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BIOSYNTHESIS OF PORPHYRINS BY BACTERIA

Synthesis of Porphyrins From δ-Aminolaevulic Acid by Cell-free Lysate of Micrococcus colpogenes

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The results of the synthesis of porphyrins from δ-aminolaevulic acid (ALA) by the whole and lysed cells of *Micrococcus colpogenes* are presented. Whereas the lysate metabolized ALA at a rate faster than the whole cells, the amount of coproporphyrin formed by it was much less than the amount formed by the cells. Under aerobic incubation the lysate formed mainly uroporphyrin. Anaerobic incubation of the lysate resulted in marked increase in the formation of coproporphyrin, but the ratio of coproporphyrin to uroporphyrin formed by it was still less than the ratio of the porphyrins formed by the whole cells. Porphyrins with intermediate number of carboxyl groups between uroporporphyrin and coproporphyrin were detected only in the case of incubation mixture containing the lysates. The difference in the metabolic operation of the pathway due to the difference in the organization of a system has been discussed.

The effect of metal ions on the synthesis of porphyrins from ALA by cells of *M. colpogenes* was reported earlier.[1] This paper describes the ability of the cell-free lysate of the bacteria to synthesize porphyrins from ALA.

Extracts of animals, plants and microorganisms have been shown to convert ALA to porphobilinogen (PBG).[2] Enzymic conversion of PBG to porphyrins including protoporphyrin has been demonstrated to occur in the presence of cell-free preparations from *Chlorella*[3] and *Euglena*,[4] spinach leaf and wheat germ,[5] and other materials.[6] Among the bacteria, cell-free extracts of the Gram negative bacteria, *Rhodopseudomonas spheriodes*[7] and *Haemophilus* sp.[8], have exhibited enzymic activities responsible for the formation of porphyrins up to coproporphyrin from ALA. Townsley and Neilands[9] have demonstrated the synthesis of porphyrins from ALA by lysate of the Gram positive bacteria, *Micrococcus lysodeikticus*. Their study was concerned, mainly, with the types and amounts of porphyrins formed by lysates made from the organisms cultivated on medium containing 'low' and 'adequate' iron levels.

The present study gives a comparative account of porphyrin-synthesizing ability of intact and lysed cells and presents evidence of the importance of 'organization' of cellular components within an organism in the regulation and smooth operations of normal metabolic processes.

Experimental

Materials.—The reagents and the strain of *M. colpogenes* used in the study were the same as described in the previous paper.[1]

Methods

Growth and Harvesting Conditions of the Bacteria.—The organism was grown and harvested as described earlier.[1]

Preparation of Cell-lysate.—Cell lysate was made by the treatment of cells with lysozyme essentially by the method of Salton and Champman.[10] Cells were suspended in 0.1M potassium phosphate buffer, pH 7.5, and lysed with Armour Laboratories, crystalline egg-white lysozyme (100 μg lysozyme/ml of cell suspension containing 8–10 mg/ml of dry wt bacteria) at 30°C. Under these conditions lysis of cells occurred in approximately 1 hr. Unlysed cells, if any, and cell debris were separated by slow centrifugation. The supernatant represented cell-free lysate.

Incubation of reaction mixtures (see the text) and oxidation of porphyrinogens formed in the mixtures followed the method described in the previous paper.[1] Incubation under vacuum was carried out in 50 ml Thunberg tubes. For the study of the amounts and types of porphyrins formed at different periods of incubation, the incubating vessels were taken out from the shaker at intervals, and placed directly into crushed ice and subsequently frozen at–10°C.

Fractionation and Determination of Porphyrins.—Porphyrins present in the reaction mixtures were fractioned and determined by the method of Dresel and Falk.[11] Since very little protoporphyrin is formed by the bacterial system under the conditions of incubation, coproporphyrin was extracted from ether with 5% HCl in all the experiments. The amounts of uroporphyrin and coproporphyrin in 5% HCl (w/v) separately were calculated from the expressions described by Cornford.[12]

Chromatography.—Samples of porphyrins were identified by the ascending chromatographic method of With[13] on Whatman paper No. 1. Chromatograms were developed in dark room at ambient temperature for 90 min, dried and

checked for spots of porphyrins under UV light.

Protein Determination.—Protein was determined by Biuret method[14] using crystalline bovine plasma albumin (Armour Laboratories, Sussex) as the standard. For the determination of protein in bacteria, digestion of the organism was first carried out with 0.1N NaOH at about 100°C for 5 min.

Results

Rate of Formation of Porphyrins from ALA by Cells and Cell-free Lysates.—The amounts of different porphyrins extracted from reaction mixtures after different periods of incubation are diagramatically shown in Fig. 1. The ability of the lysate to metabolize ALA under the condition is clearly demonstrated by the results. In fact, rate of synthesis of total porphyrins by the lysate was even faster than by the intact cells. The notable difference, however, lied in the amounts of uroporphyrin and coproporphyrin formed by the two systems. Bulk of the porphyrins formed by the lysate was present as uroporphyrin at any stages of incubation periods. Out of the total of 24 μm-moles of porphyrins, only about 1 μm-mole of coproporphyrin accumulated in the reaction mixture containing the lysate at the end of 9 hr of incubation. In contrast, almost two third of the total porphyrins was present as coproporphyrin in the incubation mixture containing intact cell, under identical conditions.

Effect of Anaerobic Incubation on the Formation of Porphyrins From ALA by Cell-free Lysates.—Lysates were incubated aerobically in flasks and under vacuum in Thunberg tubes in duplicates. Glutathione (10^{-3}M) was added to one set of the anaerobic reaction mixtures in order to study the performance of the enzymes in the presence of a sulphydryl compound. This was considered necessary particularly in view of the fact that the enzyme, urogen decarboxylase, responsible for the decarboxylation of uroporphyrin to coproporphyrin, has been shown to be a sulphydryl enzyme (Hoare and Heath[15]). The amounts of different porphyrins formed by intact cells and by the lysates under different conditions of incubation are summarized in Table 1. Values of porphyrins expressed have been corrected for cell protein and have been calculated for reaction mixtures containing 4 mg protein/ml.

It is seen from the results that the lysates formed more coproporphyrin under anaerobic incubation. The ratio of coproporphyrin to uroporphyrin increased from 1:24 under aerobic condition to 1:5 and 1:6 under anaerobic incubation with and without glutathione respectively. Presence of glutathione had stimulatory effect on the formation of both coproporphyrin and total porphyrins. However, the ratio of coproporphyrin to uroporphyrin even under this condition of incubation was still much less than the ratio of the porphyrins formed by the intact cells. Heated lysate was without any activity and no porphyrin was detected at all in the incubation mixture containing heated lysate.

On paper chromatogram (Fig. 2) developed with aquous lithium chloride in an atmosphere of NH_4OH, porphyrins formed by the cells were separated as uroporphyrin, coproporphyrin and protoporphyrin IX. A very faint spot slightly above the spot of coproporphyrin was also de-

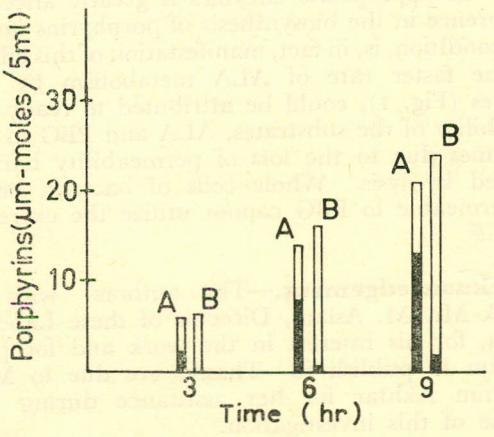

Fig. 1.—Rate of formation of porphyrins by cells (A) and lysates (B): □uroporphyrin;■coproporphyrin.

(Incubation mixture: Samples, cell suspension or lysate, 5 ml, in 0.1M phosphate buffer, pH 7.5; ALA, 2μ-moles (250 μm-moles porphyrin equivalent); $MgSO_4$, 10^{-4}M in final volume of 5.3 ml. Mixtures were shaken aerobically in flasks on the oscillatory shaker at the speed of 60 oscillations/min for different periods of time.

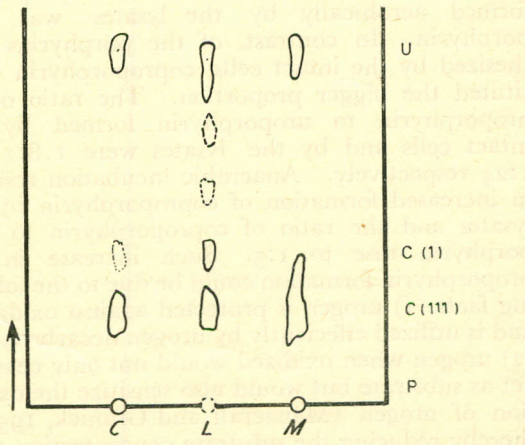

Fig. 2.—Paper chromatogram (ascending, LiCl (0.1M)-NH_4OH) relating to the chromatographic behaviour of porphyrins formed by cells (c) and lysate (L) of *M. colpogenes.*(M), is the mixture of the authentic markers, uroporphyrin(v), coproporphyrin isomers I, C(I) and III, C(III) and protoporphyrin IX (P). Faint spots on the chromatogram are marked with dashed lines.

Table 1.—Effect of Oxygen Tension During Incubation on the Formation of Porphyrins From ALA by the Lysates of *M. colpogenes*.

(Incubation mixture: samples, 5 ml in 0.1 M phosphate buffer, pH 7.5; ALA, 2 μ-moles (250 μm-moles porphyrin equivalent[1]); $MgSO_4$, 10^{-4}M in final volume of 5.3 ml. Mixtures were shaken aerobically in flasks and anaerobically under vacuum in Thunberg tubes, on the oscillatory shaker at the speed of 60 oscillations/min.)

Samples	Incubation conditions	Porphyrins (μm-moles/5m)			Ratio
		Uro	Copro	Total	Copro/ Uro
Intact cell	Aerobic	10	18	28	1.8:1
Lysate	,,	36	1.5	37.5	1:24
,,	Anaerobic	30	5.0	35.0	1:6
,,	,, +GSH	33.5	6.6	40.1	1:5
,, (heated)	Aerobic	0	0	0	—

Uro, uroporphyrin; Copro, coproporphyrin

tected. With the lysate under anaerobic incubation an array of porphyrins spots including porphyrins with intermediate number of carboxyl groups between uroporphyrin and coproporphyrin. were detected on the chromatogram. A very faint spot corresponding to that of protoporphyrin IX was also detected. The spots slightly above the spots of coproporphyrin in both the cases were most probably of coproporphyrin isomer I.

Discussion

The lysates of *M. colpogenes* exhibited the ability to metabolize ALA under both aerobic and anaerobic conditions. Bulk of the porphyrins formed aerobically by the lysates was uroporphyrin. In contrast, of the porphyrins synthesized by the intact cells, coproporphyrin constituted the bigger proportion. The ratio of coproporphyrin to uroporphyrin formed by the intact cells and by the lysates were 1.8:1 and 1:24 respectively. Anaerobic incubation resulted in increased formation of coproporphyrin by the lysates and the ratio of coproporphyrin to uroporphyrin rose to 1:5. Such increase in coproporphyrin formation could be due to the following facts:(1) urogen is protected against oxidation and is utilized effeciently by urogen decarboxylase, (2) urogen when oxidized would not only cease to act as substrate but would also sensitize the oxidation of urogen (Mauzerall and Granick, 1958)[16] thereby reducing the substrate concentration and; (3) the partially oxidized form of urogen may also be inhibitory for urogen decarboxylase. The results of the stimulatory effect of GSH on the formation of coproporphyrin are in agreement with the findings of Hoare and Heath[15] showing that urogen decarboxylase is a sulphydryl enzyme. The stability of such enzymes is dependent upon SH group liable to undergo oxidation during aerobic incubation.

It is interesting, however, that even the anaerobic incubation plus GSH did not restore the activity of the enzyme present originally in the cells. Lysis obviously brings about certain irreversible changes affecting the ability of the lysed system to carry on the normal metabolic process of porphyrin pathway. The important change is the disruption of organization affecting the spatial arrangement of cellular organelles and enzymes. In a complex metabolic process like this, it is necessary that the system of the enzymes involved in the process occurs as an integral unit with each component or enzyme of the system in close and fixed proximity. If by any means the system falls apart, it would no longer be able to carry on the normal metabolic operation as efficiently and smoothly as a well-organized system like intact cells. This is further confirmed by the chromatographic analysis of porphyrins formed by the intact and lysed cells. Whereas incubation mixture containing intact cells accumulated porphyrins corresponding to uroporphyrin, coproporphyrin and protoporphyrin, mixture with lysate, in addition, contained porphyrins with intermediate number of carboxyl groups between uroporphyrin and coproporphyrin. Under normal metabolic condition, porphyrins with intermediate number of carboxyl groups are not allowed to accumulate as they are instantaneously decarboxylated by the appropriate enzyme(s). With the disruption of the right arrangement and sequence of the enzyme system due to lysis, the smooth and orderly contact of the metabolites with the appropriate enzymes is greatly affected. Difference in the biosynthesis of porphyrins under the condition, is, in fact, manifestation of this effect.

The faster rate of ALA metabolism by the lysates (Fig. 1), could be attributed to ready accessibility of the substrates, ALA and PBG to the enzymes due to the loss of permeability barrier caused by lysis. Whole cells of bacteria being impermeable to PBG cannot utilize the excreted PBG.[17]

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