14}C was added; it was calculated from the composition of Bactoneopeptone that 130 mg of glycine was available in the medium.
+1.4 mg of D-glucosamine-1-^{14}C was added plus 50 mg of D-glucosamine as carrier.

"Cell protein" was obtained by making an alkaline extract (1 N NaOH) of the cells to which isotope was added, neutralizing the extract, and treating 5 ml aliquots with 20 ml of cold 10% trichloroacetic acid. The precipitate was washed with ethanol and ether and dissolved in formic acid for counting. Samples of prodigiosin and cell protein were counted on planchets with a windowless gas-flow counter, Nuclear Chicago Model D-47 sample changer, and Model 161A scaler. Counting efficiency was 39%. No corrections were made for geometry or self-absorption. Labeling of cell protein of the strain to which no isotope was added was below 300 c/min/mg except for labeling of strain WF protein by glucosamine added to strain 933 (612 c/min/mg). These low values show that only a minute amount of radioactivity passed through the vapor space in the form of some generally metabolizable volatile compound such as CO_2.

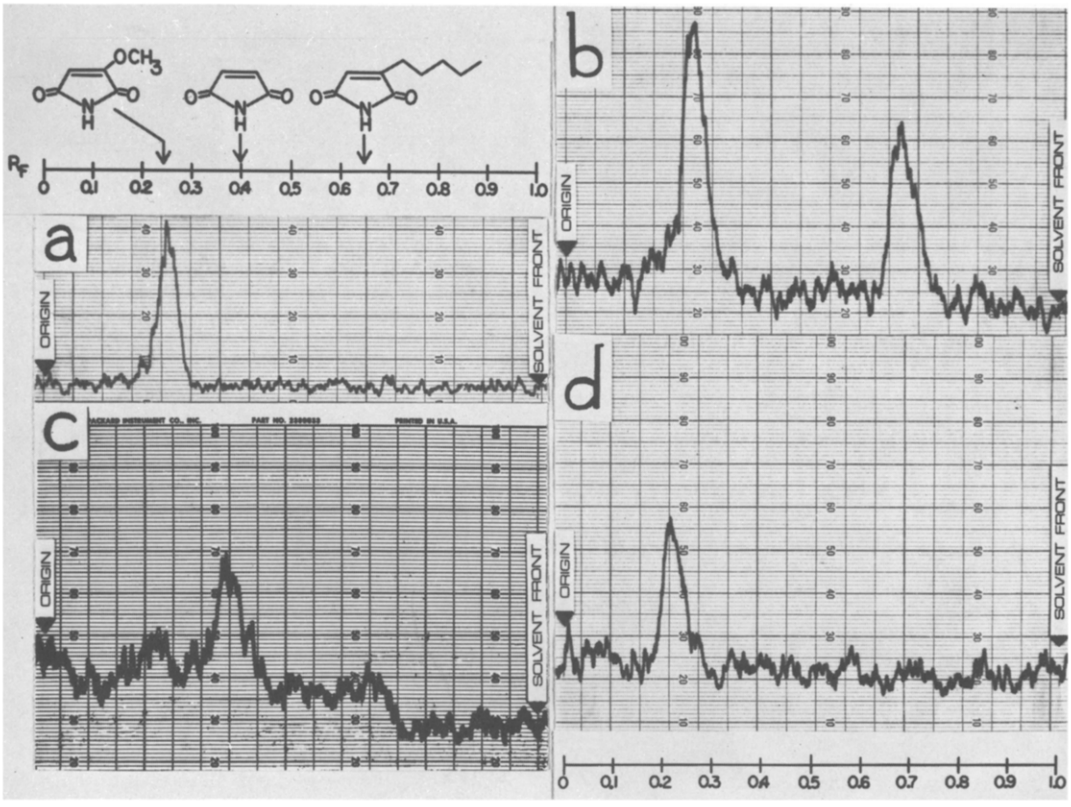


Fig. 1. Radiochromatoscan of chromic acid oxidation products of prodigiosin in System II after incorporation of isotope from:

1a. L-Methionine-methyl-^{14}C 1b. Glycine-2-^{14}C

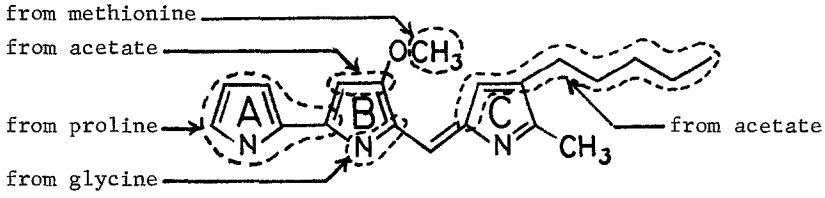
1c. DL-Proline-5-^{14}C 1d. DL-Proline-1-^{14}C

Extracts of the oxidation mixture were chromatographed on MN-Polygram Sil G/UV_{254} precoated plates (Brinkmann Instruments) using hexane:ethyl acetate:2-propanol (76.7:8.8:14.5) as solvent. Radioactive dye markers (not shown) spotted at each end of the plates served as reference points in matching plates with the radiochromatoscans. All radioactive peaks were associated with uv-absorbing spots. Scanning was accomplished with a Packard Model 7201 radiochromatogram scanner set at 100 c/min linear range except for Fig. 1a. which was obtained with a 1000 c/min range.

is labeled by the proline carboxyl. But proline-5-^{14}C also labeled the pyrrole rings contributed by MBC, contradicting the conclusions of Shrimpton et al. Although proline-5-^{14}C labeled both methoxymaleimide and amylmaleimide to some extent, its isotope went predominantly into unsubstituted maleimide. These labeling patterns suggest strongly that the proline ring

goes intact into ring A of MBC, with its carboxyl group becoming the adjacent alpha-carbon of ring B.

Both systems give information on incorporation of isotope from glycine--2-^{14}C, which has already been shown to label prodigiosin (5,11) and MBC (12, 13). Failure of glycine to label the unsubstituted maleimide in System II strengthens our conclusion that ring A is formed directly from the proline ring. If the N and C_2 of glycine go into MBC as a unit, as they seem to in prodigiosin (5), both N atoms and seven of the ten carbon atoms of MBC are accounted for. Our data complement recent findings on incorporation of acetate-1-^{13}C and acetate-2-^{13}C into prodigiosin. We are indebted to H. H. Wasserman for a preprint of the Yale report (14). Our conclusions from the two studies are illustrated in the drawing.



Our experiments do not clarify the labeling pattern in ring C. Data from both systems give a qualitative impression that glycine is a more direct precursor for MBC than for MAP. Similar utilization of isotope from L-proline-U-^{14}C, glycine-2-^{14}C, and D-glucosamine-1-^{14}C via MAP in System I suggests that perhaps none of these compounds is a direct precursor of ring C. In System II, both glycine-2-^{14}C and proline-5-^{14}C labeled amylmaleimide to some extent.

We investigated glucosamine because in our hands it stimulated prodigiosin production in temperature-inhibited cultures of S. marcescens (15). Also, glucosamine is converted into pyrrolic compounds by known in vitro (16) and in vivo (17) reactions. Our findings indicate that it is not a direct precursor of prodigiosin.

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