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BIOCHEMICAL CHANGES OCCURRING DURING MICROCYCLE SPOROGENESIS OF *Bacillus cereus* T

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SUMMARY: The biochemical changes occurring during microcycle sporogenesis of B.cereus T in glucose enriched Mackechnie and Hanson medium(1) was studied by using alpha picolinic acid and ethyl picolinate. Alpha picolinic acid inhibited microcycle sporogenesis by chelating with some metal essential for the transition of a vegetative cell to a sporulating cell probably by suppressing aconitase. The mode of action of ethyl picolinate did not seem to be metal chelation as its effects could not be reversed by zinc sulphate.

INTRODUCTION:

Microcycle sporogenesis, where spores germinated,elongated and resporulated without any intervening cell division has been reported by Vinter and Slepecky(2) in *Bacillus cereus* (NCIB 8122), Mackechnie and Hanson(1) in *Bacillus cereus* T and Holmes and Levinson(3) in *Bacillus megaterium* QMB 1551.

During the present study, B.cereus T was selected because of the relative abundance of data concerning outgrowth and sporogenesis of this organism. Microcycle sporogenesis has been studied for two reasons, primarily because of paucity of information and also because it gives a system uncomplicated by cell division for investigating the biochemical process associated with the conversion of a vegetative cell to a spore. Alpha picolinic acid(APA) and ethyl picolinate are the inhibitors of normal sporogenesis

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with widely different sites of action. The effects of these inhibitors on microcycle sporogenesis have been compared with those of normal sporogenesis.

MATERIALS AND METHODS:

Media and culture condition: The microcycle medium described by Mackechnie and Hanson (1) enriched with 0.1% glucose was used in these investigations. Spores for microcycle cultures were suspended in sterile distilled water to give approximately 4.69×10^9 spores/ml and heat activated for two hours at 65°C. They were washed and resuspended in sterile distilled water and used to inoculate 10 ml of the microcycle medium to give approximately 6.7×10^7 spores/ml. The cultures were incubated at 30°±1°C on a rotary shaker (160 r.p.m.). Under these conditions, aeration of the culture was adequate for resporulation. The number of cells completing microcycle sporogenesis was determined by examining wet mounts under phase microscope and counting the percentage of refractile spores. Total viable count(TVC), heat stable counts (HSC-30 mts at 65°C) and octyl stable counts (OSC) were measured during microcycle sporogenesis by using standard plating techniques. Optical density(O.D.) of the culture was determined by 'Spectronic 20' spectrophotometer at 440 mμ.

Preparation of spores: The spores of Bacillus cereus T were prepared by active culture technique described by Collier(4) with suitable modification by Halvorson(5). The cultures were incubated at 30°C±1°C on rotary shaker at 160 r.p.m. until a large percentage of free spores were formed (25-30 hrs). The spores were harvested in refrigerated centrifuge, washed 8 to 10 times with approximately 3 liters of sterile distilled water mixed with 1 to 2 ml of octyle alcohol to prevent germination of spores during washing. The final 3 to 4 washings were done with only sterile distilled water when the spores were to be used immediately. Before use, the stored spore suspensions were washed a couple of times to remove the octyl alcohol.

Chemicals used: The chemicals used in these studies were of Analytical Reagent(A.R.) grade except ethyl picolinate which was prepared in the laboratory. Ethyl picolinate was prepared by the method described by Ulrich, Haug and Hanse(6).

Inhibition studies: The inhibitors were added at the times denoted to give the desired concentrations. Stock solution of APA was prepared by dissolving it in water, adjusting the pH to 7.0 and then autoclaving. Ethyl picolinate was added without any pretreatment.

Reversal studies: The reversal agent used in the present study was added to the cultures at the appropriate time to give the desired concentration.

RESULTS AND DISCUSSION

Nakata and Halvorson(7) have shown that during normal sporogenesis of B.cereus T in a medium containing glucose, acetic and pyruvic acids are accumulated during logarithimic growth that

causes lowering the pH of the medium. These accumulated acids are further metabolised during transition from vegetative growth to sporulation and the pH of the medium rises.

Mackechnie and Hanson(1) reported microcycle sporogenesis of B.cereus T in a chemically defined medium which did not contain glucose. In the absence of glucose, the pH of the culture continuously rises and most inhibitors of normal sporogenesis which are effective in the presence of glucose, lose their activity in the absence of glucose. The biochemical changes occurring in the presence of glucose may, therefore, be different from those in the absence of glucose.

In the present study, it has been found that addition of glucose to the microcycle medium did not affect microcycle sporogenesis in B.cereus T. In the presence of glucose, the pH dropped from 7.2 to 6.95 by 8th hour and subsequently increased to 7.25 by the 30th hour of incubation (Table-1). Upto 5% of cells showed the evidence of single division.

Addition of APA (3.25×10^{-3}M) at different time intervals showed that APA inhibited the microcycle sporogenesis only upto 6 hrs. The pH of the culture stayed at 6.5 in the presence of the inhibitor. The O.D. of the culture did not show significant rise beyond the level reached during outgrowth whereas, the O.D. in the absence of APA increased steadily even after outgrowth was complete. It indicated that APA was effective only if added before the pH started rising. Similar results were obtained by Gollakota and Halvorson(8) who studied the effects of APA on the normal sporogenesis of this organism.

Hanson(9) found that APA specifically inhibited the formation of aconitase. Szulmajester and Hanson(10), Fortnagel and Freese (11) found that mutants of B.subtilis devoid of aconitase

TABLE-1

EFFECTS OF TIME OF ADDITION OF APA (3.25×10^{-3}M) ON MICROCYCLE SPORULATION OF *Bacillus cereus* T IN MICROCYCLE MEDIUM WITH GLUCOSE

AGE OF CULTURE IN HOURS	AT THE TIME OF ADDITION OF APA		AFTER 30 HOURS OF INCUBATION					
	pH	O.D.	pH	O.D.	SLIDE STUDIES	TVC/ml	OSC/ml	HSC/ml
CONTROL	7.2	0.38	7.25	0.75	Spores	7.1×10^7	8.1×10^7	7.9×10^7
0	7.2	0.38	6.5	0.49	Veg.cells	-	-	-
2	7.2	0.32	6.5	0.48	Veg.cells	-	-	-
4	7.1	0.31	6.55	0.46	Veg.cells	-	-	-
6	7.1	0.43	6.5	0.50	Veg.cells	-	-	-
8	6.95	0.45	6.6	0.53	20-30% Spores	5.9×10^7	5.1×10^6	4.2×10^6
10	7.0	0.46	6.8	0.53	60-70% Spores	6.1×10^7	2.5×10^7	2.0×10^7
11	7.1	0.57	6.95	0.67	90-95% Spores	6.1×10^7	7.1×10^7	8.8×10^7
12	7.1	0.63	7.2	0.74	90-95% Spores	-	-	-
14	7.15	0.63	7.2	0.74	90-95% Spores	5.9×10^7	6.1×10^7	7.1×10^7
18	7.2	0.72	7.25	0.75	90-95% Spores	-	-	-
25	7.25	0.74	7.25	0.74	90-95% Spores	-	-	-
30	7.25	0.75	7.25	0.75	90-95% Spores	-	-	-

were asporogenic and conditions that caused the repression of the synthesis of aconitase resulted in inhibition of sporulation. Gollakota and Halvorson(12) showed that fluoroacetic acid(FAA) specifically inhibited normal sporogenesis of *B.cereus* T in the presence of glucose and suggested that this inhibition was due to the inhibition of aconitase.

TABLE-II

EFFECTS OF DIFFERENT CONCENTRATION OF ZINC SULPHATE ON REVERSAL OF APA (3.25×10^{-3}M) INHIBITION*

TREATMENTS	AFTER 30 HOURS OF INCUBATION			
	SLIDE STUDIES	TVC/ml	OSC/ml	HSC/ml
CONTROL	Spores	5.2×10^{7}	7.1×10^{7}	6.5×10^{7}
" + APA	Veg.cells	2.1×10^{5}	5.1×10^{4}	3.9×10^{4}
" + " +$ZnSo_4$ (1.0×10^{-4}M)	Veg.cells + Spores	4.1×10^{5}	5.3×10^{4}	4.1×10^{7}
" + " +$ZnSo_4$ (4.5×10^{-4}M)	Spores	6.4×10^{7}	5.9×10^{7}	6.2×10^{7}
" + " +$ZnSo_4$ (1.0×10^{-3}M)	Spores+ unelongated vegetative cells	3.2×10^{5}	3.5×10^{4}	2.1×10^{4}

* APA and zinc sulphate were added at zero hour.

It can,therefore, be concluded that for microcycle sporogenesis of B.cereus T in the presence of glucose aconitase is indispensable.

Though, zinc sulphate (4.5×10^{-4}M) itself inhibited microcycle sporogenesis, it could completely reverse the effects of APA if added at zero hour(Table-2). This indicated that APA inhibits microcycle sporogenesis by metal chelation leading to the suppression of aconitase.

Ethyl picolinate (4.38×10^{-2}M) added at 4 hour or later inhibited the microcycle sporogenesis(Table-3). Ethyl picolinate was found to be effective upto 12 hrs, even after the recovery of pH to neutrality-a time when APA became ineffective. This shows that it did not interfere with the utilisation of the acid intermediates. Similar results were obtained by Upreti et al

TABLE-III

EFFECTS OF TIME OF ADDITION OF ETHYL PICOLINATE (4.38×10^{-2}M) ON MICROCYCLE SPORULATION OF Bacillus cereus T IN MICROCYCLE MEDIUM WITH GLUCOSE

AGE OF CULTURES (IN HOURS)	AT THE TIME OF ADDITION OF ETHYL PICOLINATE		AFTER 30 HOURS OF INCUBATION					
	pH	O.D.	pH	O.D.	SLIDE STUDIES	TVC/ml	OSC/ml	HSC/ml
CONTROL	7.2	0.38	7.25	0.75	Spores	7.8×10^7	8.1×10^7	7.9×10^7
4	7.1	0.31	7.05	0.59	Veg. cells	-	-	-
8	7.0	0.46	7.0	0.57	Veg. cells	-	-	-
12	7.1	0.61	7.0	0.65	3-5% spores	6.7×10^7	2.3×10^6	1.8×10^6
14	7.2	0.69	7.1	0.71	30-40% spores	6.1×10^7	3.0×10^7	2.3×10^7
18	7.25	0.74	7.2	0.71	90-95% spores	6.6×10^7	6.9×10^7	7.1×10^7

TABLE-IV

EFFECTS OF DIFFERENT CONCENTRATIONS OF ZINC SULPHATE ON REVERSAL OF ETHYL PICOLINATE (4.38×10^{-2}M) INHIBITION*

TREATMENTS	AFTER 30 HOURS OF INCUBATION			
	SLIDE STUDIES	TVC/ml	OSC/ml	HSC/ml
CONTROL	Spores	7.1×10^7	6.8×10^7	8.1×10^7
CONTROL+ETHYL PICOLINATE	Veg.cells	6.1×10^7	4.9×10^4	3.9×10^4
" +$ZnSo_4$ (5.0×10^{-5}M)	Veg.cells	5.1×10^7	2.9×10^4	3.1×10^4
" +$ZnSo_4$ (1.0×10^{-4}M)	Veg.cells	5.7×10^7	$< 10^5$	$< 10^5$

* Zinc sulphate and ethyl picolinate were added in the cultures at 4 hours of incubation.

(13) where ethyl picolinate inhibited the normal sporogenic cultures. The effects of ethyl picolinate on microcycle sporogenesis could not be reversed by zinc sulphate(Table-4). It suggests that ethyl picolinate inhibition of microcycle sporogenesis of B.cereus T may not be due to metal chelation and but ethyl picolinate may be interfering with the metabolism of some amino acids.

It can, therefore, be concluded that in B.cereus T in the presence of glucose, formation of a new spore from a germinated spore is characterised by the same biochemical changes whether there is intervening cell division or not - normal or microcycle sporogenesis.

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REFERENCES:

1. Mackechnie,I. and Hanson,R.S.(1968)J.Bacteriol.95,355-359
2. Vinter,V.,and Slepecky,R.A.(1965) J.Bacteriol.90,803-807
3. Holmes,P.K., and Levinson,H.S.(1967)J.Bacteriol.94,434-440
4. Collier,R.E.(1957) Spores I, pp 10-14, Inst.Biol.Sci.Symp.Series.
5. Halvorson,H.O.(1957) J.Appl.Bacteriol.20,305-314
6. Ulrich,Haug and Hanse,E.(1960)Chem.Ber.93,594-598
7. Nakata,H.M. and Halvorson H.O.(1960) J.Bacteriol.80,801-810
8. Gollakota,K.G. and Halvorson,H.O.(1960)J.Bacteriol.79,1-8
9. Hanson,R.S.(1962) Ph.D.thesis submitted to University of Illinois
10. Szulmajester,J. and Hanson,R.S.(1965) Spores III,pp 162-173, Am.Soc.Microbiol,Michigen
11. Fortnagel,P. and Freese,E.(1968) Bacteriol.Proc.37,117
12. Gollakota,K.G., and Halvorson,H.O.(1963) J.Bacteriol.85, 1386-1393
13. Upreti,G.C.,Singh,R.S.,Verma,J., Bhatia,P.L. and Gollakota, K.G.(1969) Biochim.Biophys.Res.Commun.34,611-618