

THE EFFECTS OF SOME ALPHA PICOLINIC ACID DERIVATIVES  
ON GROWTH AND SPORULATION OF BACILLI

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The effects of ethyl picolinate and picolinamide on growth and sporulation of B. megaterium and B. subtilis have been studied in synthetic medium containing glucose. Ethyl picolinate inhibits sporulation probably by undergoing hydrolysis intracellularly, the acid produced chelating with some metal essential for utilization of the neutral intermediates. Picolinamide gives heat labile spores but the mechanism does not appear to be metal chelation. Picolinamide may interfere with the role of dipicolinic acid as an activator of NADH<sub>2</sub> oxidase. Different sites of action of alpha picolinic acid, its ester and amide have been proposed.

Dipicolinic acid (DPA) first isolated from spores of Bacillus megaterium by Powell (1953) plays a vital role in heat resistance of bacterial spores. Its role as an electron acceptor has also been emphasized. Various inhibitors which interfere with the formation of spores or which result in production of DPA deficient heat labile spores have been extensively studied by Gollakota and Halvorson (1963). Alpha picolinic acid (APA), a structural analogue of DPA has been found by them to specifically inhibit sporulation by interfering with the formation of enzymes involved in utilization of intermediate acids probably by chelating with some metal ion because APA inhibition could be reversed by Zn<sup>++</sup>, Co<sup>++</sup> or Ni<sup>++</sup>. Succinic and malonic acids were also found to reverse the

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effects of APA. On the other hand, diethyl succinate and diethyl malonate failed to reverse the effects of APA. Further, these esters themselves specifically inhibited sporulation. Masking of the carboxyl groups by esterification thus resulted in a radical change of biological activity. Therefore, a study of the effects of ethyl picolinate and nicolinamide, in which the carboxyl group essential for chelation has been masked, was undertaken.

#### Materials and Methods:

B. megaterium 753 and B. subtilis 15u were cultured in sterilized media, whose compositions are given in Table 1. The active culture technique developed by Collier (1957) was used for growth of these organisms. The time of final transfer was called as zero hour. The cultures were incubated at  $30^{\circ} \pm 1^{\circ}\text{C}$  on a rotary shaker (160 rpm). Total viable, heat or octyl alcohol stable counts were made by plating on starch nutrient agar appropriate dilutions of culture as such, after heat ( $80^{\circ}\text{C}$  for 30 mts) or octyl alcohol (0.5% octyl alcohol for 15 mts) treatment respectively. Ethyl picolinate was prepared by the method of Ulrich and Hans (1960). Picolinamide was prepared by treating ethyl picolinate with cold liquor ammonia in the presence of absolute alcohol. Ethyl picolinate and picolinamide were added to the culture as such. APA was added after adjusting the pH to 7.0 with NaOH.

#### Results

Inhibitory effects of alpha picolinic acid and its derivatives: Added at zero hour  $1.21 \times 10^{-3}\text{M}$  APA specifically inhibited sporulation of these organisms. However,  $1.62 \times 10^{-3}\text{M}$  APA was routinely used in these studies.

Minimum concentration of ethyl picolinate inhibitory to

TABLE I

Synthetic media for B. megaterium 753 and  
B. subtilis 15 $\mu$

Components	<u>B. megaterium</u> 753 gm/litre	<u>B. subtilis</u> 15 $\mu$ gm/litre
FeCl <sub>3</sub>	0.0005	0.0005
MgSO <sub>4</sub>	-	0.0040
MgCl <sub>2</sub>	0.0040	-
MnCl <sub>2</sub>	0.0125	0.0125
NH <sub>4</sub> NO <sub>3</sub>	0.0950	0.1000
NH <sub>4</sub> Cl	0.0550	-
Na <sub>2</sub> SO <sub>4</sub>	0.0500	0.0500
KH <sub>2</sub> PO <sub>4</sub>	1.3600	1.000
K <sub>2</sub> HPO <sub>4</sub>	-	3.000
CaCl <sub>2</sub>	0.0100	0.0800
L-alanine	-	0.375 **
L-asparagine	-	0.375
Sodium citrate	-	1.000
D-Glucose	1.800	2.000
Sodium glutamate	1.500 *	1.500
pH	7.1 - 7.2	7.1 - 7.2

\* Sodium glutamate was omitted in final transfer in  
B. megaterium 753.

\*\* L-alanine omitted in first and subsequent transfers in  
B. subtilis 15 $\mu$

sporulation of these organisms was found to be  $2.22 \times 10^{-2}$ M.  
Studies on the effects of time of addition of APA and ethyl

TABLE 2

Effect of time of addition of ethyl picolinate  
and APA on sporulation of B. megaterium 753.

Time of addition	pH before addition	After incubation for 40 hr.	
		Acid (APA)	Ethyl picolinate
0	7.2	-	-
2	6.9	-	-
6	6.6	-	-
9	6.4	-	-
10	6.5	+	-
12	6.7	+	-
15	6.8	+	-
17	6.9	+	-
19	7.0	+	-
20	7.1	+	+
22	7.2	+	+
30	7.6	+	+
36	7.6	+	+

+ sporulation

- no sporulation

picolinate in B. megaterium (Table 2) indicate that alpha picolinic acid is effective only if added before the pH begins to rise. Ethyl picolinate was found to be effective even when the pH had recovered upto 7.00 .

These organisms formed heat labile spores in the presence of picolinamide. The proportion of heat stable spores consist-

ently decreased with increasing picolinamide concentration and at a concentration  $2.02 \times 10^{-2}M$  to  $2.43 \times 10^{-2}M$  of picolinamide, only 4% heat stable spores were formed, the rest being heat labile.

Reversal of Inhibition by zinc sulphate: Zinc sulphate ( $7 \times 10^{-4}M$ ) which inhibited sporulation if added alone was found to completely reverse the inhibition caused by alpha picolinic acid when added together with the inhibitor at zero hour.

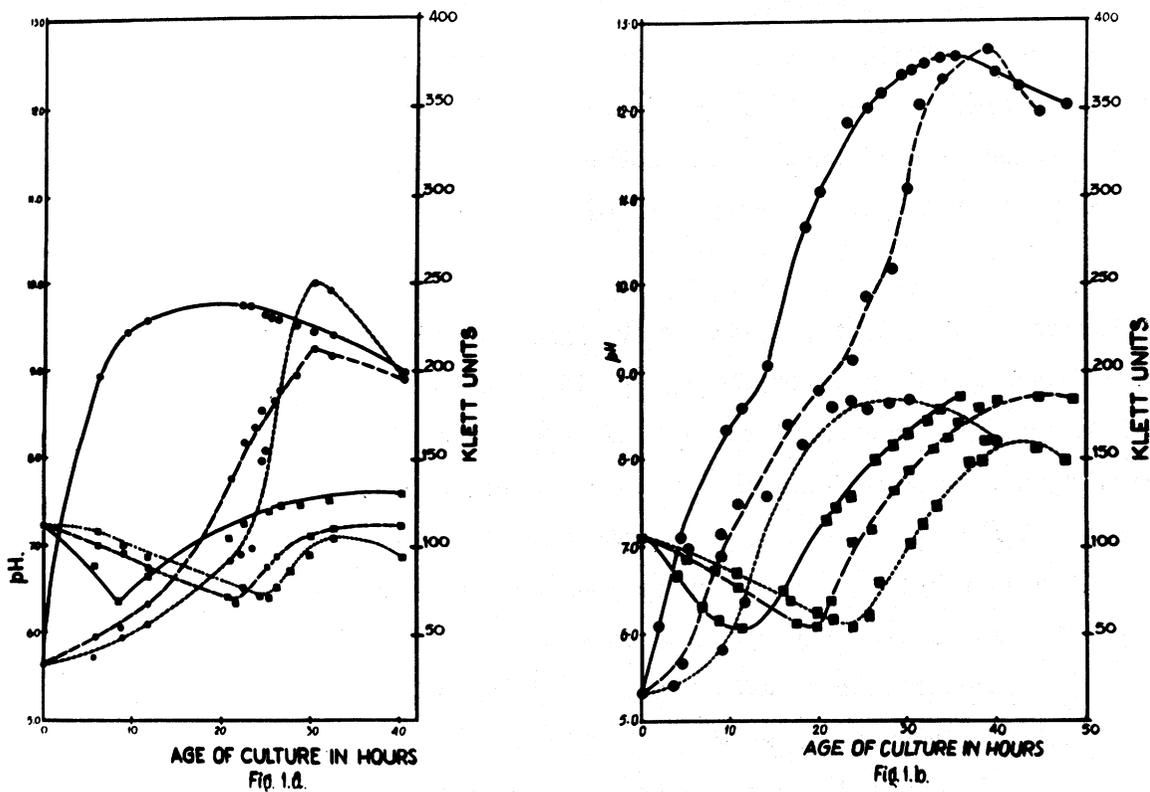


Fig. 1 Growth and pH changes in *B. megaterium* 753 (1a) and *B. subtilis* 15u (1b). Klett readings, control  $\bullet-\bullet-$  in presence of ethyl picolinate  $\cdots-\bullet-\cdots$  and in presence of ethyl picolinate and reversing concentration of Zinc sulphate  $\bullet-\cdots-\bullet-$  pH changes, control  $\blacklozenge-\blacklozenge-$ , in presence of ethyl picolinate  $\cdots-\blacklozenge-\cdots$  and in presence of ethyl picolinate and reversing concentration of Zinc sulphate  $\blacklozenge-\cdots-\blacklozenge-$

Inhibition caused by ethyl picolinate ( $2.22 \times 10^{-2}M$ ) was reversed by comparatively lower concentration of zinc sulphate ( $3.5 \times 10^{-5}M$ ). Fig. 1 shows the growth and pH curves of B. megaterium and B. subtilis in control, in presence of ethyl picolinate and reversing concentrations of zinc sulphate added together at zero hour. Growth was slower in initial stages in ethyl picolinate inhibited cultures but pH finally reached neutrality. In zinc sulphate reversed cultures the final O.D. and pH reached nearly the same level as in control although the initial lag did not totally disappear.

Different concentrations of zinc sulphate added along with  $2.43 \times 10^{-2}M$  nicotinamide failed to increase the thermoresistance of the spores. Hence zinc sulphate could not reverse the effects of nicotinamide on heat resistance of the spores.

#### Discussion.

Sporulation of B. megaterium 753 and B. subtilis 15u has been found to be specifically inhibited by APA ( $1.62 \times 10^{-3}M$ ) when grown in a synthetic medium containing glucose if added before the pH began to rise. This inhibition could be reversed by zinc sulphate ( $7 \times 10^{-4}M$ ). Ethyl picolinate also inhibited sporulation of these organisms but its minimum effective concentration ( $2.22 \times 10^{-2}M$ ) was roughly 15 times more than the minimum effective concentration of APA. Ethyl picolinate seems to undergo only mild hydrolysis as shaking the blank medium with ethyl picolinate for 40 hours did not result in any significant pH change. Further, the concentration of zinc sulphate ( $3.5 \times 10^{-5}M$ ) required to reverse its effect was much lower than that required to reverse the effects of APA. Therefore, the effects of ethyl picolinate may be due to the picolinic acid produced from it on hydrolysis. However, there are two significant

differences: (1) the disproportionately smaller amount of zinc sulphate required to reverse the effects of ethyl picolinate. APA may be acting by chelating with the metal extracellularly while the APA produced intracellularly from the ethyl picolinate may be active. This can explain the differences in the minimum inhibitory concentration of these two compounds. (2) the fact that in ethyl picolinate inhibited cultures the pH reaches neutrality whereas in APA inhibited cultures the pH stays at the low level reached during vegetative growth. The enzyme systems inhibited by APA and ethyl picolinate may be different although metal involvement is indicated in both cases.

The metals involved may be the same or different. In either case, the metal involved in utilization of acid intermediates must be chelating more strongly with the enzyme concerned than with APA. Similarly the binding between APA and the metal involved in utilization of neutral intermediates must be stronger than that between the metal and the concerned enzyme. The identity of the enzyme systems involved is being investigated. Hanson et al (1963) found that APA specifically inhibited the formation of aconitase in B. cereus T. Recently Fortnagel & Freese (1968) have shown that APA inhibits aconitase noncompetitively in B. subtilis.

With picolinamide ( $2.43 \times 10^{-2}M$ ) heat labile spores were formed. Thus the effects of picolinamide on sporulation are totally different from those of ethyl picolinate and alpha picolinic acid. Further, the mode of action of picolinamide does not seem to be due to its hydrolysis into APA as  $Zn^{++}$  could not reverse the effect of picolinamide. DPA was shown to activate the  $NADH_2$  oxidase of spores by an electron accepting mechanism (Halvorson et al 1958). Therefore, picolinamide, a structural analogue of nicotinamide which is a constituent of NAD, may

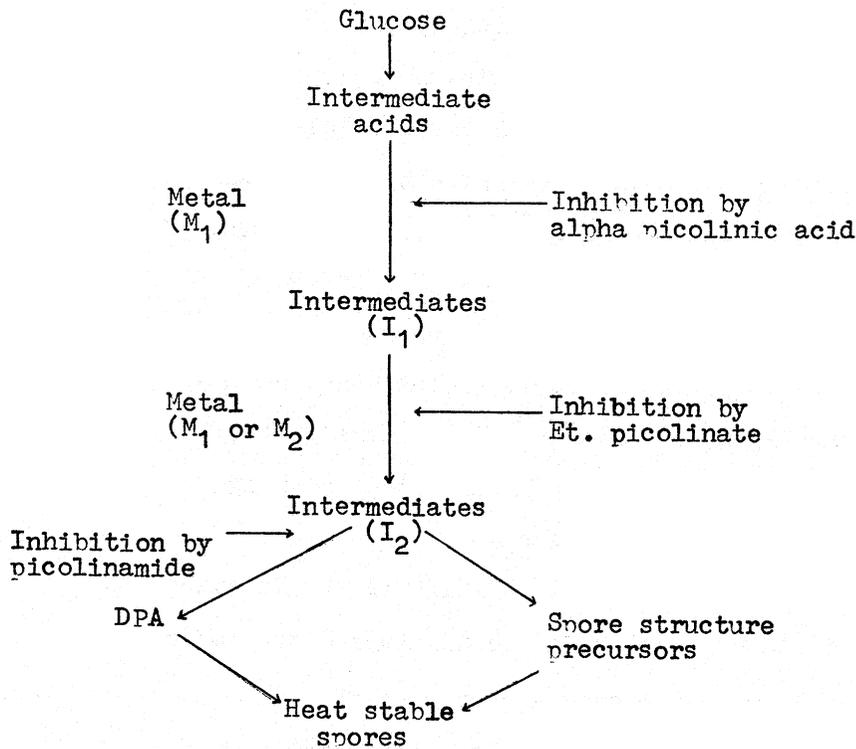


Fig. 2. Model showing the probable sites of inhibition by APA, ethyl picolinate and picolinamide.

be interfering with the above role of DPA in the spores. Thus picolinamide may be effective even when ethyl picolinate has ceased to act. Fig. 2 shows the proposed model indicating the sites of inhibition. Metals  $M_1$  and  $M_2$  shown therein may be same or different.

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