DIFFERENTIATION BETWEEN HEAT RESISTANCE AND OCTYL ALCOHOL RESISTANCE OF THE CELLS OF BACILLUS CEREUS T.[1]

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Employing cultures capable of rapid growth and simultaneous sporulation, it was found that octyl alcohol resistance in B. cereus T developed earlier than heat resistance. Heat resistant cells were also resistant to octyl alcohol; but octyl alcohol resistant cells were not necessarily resistant to heat and were deficient in DPA. Heat sensitive but octyl alcohol resistant cells developed to vegetative form without going through the state of complete spore. Presence of octyl alcohol in the culture inhibited synthesis of DPA and the morphological development of those cells which survived treatment with the inhibitor.

Vegetative cells and germinated spores of sporeforming bacilli lose their viability when exposed to 80°C for 30 min., whereas mature spores survive this treatment. During a study on the germination of spores of Bacillus cereus T in milk, Gollakota and Halvorson (1963) encountered the problem of foaming when making dilutions for plate counts. Octyl alcohol was one of several antifoaming agents employed by these investigators to overcome the problem of foaming. However, it was found that octyl alcohol killed germinated spores and vegetative cells, but did not kill mature spores of the bacterium.

The purpose of this study was: (a) To determine the time during the sporulation cycle at which cells of B. cereus T become resistant to octyl alcohol and to heat; (b) to study the effect of octyl alcohol on dipicolinic

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acid (DPA) synthesis and the relation of DPA to octyl alcohol resistance (Powell, 1953); and (c) to study the germination characteristics of octyl alcohol resistant cells.

Materials and Methods:

B. cereus T was grown and maintained according to the methods described by Gollakota and Halvorson (1960). Cultures were incubated at 30 ± 1°C on a gyrotary shaker. The active culture technique of Collier (1957), modified for the growth of B. cereus T by Halvorson (1957), was used in all the experiments. Unless otherwise indicated, 0.05 ml n-octyl alcohol (Chemisch Fabrick, London) was added to 5 ml sample of the culture. The treated sample was shaken vigorously for 2 min. Total viable, octyl alcohol resistant and heat resistant counts were determined by serial dilution and plating on nutrient agar. DPA was measured by the method of Janssen et al. (1958).

Results:

Time of occurrence of octyl alcohol resistance and heat resistance during growth and sporulation: Table 1 shows changes in pH, morphology of cells, and numbers of viable cells in heat and octyl alcohol treated cultures of B. cereus T. During vegetative growth, the pH of the culture dropped from 6.7 to 4.7 after 3 hours incubation. Subsequently it increased to 7.8 by 24 hours of incubation. The changes in pH have been shown to be due to accumulation and subsequent utilization of organic acids by the bacterium (Nakata and Halvorson, 1960). The cells became resistant to octyl alcohol at about 11 hours of incubation. Heat resistance occurred 1 to 2 hours after the appearance of octyl alcohol resistant forms. Cells that were octyl alcohol resistant but heat sensitive, were stainable with crystal violet.

When a 12-hour old culture, containing about 4×10^3/ml octyl alcohol resistant cells and no heat resistant cells was incubated for an additional 24 hours in the presence of 0.05 ml octyl alcohol/5 ml of culture medium, the octyl alcohol resistant forms increased to 6×10^3/ml but no heat resistant cells were detected. This indicated that the surviving cells did

TABLE 1

pH, MORPHOLOGY AND APPEARANCE OF OCTYL ALCOHOL AND HEAT RESISTANT FORMS

Age of Culture in Hr.	pH	Morphology*	Plate Counts/ml	
			Octyl Alcohol Resistant	Heat Resistant
0	6.7	Vegetative cells in long chains	--	--
3	4.7	Slightly granulated cell	Nil	Nil
4	4.1	Granulated cells	Nil	Nil
5	6.1	Much granulation, length of chains reduced	Nil	Nil
6	6.7	Heavy granulation, clumping started	Nil	Nil
7	7.0	Heavy granulation, chains and clumps	Nil	Nil
8	7.3	Cells reduced to the shape of spores with heavy granulation, remaining in clumps	Nil	Nil
9	7.3	Same as at 8 hr.	Nil	Nil
10	7.3	Same as at 8 hr.	Nil	Nil
11	7.4	Cells took the shape of spore but stainable	5.0×10^6	Nil
12	7.4	Spores in clumps and chains, unstainable	1.7×10^7	1.0×10^6
13	7.5	Same as at 12 hr.	2.0×10^7	9.0×10^6
24	7.8	Spores in the chains of 2 to 4	2.5×10^8	1.4×10^8

*Observed microscopically from crystal violet stained smears.

not become heat resistant in the presence of octyl alcohol.

The changes in pH and turbidity of the culture when incubated for 24 hours after addition of octyl alcohol are illustrated in Fig. 1. The pH remained unchanged but the turbidity of young culture was reduced. As the age of the culture increased the reduction of turbidity was less and octyl alco-

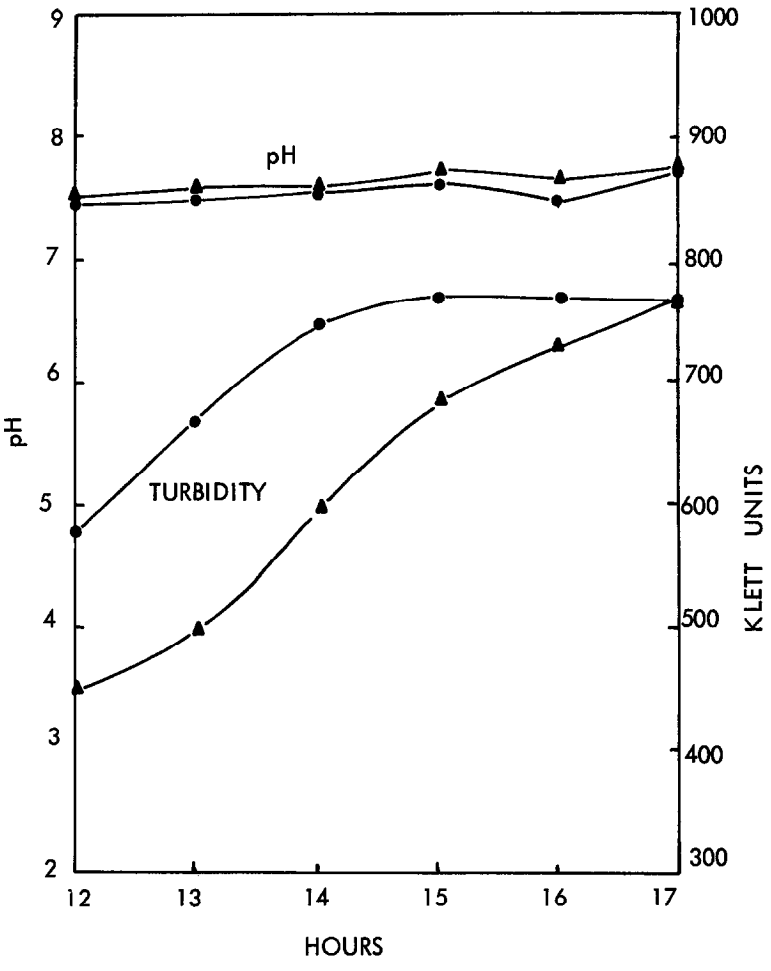

Fig. 1: Effect of octyl alcohol on pH and turbidity of the cultures. Octyl alcohol was added to samples of the culture at different time intervals and incubated for 24 hours. ●, at the time of addition of octyl alcohol; ▲, after 24 hours following the addition of octyl alcohol.

hol had no effect on the turbidity of 17-hour old culture. The reduction in turbidity of the culture was due to lysis of octyl alcohol sensitive cells as evidenced by crystal violet stain preparations.

DPA content of 12-hour old culture containing octyl alcohol resistant cells but no heat resistant cells, was about 6.4 μg/ml (Fig. 2). Subsequently the amount of DPA increased linearly reaching a maximum of 59.2 μg/ml at the age of 17 hours. This increase in DPA was accompanied by an increase in the

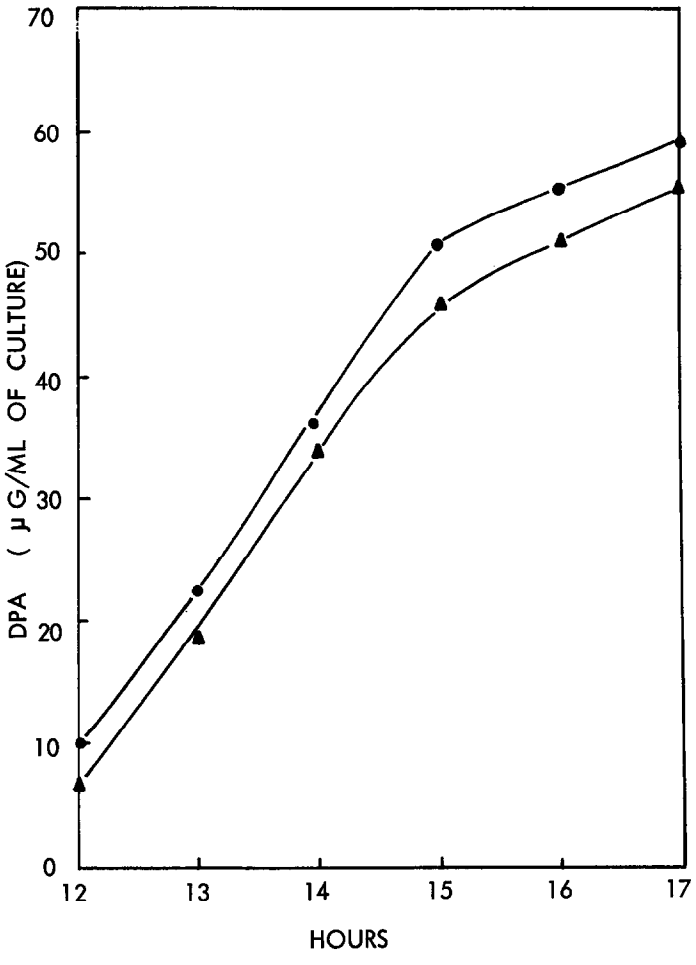

Fig. 2: Effect of octyl alcohol on DPA synthesis when the culture was incubated for 24 hours in presence of the compound. ●, at the time of addition of octyl alcohol; ▲, after 24 hours following the addition of octyl alcohol.

number of heat resistant cells. After 24 hours incubation following the addition of octyl alcohol no increase in DPA content or heat resistant forms was observed. It was, therefore, evident that the presence of octyl alcohol arrested metabolic activity of the cells, without regard to their resistance or sensitivity to octyl alcohol.

Response of octyl alcohol treated cells in a non-growth medium: Twelve, 13 and 14-hour old cultures were treated with octyl alcohol (0.05 ml/5 ml culture) for 1 hour at 30° C. The cells were then washed two times, resus-

pended in a non-growth medium (the growth medium without glucose or yeast extract) and incubated on the shaker. Crystal violet smears were prepared every hour for microscopic examination.

Cells from a 12-hour old culture at 0 time in the non-growth medium were heavily granulated and heat labile, but many were also octyl alcohol resistant. The 13-hour old culture contained a few spores while the 14-hour culture contained only spores. Four hours after resuspension in the non-growth medium, cells from the 12- and 13-hour cultures developed into vegetative cells. After 6-7 hours in the non-growth medium the cells were granulated and in chains. After 24 hours in the non-growth medium chains of spores and clumps of heavily granulated cells were observed in 12-hour old culture, while 13-hour culture exhibited, in addition to these morphological types, some old spores. Old spores were distinguishable from new ones on the basis of their degree of stainability (new spores were somewhat clearer

TABLE 2

EFFECT OF SUSPENDING THE OCTYL ALCOHOL TREATED CULTURE IN NON-GROWTH MEDIUM

Age of culture (Hours)	Plate counts/ml at the time of treatment		Plate counts/ml 24 hrs. after treatment and incubation in non-growth medium	
	Octyl Alcohol	Heat	Octyl alcohol resistant plate counts/ml	Heat resistant plate counts/ml
12.00	4.0×10^3	100	8.0×10^7	3.4×10^7
13.00	9.2×10^6	1.3×10^8	1.8×10^8	5.4×10^7
14.00	9.2×10^6	6×10^6	1.7×10^8	3.9×10^7

Note: The samples were treated with octyl alcohol for one hour, washed twice and resuspended in non-growth medium; then incubated for 24 hours at 30°C on a gyrotary shaker.

compared to old spores). The 14-hour old culture showed no changes even after 24 hours incubation in the non-growth medium.

Octyl alcohol and heat resistant counts of the cultures at 0 time and 24 hours after incubation in non-growth medium are recorded in Table 2. Counts, approaching maximum population, of heat and octyl alcohol resistant cells were obtained when these cultures were plated on nutrient agar after 24 hours incubation from the time of octyl alcohol treatment (Table 2). Most probably, this increase in octyl alcohol resistant forms and appearance of heat resistant forms in 12- and 13-hour cultures were due to growth and sporulation of octyl alcohol resistant cells at the expense of nutrients released from dead cells. Increase in the counts of 14-hour old culture was perhaps due to separation of clumps and chains of spores. The spores of B. cereus T did not germinate in non-growth medium.

Discussion:

Gollakota and Halvorson (1963) reported that germinated cells and vegetative cells of B. cereus T were killed immediately when exposed to octyl alcohol. Our data show that octyl alcohol resistant cells were present in 12 ± 1 hour cultures of the bacillus, a time when complete spores were not yet in evidence. Such cells were practically DPA-deficient. This latter observation is in agreement with that reported by Gollakota and Halvorson (1963) who observed that the DPA content of the spores formed in the presence of ethyl oxamate and diethylpimelate was about 10% of that of normal spores. Such spores were octyl alcohol resistant but heat sensitive.

The octyl alcohol resistant cells incubated for 24 hours in the presence of octyl alcohol were unable to synthesize DPA and, therefore, did not acquire heat-resistance. In general octyl alcohol is known to interfere with L-amino acid oxidases. Gollakota and Halvorson (1963) reported that inhibition of germination of spores of B. cereus T by octyl alcohol might be due to these properties of octyl alcohol. Probably DPA synthesis was inhibited in the presence of octyl alcohol due to its interference with amino acid

oxidases. Martin and Foster (1958) have reported that various amino acids are incorporated into the DPA of sporulating cells. They further concluded that DPA is synthesized by the condensation of aspartate with pyruvate or oxaloacetate with alanine. Therefore, it may be possible that due to the inactivation of amino acid oxidases, the required precursors were not available for DPA synthesis.

Studies with octyl alcohol treated, washed, and resuspended cells into non-growth medium revealed that the octyl alcohol resistant (heat sensitive) cells did not sporulate but did develop to vegetative cells. These cells probably developed to vegetative cells at the expense of nutrients available from dead cells. On prolonged incubation some of these newly developed cells sporulated in a normal way. Therefore, the mechanism of octyl alcohol resistance is different from that of heat resistance.

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