BIOSYNTHESIS OF DIPICOLINIC ACID IN BACILLUS SUBTILIS

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In the form of its calcium salt, dipicolinic acid (DPAc) is invariably a major constituent of bacterial endospores. Undetectable in vegetative cells, it is rapidly synthesized during the course of sporulation. The physiological function of DPAc is not yet clear, but several observations show that the thermal resistance of endospores is closely correlated with their calcium dipicolinate content (Church and Halvorson, 1959 ; Black et al., 1960 ; Aronson et al., 1967). The pathway of DPAc biosynthesis in bacteria was completely unknown until very recently. This component is closely related in structure to dihydrodipicolinic acid, an intermediate in the biosynthesis of lysine by E.coli (Yugari and Gilvarg, 1965), a fact which suggests that its synthesis might occur by a slight modification of the reaction sequence responsible for lysine formation (Fig. 1). Bach and Gilvarg (1966) have demonstrated that a net synthesis of DPAc from L-aspartic-β-semialdehyde (ASA) and pyruvate, the immediate precursors of dihydrodipicolinic acid in E.coli, can be catalyzed by extracts from sporulating cells of B.megaterium. We have developed a more sensitive and convenient method for studying this reaction, which we have used to investigate the development of the specific enzyme system concerned with DPAc formation during the sporulation of B.subtilis.

It is difficult to demonstrate the conversion of dihydrodipicolinic acid to DPAc as an isolated reaction, since the substrate is extremely unstable. We have therefore coupled this reaction with the enzymatic generation of dihydrodipicolinic acid from ASA and sodium pyruvate, catalysed by the condensing enzyme purified either from B.subtilis or E.coli (Yugari and Gilvarg, 1965). A saturating amount of the

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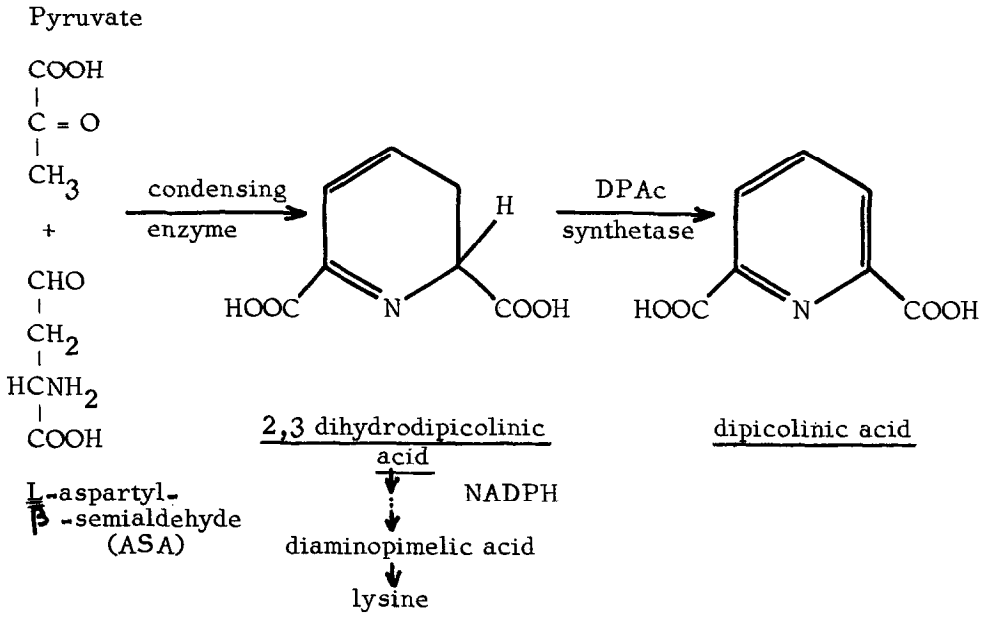

Fig. 1. Postulated pathway of dipicolinic acid synthesis.

condensing enzyme was incubated for 20 min. at 30°C with aspartic semialdehyde (Black and Wright, 1955), sodium pyruvate-3-^{14}C and the extract to be tested. The precise composition of the incubation mixture is described in tables 1-3. The radioactive DPAc formed was isolated by adding 1.0 ml of a cold solution of 0.2 M potassium dipicolinate pH 7.2 as carrier, followed by 0.2 ml of 5M H_2SO_4. When CETAB (cetyl trimethyl ammonium bromide) treated cells were used, they were removed from the incubation mixture by centrifugation in the cold before the addition of acid. The precipitated DPAc was collected on a millipore filter, washed five times with 1.0 ml of 0.75M H_2SO_4, and transferred to a plastic scintillation vial. The precipitate was then redissolved by the addition of 2.0 ml of 0.25M NaOH. At this point, an aliquot of 0.015 ml of the solution was diluted with 3 ml of 0.5M HCl and the amount of DPAc precipitated was calculated by measuring the absorbance of this solution at 272 mμ. Under these conditions, the final recovery of DPAc was about 50 per cent of the total DPAc present after addition of carrier. To the remainder of the solution, 16 ml of scintillation fluid (Bray, 1960) was added and the radioactivity of the resulting suspension was determined after vigorous shaking by counting for one minute intervals in a Packard Tri-Carb scintillation counter. The actual amount of radioactive DPAc

produced is calculated by normalizing the number of counts finally obtained on the basis of the recovery of carrier in each experiment.

Using this assay, we found (Table 1) that the addition of the condensing enzyme to the two substrates results in the formation of a small but easily measurable quantity of DPAc.

Table 1. SPONTANEOUS FORMATION OF DIPICOLINIC ACID

Additions	Product formed (mumoles)
none	< 0.5
CE of E.coli	3.6
CE + $MnCl_2$ (1 mM)	43.5
CE + EDTA (5 mM)	2.6
CE + EDTA + $MnCl_2$	2.5

The reaction mixture contained : Tris-HCl buffer, pH 8.1, 0.2 M ; L-ASA, 5 mM ; and sodium pyruvate-3-^{14}C, 1.3 mM, 0.2 mC/mmole ; in a total volume of 0.2 ml.

CE = 0.05 units of condensing enzyme purified about 100-fold from E.coli (Yugari and Gilvarg, 1965).

This non-enzymatic synthesis of DPAc is considerably stimulated by Mn^{++}. The latter effect can be completely eliminated by EDTA. EDTA was therefore added to the reaction mixture in all subsequent assays. The rate of the spontaneous reaction was increased by incubation at higher temperatures and lower pH values. The product of the spontaneous reaction was characterized as DPAc by recrystallization from hot water and by ion exchange chromatography on a Dowex-1 column.

Sonic extracts from sporulating cells of B.megaterium catalyze formation of DPAc at a rate far greater than that of the spontaneous reaction (Table 2).

The enzymatic synthesis is dependent on the presence of ASA and is abolished by boiling the extract. These results confirm the findings of Bach and Gilvarg (1966).

Attempts to prepare active cell free extracts of the Marburg strain of B.subtilis by lysozyme treatment, sonication or grinding with glass beads were unsuccessful. However, when sporulating cells of B.subtilis were treated with the detergent CETAB, a ten-fold

Table 2. SYNTHESIS OF DIPICOLINIC ACID BY EXTRACTS OF B.MEGATERIUM

Additions	Product formed (mumoles)
none	< 0.5
CE of E.coli	2.6
CE + extract of B.megaterium (0.6 mg prot.)	34.6
CE + boiled extract of B.megaterium	3.7
CE + extract of B.megaterium, L-ASA omitted	< 0.5

The reaction mixture was the same as that in Table 1, except that it included EDTA, 5 mM.

Table 3. SYNTHESIS OF DIPICOLINIC ACID BY DETERGENT-TREATED CELLS OF B.SUBTILIS

Additions	Product formed (relative quantity)
none	< 2
CE of E.coli	9
CE + treated cells of B.subtilis (0.7 mg prot.)	100
CE + boiled treated cells	11
CE + treated cells, L-ASA omitted	< 2
CE + treated cells, NAD^+ omitted	53

The reaction mixture was the same as that in Table 2, except that the buffer was potassium phosphate, pH 7.0, 0.2M ; and NAD^+ was included at a concentration of 0.5 mg/ml. A suspension of cells was treated with detergent by adding an equal volume of a solution of CETAB (1 mg/ml) and shaking vigorously in the cold. A relative quantity of 100 represents approximately 43 mumoles of DPAc.

stimulation of DPAc production over the spontaneous level was observed, as shown in Table 3. The catalytic activity is again dependent on the presence of ASA and is destroyed by heating. The product was again characterized chemically as DPAc. The presence of a cofactor is necessary for maximal activity. The omission of NAD^+ results in a two-fold decrease in the amount of DPAc formed. FAD is also active as a cofactor.

Figures 2a and b show that the reaction catalyzed by the CETAB-treated cells of B. subtilis proceeds at a constant rate for approximately 20 minutes at 30°C, and is proportional to the concentration of cells added.

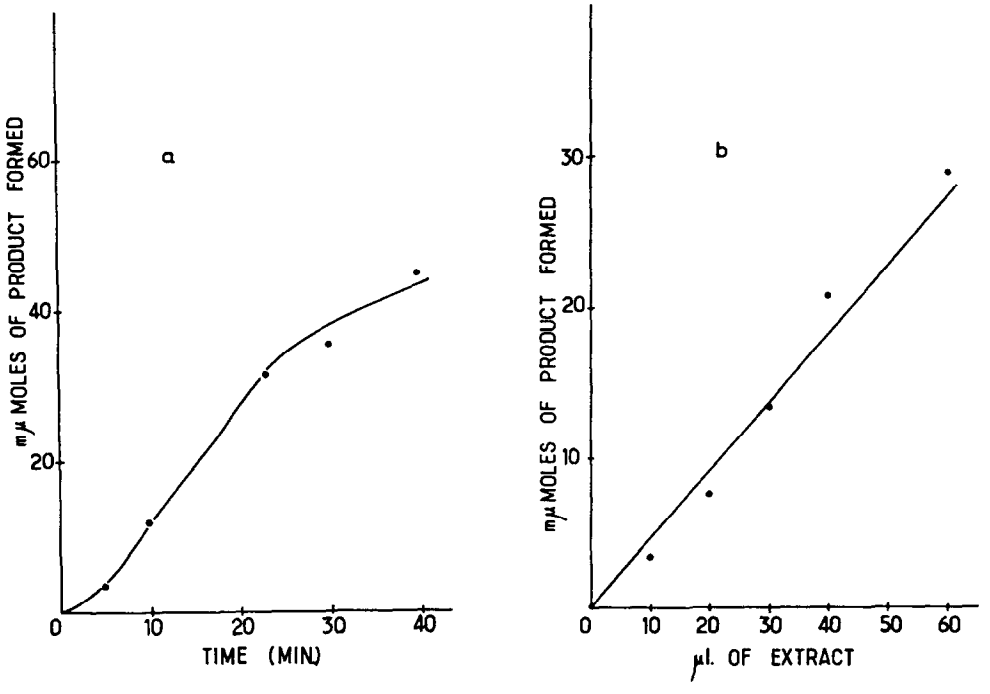

Fig. 2. Dependence of activity on (a) time (b) concentration of cells added.

The proposed pathway for DPAc biosynthesis (fig. 1) is supported by these observations with extracts and detergent-treated cells. The operation of the same pathway in vivo is indicated by the fact that a recently isolated mutant of B. lichenformis, blocked at an early step in lysine biosynthesis, produces spores containing very little DPAc (Aronson et al., 1967).

We then undertook a study of the kinetics of appearance of the enzymatic activity, called for convenience DPAc-synthetase, catalyzing the last step in DPAc biosynthesis in B. subtilis. As shown in Figure 3a, there is a sharp increase of about four-fold in the specific activity of the DPAc-synthetase over the activity apparently

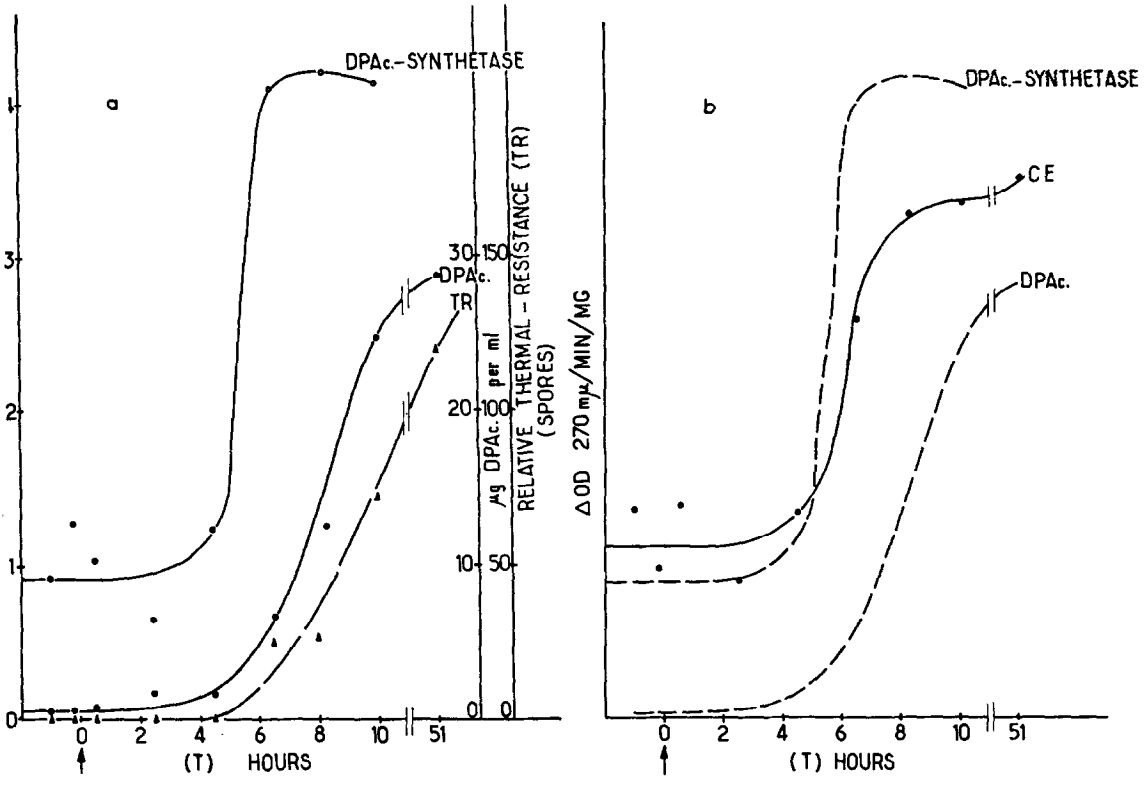

Fig. 3. Kinetics of events during sporulation. The cells were grown in a Bacto-peptone medium to which were added 10^{-4}M $MnCl_2$, 10^{-3}M $CaCl_2$; 10^{-3}M $MgSO_4$; 10^{-6}M $FeSO_4$ and 10^{-2}M KCl. The condensing enzyme was assayed in lysozyme lysates by the imidazole method of Yugari and Gilvarg (1965). a) ▲—▲, relative thermal resistance ; ●—●, DPAc ; o—o, DPAc-synthetase. b) ●—●, condensing enzyme.

present in exponentially growing cells. This increase occurs 5 hours after the cessation of exponential growth (T_5), coincident with the development of thermal resistance in the bacterial population and with the initiation of DPAc synthesis in the developing spores. This increase in activity, and the time at which it occurs, are further indications that the reaction measured is physiologically significant.

We also measured the activity of the condensing enzyme catalyzing the step in DPAc formation immediately preceding that of the DPAc-synthetase, i.e., the condensation of ASA with pyruvic acid to form dihydrodipicolinic acid. As can be seen in Figure 3b, the specific activity of this enzyme also increases at T_5. We have found that the condensing enzyme in exponentially growing cells is

neither repressed nor inhibited by lysine and L-diaminopimelic acid. Consequently, the increase in the specific activity of this enzyme observed during sporulation cannot be attributed to an effect by these end-products.

The increase of these two enzymatic activities can explain the de novo synthesis of DPAc during the course of sporulation. Although the two enzymes studied are involved in the same biosynthetic pathway, they pose two different regulatory problems for the cell. The DPAc-synthetase is an enzyme exclusively concerned with the production of a specific spore component, while the condensing enzyme is implicated both in the vegetative life of the cell and in spore formation. A further investigation of these and related enzyme activities during sporulation in B. subtilis is underway in the hope of determining the nature of the regulatory mechanisms involved.

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