INHIBITION BY TEMPERATURE OF THE TERMINAL STEP IN BIOSYNTHESIS OF PRODIGIOSIN

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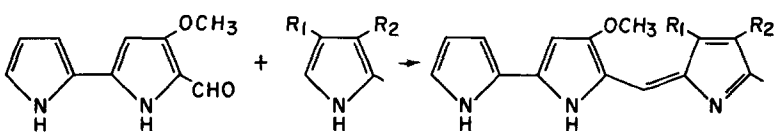
Figure 1 shows the terminal step in biosynthesis of prodigiosin (I), the red pigment produced by Serratia marcescens (Wasserman et al., 1960). Although the condensation occurs chemically at low pH (Rapoport and Holden, 1960), in living cells the reaction appears to be enzymatically catalyzed. An analogue of prodigiosin (II) forms if 2,4-dimethylpyrrole (DMP) is substituted for the natural monopyrrole (Wasserman et al., 1960). The addition of DMP to colorless mutants of S. marcescens that produce the bipyrrole results in formation of II. We have used this reaction to study the effect of temperature upon formation of pigment by living cells. Higher temperatures of incubation inhibit production of pigment (Williams and Gott, 1962). One of the reactions inhibited by temperature appears to be coupling of monopyrrole to bipyrrole.

Mutant 933 (Santer and Vogel, 1956; Wasserman et al., 1960) that produces only the bipyrrole but forms pigment if provided with the mono-



I. $R_1 = H$; $R_2 = C_5H_{11}$: Prodigiosin

II. $R_1 = CH_3$; $R_2 = H$: analogue of I

Fig. 1. Terminal step in biosynthesis of prodigiosin.

pyrrole was grown with vigorous shaking for 24 hr at 27 C in a medium containing 0.5% peptone and 1.0% glycerol. The cells, harvested by centrifugation and washed once with 0.1 M phosphate buffer, pH 7.0, provided the coupling enzyme. Protein in the cells was measured by the method of Lowry et al. (1951). The amount of bipyrrole in the supernatant fluid was determined by extracting the compound with $CHCl_3$ and then measuring the absorption of the extract at 363 mu (Santer, 1958; Williams and Gott, 1964). The supernatant was used directly as the source of bipyrrole.

The coupling enzyme was assayed in a 250 ml Erlenmeyer flask containing, in 5 ml of 0.1 M phosphate buffer, pH 7.5, 4.0 mg/ml of cellular protein, 10 ug/ml of bipyrrole and 1 mg/ml of DMP (K and K Laboratories, Plainview, N. Y.). Incubation was aerobic with shaking in a waterbath for the time and temperatures indicated in the figures. The pigment formed was extracted with acetone, evaporated almost to dryness and the residue dissolved in 10 ml of ethanol containing 1 ml of 1 N HCl. The absorption of the ethanolic extract was measured at 527 and 655 mu; the amount of pigment in the extract was determined by subtracting the latter value from the former. The measure of enzyme

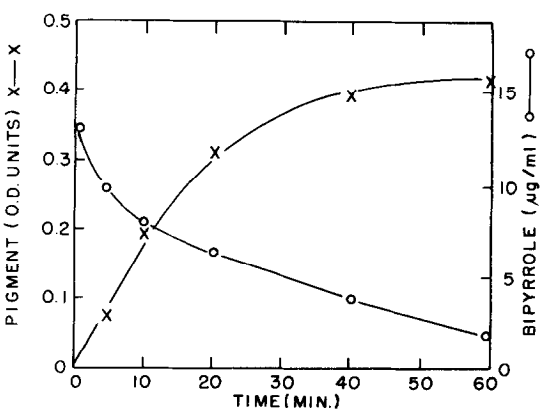


Fig. 2. Formation of pigment (X—X) and disappearance of bipyrrole (o—o) during the course of the coupling reaction carried out at 27 C. Assay conditions were as described in the text. Pigment values represent amount extracted from 5 ml of reaction mixture.

activity was the amount of pigment formed/mg cellular protein/hr.

Chromatography of I and II in several solvent systems showed similar Rf values. In acidified ethanol, I had a peak at 537 mu; II at 527 mu. The latter value was used for quantitative estimation of II.

The time-course of coupling at 27 C is shown in Fig. 2. The reaction was almost 50% complete at 10 min. As pigment formed, bipyrrole disappeared from the suspension. No pigment formed in the absence of either substrate, nor did cells heated to boiling produce pigment.

The effect of temperature upon coupling is shown in Fig. 3. Cells of 933 grown at 27 C were mixed with bipyrrole and the suspensions permitted to equilibrate at the temperatures indicated for 10 min. DMP was then added, and the suspensions were assayed for coupling activity. Maximal activity occurred over a range between 15 to 35 C. Pigment formation decreased sharply above 35 C. At 40 C and above, no pigment formed. Lower temperatures also affected coupling, but some pigment still formed at 0 C.

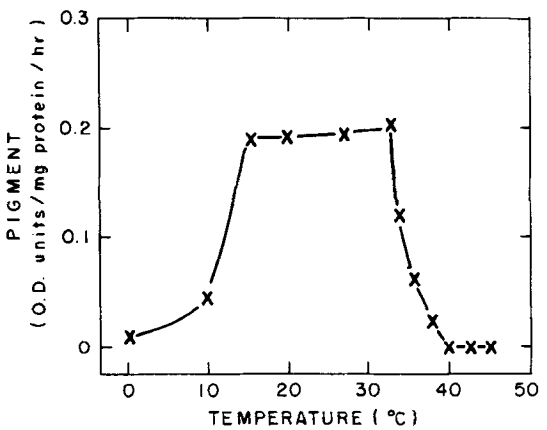


Fig. 3. Effect of temperature upon the coupling reaction. Assays for coupling activity were carried out at the indicated temperatures.

Bipyrrole and DMP were not affected by the temperatures and times used in these experiments. There also was no decrease in the number of viable cells of 933. At temperatures where no pigment formed, no bipyrrole

disappeared from the reaction mixture. Assays carried out at 38 C, where little pigment formed, gave the results presented in Fig. 4. The data in Fig. 4 also indicated that absence of pigment at the end of 1 hr was not due to dissociation by the higher temperature of a pigmented complex formed early during the incubation period.

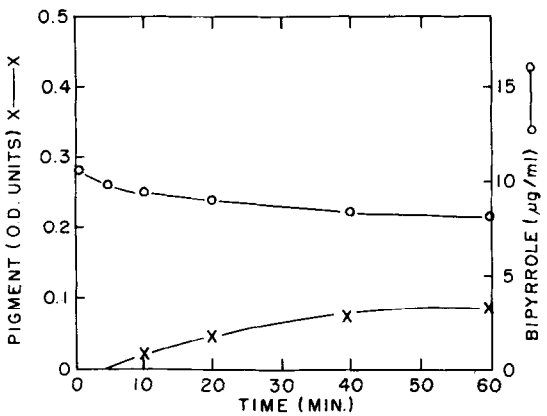


Fig. 4. Effect of incubation at 38 C upon formation of pigment (X—X) and disappearance of bipyrrole (o—o). Assay mixture was identical to that used in Fig. 1.

Failure of pigment formation might be due to destruction of the enzyme, and thus its activity, or to inability of the enzyme to combine with substrate at certain temperatures. In the latter case, enzymatic activity should be restored by returning the cells to 27 C following incubation at other temperatures. To evaluate these possibilities, cultures of 933 were grown at 27 C. The cells were harvested and stored at different temperatures for periods of time. The suspensions then were brought to 27 C, permitted to equilibrate for 30 min and assayed for coupling activity. The amount of pigment formed in each assay was compared to the amount formed by control suspensions held at 27 C for the entire period of time. As shown in Fig. 5, cells stored for 1 hr at 40 C lost 60% of their activity; those stored at 45 C, 100%. Suspensions stored at 0 and 10 C lost about 25% of activity. Suspensions held overnight in a dry-ice chest at -68 C lost no activity.

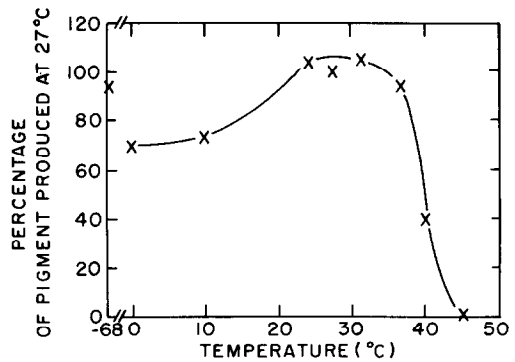


Fig. 5. Effect of storage at various temperatures upon coupling activity at 27 C. Cells of 933 were stored overnight at 10, 0 and -68 C; for 1 hr at other temperatures. Suspensions were permitted to equilibrate for 30 min at 27 C before addition of DMP. Final mixture for assay was the same as in Fig. 1.

These data indicate that the enzyme responsible for the final step in biosynthesis of prodigiosin is sensitive to heat. Higher temperatures apparently destroy the enzyme; lower temperatures do not destroy the enzyme but reduce its activity. Thus, one explanation for the failure of S. marcescens to form pigment at higher temperatures of incubation is the sensitivity of the coupling enzyme to heat.

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