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Anaerobic Formation of Protoporphyrin IX from Coproporphyrinogen III by Bacterial Preparations

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The conversion of coproporphyrinogen III into protoporphyrin IX requires the transformation of two propionic acid side chains of positions 2 and 4 of the rings into vinyl groups, a process involving both decarboxylation and oxidation. The enzyme (coproporphyrinogen oxidative decarboxylase or coprogenase) responsible for the conversion is inhibited by anaerobiosis (Falk, Dresel & Rimington, 1953; Porra & Falk, 1961). Molecular oxygen is essential for its activity and cannot be replaced by FAD, oxidized cytochrome *c* or any artificial electron acceptors (Sano & Granick, 1961; Porra & Falk, 1964). In contrast, the results presented here show that the enzyme from a bacterial preparation is capable of forming protoporphyrin IX under anaerobic conditions. Cellular fractions of a *Pseudomonas* sp. formed protoporphyrin from both δ-aminolaevulic acid and coproporphyrinogen III on incubation under vacuum in the presence of GSH.

Methods. A species of *Pseudomonas* (Brown, 1957) was grown with rotatory agitation for 15hr. at 30° in a medium composed of Bactopeptone (1%, w/v), yeast extract (0·1%, w/v) and NaCl (0·5%, w/v), adjusted to pH7·5. The organisms were harvested by centrifugation at 4° and were washed twice with distilled water and once with 0·05M-potassium phosphate buffer, pH7·5. Washed organisms were suspended in 0·1M-potassium phosphate buffer, pH7·5, and were disrupted in an MSE type M.T. 20 (60w) ultrasonic disintegrator, with probe diameter $\frac{3}{8}$in. at 2–4° for three 1min. periods; intervals of 2–3min. were allowed between periods of exposure to ultrasonic vibration to facilitate cooling. Unbroken cells and cell debris were first removed by centrifugation at 3000g for 15min. at 4°. The lysate was then centrifuged at 0° for 90min. at 90000g; the supernatant solution so obtained was used as the source of enzyme.

The reaction mixture (see below) was incubated aerobically in 100ml. conical flasks plugged loosely with cotton and under vacuum in Thunberg tubes at 30° in the dark with shaking. Flasks and the tubes laid horizontally were shaken at 60 oscillations/min. with an amplitude of 9cm. for 4hr. Both the flasks and the tubes were covered with aluminium foil to prevent photochemical oxidation of the porphyrinogen. Thunberg tubes containing the mixture were flushed with oxygen-free nitrogen for 4–5min. before evacuation.

Coproporphyrinogen III was prepared by reduction of coproporphyrin III with sodium amalgam (Mauzerall & Granick, 1958). The coproporphyrin was checked for purity and found to be free from protoporphyrin. The yield of coproporphyrinogen, calculated by the difference in porphyrin content before and after oxidation, was never less than 95·5% of the total 'porphyrin' added as substrate. At the end of incubation period, porphyrins were separated and determined by the methods of Dresel & Falk (1956) and Porra & Falk (1964). Chromatographic analysis of the free acids of porphyrins was performed by the method of Falk, Dresel, Benson & Knight (1956).

Results. The results of the effect of incubation in air and under vacuum, in the presence of GSH, on the formation of protoporphyrin IX from coproporphyrinogen III are shown in Table 1. In both the experiments protoporphyrin IX was formed even under vacuum in the presence of GSH. Formation of protoporphyrin IX from coproporphyrinogen III was about 20% aerobically and 10% under vacuum. Except for the quantity, there was no difference between the protoporphyrin IX formed under the two incubation conditions. Both had Soret maxima at 408mμ in 10% (w/v) HCl and behaved like authentic protoporphyrin IX on chromatograms developed with 2,6-lutidine. Protoporphyrin with similar characteristics was also obtained when the cytoplasmic fraction of the bacteria was incubated with δ-aminolaevulic acid under vacuum in the presence of GSH (Uddin, 1966).

It is thus evident that anaerobic conditions, though unfavourable, were not completely inhibitory for the reaction, as was found in animal systems by Sano & Granick (1961) and Porra & Falk (1964). It is unlikely that the results were caused by traces of residual oxygen in the Thunberg tubes. Under similar conditions of incubation mammalian liver coprogenase was completely inactive (Porra & Falk, 1964). The present results

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Table 1. *Effect of aerobic and anaerobic incubation on the conversion of coproporphyrinogen III into protoporphyrin IX by cytoplasmic fraction of* Pseudomonas *sp.*

Enzyme preparations (5ml.) in 0·1M-potassium phosphate buffer, pH 7·5, were incubated for 4hr. at 30° with coproporphyrinogen III (21mμmoles), $MgSO_4$ (0·1mM) and GSH (1·0mM) in a final volume of 6ml. Enzyme preparations used in the two experiments were prepared from different batches of organisms and contained 3·1 and 3·4mg. of protein/ml. in Expts. I and II respectively. Controls containing heat-inactivated enzyme were run simultaneously with the experiments; no protoporphyrin was formed under either of the experimental conditions.

Expt. no.	Incubation condition	Porphyrins recovered (mμmoles/6ml. sample)		
		Coproporphyrin	Protoporphyrin	Total
I	Aerobic	9·0	3·9	12·9
	Vacuum	17·0	2·0	19·0
II	Aerobic	7·8	4·0	11·8
	Vacuum	16·5	2·1	18·6

are more likely to be explained by peculiarities of the bacterial electron-transfer system. Some oxidized components might serve as alternative electron acceptors in the bacterial system under anaerobic conditions. Lascelles (1956) has, in fact, demonstrated anaerobic formation of protoporphyrin from δ-aminolaevulic acid in light by cell suspensions of *Rhodopseudomonas spheroides* in the presence of an iron salt and an oxidizable substrate. Moreover, some micro-organisms have been reported to form cytochromes under anaerobic growth condition. Cytochrome *b* has been detected in anaerobically grown cells of *Bacillus cereus* (Schaeffer, 1952). Similarly bands of cytochromes a_1 and b_1 have been detected in yeast cells grown anaerobically (Chin, 1950; Ephrussi & Slonimski, 1950). According to Tustanoff & Bartley (1964), yeast cells, when grown on galactose under continuous flushing with nitrogen, have considerable respiratory as well as cytochrome oxidase activity. Cytochromes have been found in some obligate anaerobes such as *Chromatium* sp. and *Desulfovibrio desulfuricans* (Newton & Kamen, 1961). All these observations clearly indicate that synthesis up to haemoprotein can proceed in some bacteria and yeasts in the complete absence of oxygen. A technical difficulty, though, with all such experiments lies in keeping the cells free from oxygen at all times, i.e. during growth, harvesting etc. Traces of oxygen contaminating nitrogen or contact of cells with oxygen before enzyme assay may elicit the synthesis of the respiratory enzymes. Nevertheless, in view of the significant differences in the results of the effect of anaerobiosis on the conversion of coproporphyrinogen III into protoporphyrin reported here and in the literature, an investigation into the mechanism of electron transfer from coproporphyrinogen to an acceptor, alternative to oxygen, involved in the bacteria, would be of interest.

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