Inhibition of Genetic Transformation in *Bacillus subtilis* by Phenethyl Alcohol

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SUMMARY

Phenethyl alcohol (PEA) has been found to obstruct genetic transformation in *Bacillus subtilis*. A concentration of 0.3 % (v/v) PEA prevents the development of competence and destroys predeveloped competence without causing a measurable decrease in viable cells. Treatment of competent cells with 0.3 % PEA prior to their exposure to transforming deoxyribonucleic acid (DNA) causes the cells to become incapable of irreversibly incorporating DNA. Treatment of competent cells which have irreversibly incorporated DNA with 0.3 % PEA causes a reduction in the rate of transformation. These observations suggest that PEA affects the ability of competent cells to irreversibly incorporate DNA and also affects the cells' ability to express the DNA in the form of transformed cells once the DNA is irreversibly incorporated. The possible nature of the effect of PEA on competent cells is discussed.

INTRODUCTION

Phenethyl alcohol (PEA) has been shown to be inhibitory to Gram-negative (Lilley & Brewer, 1953) and Gram-positive (Berrah & Konetzka, 1962; Mendelson, 1965) bacteria, and animal cells (Zahn *et al.* 1966; Leach *et al.* 1964; Bruchosvky, Mak & Till, 1967; Bruchovsky & Till, 1967; Rosenkranz, Mednis, Marks & Rose, 1967; Plagemann, 1968).

The nature of the inhibitory action of PEA on bacteria has been studied extensively, but the results have been somewhat contradictory. Berrah & Konetzka (1962), Treick & Konetzka (1964), and Lark & Lark (1966) report that PEA blocks deoxyribonucleic acid (DNA) synthesis. Rosenkranz, Carr & Rose (1964, 1965*a, b*) and Rosenkranz *et al.* (1967) attribute PEA action to an inhibition of ribonucleic acid (RNA) synthesis. Lester (1965) and Silver & Wendt (1967) found that PEA affected the permeability of bacterial cells. Silver & Wendt (1967) associate the permeability change with a limited breakdown of the cytoplasmic membrane. Plagemann (1968) concludes that PEA affects a major process of growth control in rat hepatoma cells, and he suggests that this effect may be the result of PEA acting on the cytoplasmic membrane.

We tested the effect of PEA on genetic transformation of *Bacillus subtilis* 168 and found the compound to be very inhibitory. This same observation was made independently and almost simultaneously in another laboratory (Leach & Richardson, 1966). We undertook an investigation of the nature of the effect of PEA on *B. subtilis* transformation and obtained the data presented in this report.

METHODS

Organisms. *Bacillus subtilis* 168 *ind*, obtained from Dr W. Romig, University of California at Los Angeles, was used as the recipient. *Bacillus subtilis* 168 *ind str-r*, obtained by u.v.-induction, served as the non-radioactive transforming DNA donor. To prepare tritium-labelled transforming DNA, we used *B. subtilis* 168 *ind thy str-r*, a strain that was acquired by transforming *B. subtilis* 168 *ind thy* (Farmer & Rothman, 1965), obtained from Dr F. Rothman, Brown University, Providence, Rhode Island, with DNA obtained from *B. subtilis* 168 *ins str-r*. Cultures were maintained as spore stocks on potato extract agar (Thorne, 1962). Each week that vegetative cells were needed they were prepared by an inoculum from the spore stock on Antibiotic Medium 3. All incubation of the growing organisms was at 37°.

Media. The salts medium (MS) of Spizizen (1958) was used as the base for all defined media and for dilution blanks. Growth medium contained MS plus 0.5 % glucose and 5.0 µg./ml. L-tryptophan. Transformation medium (TM) contained MS, 0.5 % glucose, 5.0 µg./ml. L-tryptophan, 10⁻⁴ M-ferrous ammonium sulphate, and 0.01 % acid hydrolysed casein. Growth medium, with and without L-tryptophan, additionally supplemented with 0.005 % acid hydrolysed casein, was used as a plating medium. Difco-Brain Heart Infusion (BHI) and Difco-Antibiotic Medium 3 were also used as plating media. All plating media contained 1.5 % agar.

Extraction of DNA. Deoxyribonucleic acid was extracted according to a modification of the procedure of Marmur (1961).

Procedure for attaining competence. Growth medium was inoculated with a loopful of vegetative *Bacillus subtilis* 168 *ind* and incubated until the culture reached an optical density at 600 mµ of about 0.490 on the Spectronic 20 spectrophotometer. Then the culture was monitored until 1.0 ml. added to 5.0 ml. of TM would adjust the optical density to 0.100. The TM, freshly inoculated at that rate, was divided into 5.0 ml. samples to allow easy sampling at close intervals. The samples were incubated in a gyrotory shaker and at desired intervals aliquots were removed and assayed for viable organisms and for frequency of transformation.

Transformation assay. The frequency of transformation was assayed by exposing cells to DNA in transformation tubes containing 0.3 ml. cells, 0.3 ml. DNA dissolved in Marmur's (1961) saline citrate, and 2.4 ml. MS containing 0.5 % glucose. When phenethyl alcohol (PEA) was added to transformation tubes, the volume of MS containing 0.5 % glucose was adjusted to allow a final transformation tube volume of 3.0 ml. The frequency of transformation was calculated by dividing the number of transformants appearing after a period of DNA uptake by the viable cell titre at the beginning of the uptake period. Organisms were exposed to DNA for 30 min. unless otherwise indicated. After being exposed to DNA, organisms were removed from the transformation tubes and were impinged upon membrane filters. The impinged organisms were washed with about 50 ml. MS to stop DNA incorporation (and to remove PEA when present). In some experiments, DNA incorporation was stopped with deoxyribonuclease (DNase), but organisms were still impinged upon membrane filters and washed. DNase treatment consisted of exposing organisms and DNA to 50.0 µg./ml. of DNase and 5.0 µmde-MgSO₄/ml. for 5.0 min. Membrane filters with impinged, washed organisms were transferred to streptomycin-free plates and incubated 4 hr before the membranes were transferred to plates containing

dihydrostreptomycin sulphate 450 $\mu\text{g./ml.}$ Colonies arising from transformed organisms were counted after 24 hr on BHI, or after 36 to 48 hr on L-tryptophan-free growth medium containing 0.005 % acid hydrolysed casein. Viable count plating was done at the time the organisms were added to transformation tubes. When transformants were plated to BHI, viable count plating was done on Antibiotic Medium 3, otherwise viable count plating was done on growth medium containing 0.005 % acid hydrolysed casein. DNA was used at saturating concentrations in all transformation experiments. When radioactive DNA incorporation was measured, DNase-treated organisms were washed 3 times in ice-cold MS. The washed cells were then dissolved in 0.5 ml. N-NaOH and 14 ml. scintillation fluid (Lin, Mosteller & Hardesty, 1966).

Preparation of tritium-labelled transforming DNA

Bacillus subtilis 168 *ind thy str-r* was routinely grown in MS containing 0.5 % glucose, L-tryptophan 40 $\mu\text{g./ml.}$, 0.005 to 0.01 % acid hydrolysed casein, thymidine 50.0 $\mu\text{g./ml.}$, and aminopterin 10 to 100 $\mu\text{g./ml.}$ Methyl-tritiated thymidine (sp.act. 2.0 C/mm) was added at concentrations of 50 mc/l.

RESULTS

We elected to test PEA on *Bacillus subtilis* transformation at a concentration which would permit some growth, but at a reduced rate. As can be seen from Fig. 1, 0.3 % (v/v) PEA was found to be partially inhibitory; therefore, this concentration was used in transformation studies.

Fig. 1

Fig. 2

Fig. 1. Effect of various concentrations of PEA on growth. Organisms were transferred from growth medium to transformation medium (TM) as if competence were to be induced. The freshly inoculated TM was then divided into five aliquots. To four of the aliquots the indicated concentrations of PEA were added. Growth was measured turbidimetrically.

Fig. 2. Effect of 0.3 % PEA on competence development. Organisms were transferred from growth medium to TM and the freshly inoculated TM was immediately divided into two parts. To one part PEA was added to a final concentration of 0.3 %. The other part served as a PEA-free control. At the intervals indicated both cultures were assayed for viable count and frequency of transformation. The marker transformed was *str-r*. Curve: 1, frequency of transformation, control; 1a, viable organisms control; 2, frequency of transformation, PEA added; 2a, viable organisms, PEA added.

When organisms were allowed to develop competence in TM according to our procedure, a competence development pattern like the control-frequency of transformation line in Fig. 2 was observed. When 0.3 % PEA was added to TM along with the inoculum (at 0 time), competence failed to develop and in fact organisms lost competence during the course of partially inhibited growth. The competent organisms present at the beginning of PEA exposure in Fig. 2 were reduced 100-fold during the period of the experiment.

It is clear from the data in Fig. 2 that PEA blocked transformation. To test the effect of PEA on highly competent organisms (pre-developed competence), 0.3 % PEA was added 60 min. after inoculation, the time just prior to that at which maximum competence was anticipated. The organisms were left in the presence of PEA for

Fig. 3. Effect of removal of PEA from competent organisms exposed to 0.3 % PEA for 25 min. 0.3 % PEA was added to one-half of a culture which had been in TM for 60 min. Twenty-five min. later the organisms in PEA were washed on a membrane filter (point W in figure) and resuspended in fresh TM prewarmed to about 37° (point R in figure). The other half of the 60 min. culture served as a PEA-free control and was not washed. The two cultures were assayed for viable count and frequency of transformation at the intervals indicated. The marker transformed was *str-r*. Curve: 1, frequency of transformation, control; 1a, viable organisms, control; 2, frequency of transformation, PEA added and PEA removed; 2a, viable organisms, PEA added and PEA removed.

25 min., after which the PEA was removed by washing on a membrane filter. The PEA-treated, washed organisms were resuspended in fresh TM and re-incubated. The frequency of transformation and viable organisms were assayed during the entire treatment period, and the results appear in Fig. 3. The data in Fig. 3 corroborate the data in Fig. 2. Phenethyl alcohol stopped the development of competence and destroyed predeveloped competence at an inhibitor level that did not kill but actually permitted some growth. The competent organisms which had been treated with PEA did not regain competence for at least 90 min. after PEA was removed by washing. The effect of PEA on competent organisms was very rapid. Competence increase was stopped immediately upon addition of PEA, and after only 5-6 min. competence was lost at an exponential rate until PEA was removed.

Fox (1957), Lerman & Tolmach (1957), Young & Spizizen (1961), Levine & Strauss (1965), and Kammen, Beloff & Canellakis (1966) have shown that the number of cells transformed per given amount of transforming DNA is proportional to the length of exposure of the cells to the DNA. This relationship makes it possible to measure DNA uptake by scoring for transformants after various periods of exposure of organisms to transforming DNA.

Fig. 4

Fig. 5

Fig. 4. Effect on the frequency of transformation when 0.3% PEA is added to competent organisms with DNA. Ninety min. competent organisms were added to transformation tubes. At the time of organism and DNA addition, 0.3% PEA was added to one-half of the tubes (0.3% PEA in figure). The other half of the tubes served as PEA-free controls (control in figure). Samples were taken at the times indicated and assayed for frequency of transformation. The frequency of transformation was calculated by dividing the number of transformants at each sampling point by the viable organism titre at the time of addition of the organisms to the transformation tubes. The marker transformed was *str-r*.

Fig. 5. Effect of 0.3% PEA on the uptake of tritium-labelled DNA. 9.0 ml. aliquots of 90 min. competent organisms in TM were mixed with 1.0 ml. tritium-labelled DNA. The conditions of PEA exposure and sampling were identical to those described in Fig. 4. After the samples were assayed for frequency of transformation, the remaining organisms were washed, dissolved in NaOH, and assayed for radioactivity. The control and 0.3% PEA curves in the figure correspond to the curves in Fig. 4.

Because PEA acts so rapidly (Fig. 3) it could be added to competent organisms along with transforming DNA and organisms were rendered non-transformable before any appreciable number were transformed. This permitted us to devise experiments to show that PEA blocked irreversible DNA uptake (incorporation).

Competent *Bacillus subtilis* 168 *ind* were exposed to a saturating concentration of DNA and the number of transformants appearing after various periods of DNA

exposure is shown by the 'Control' in Fig. 4. The experiment was repeated with cells to which 0.3 % PEA had been added with transforming DNA (0.3 % PEA, Fig. 4). The control shows that the number of transformants was proportional to time of exposure to DNA; therefore, the experiment measured DNA uptake. The absence of transformation in organisms treated with PEA suggests that PEA obstructed transformation by blocking DNA uptake by competent organisms. This assumption is valid only if all reactions in transformation subsequent to DNA uptake can occur without inhibition. Since it is possible that PEA blocks process(es) occurring after uptake (see Table 2) more direct evidence for uptake inhibition is required than the absence of transformation in the presence of PEA.

Such evidence was obtained from an experiment identical to the one described in Fig. 4, but using tritium-labelled transforming DNA. In this experiment, both the frequency of transformation pattern and the radioactive DNA uptake pattern were determined, but since the former was essentially identical to that in the previous figure, only the radioactive DNA uptake data are plotted in Fig. 5. It is evident from Fig. 5 that PEA blocked DNA uptake by competent *Bacillus subtilis*.

Fox & Hotchkiss (1957) consider DNA uptake (incorporation) to proceed in two steps. Competent organisms first bind DNA in a reversible fashion. Reversibly bound DNA can be removed from organisms by DNase and/or by washing. As a second step, some reversibly bound DNA becomes irreversibly bound. Irreversibly bound DNA cannot be removed from competent organisms by treatment with DNase or by washing.

Table 1. *Reversible and irreversible incorporation of ^3H DNA by competent and non-competent organisms*

Cell type	PEA addition	Incorporation (c.p.m. 10^7 viable organisms)		Frequency of transformation
		Reversible (without DNase)	Ir- reversible (with DNase)	
90 min.	None (control)	116	18	3.05×10^{-3}
Competent organisms	0.3 % with DNA	91	2.0	4.64×10^{-5}
Non-competent organisms	None	22	0.1	6.4×10^{-6}

Bacteria were exposed to radioactive DNA for 30 min. One half of each system was assayed for radioactivity before being treated with DNase; the other one half after. DNase-treated organisms were also assayed for frequency of transformation. The marker transformed was *str-r*.

The Fox & Hotchkiss (1957) model of the incorporation of transforming DNA was used to show that PEA blocks irreversible but not reversible incorporation of DNA by *Bacillus subtilis*. Competent organisms were exposed to tritium-labelled DNA in the presence and in the absence of 0.3 % PEA. One-half of the organisms in each system were assayed for radioactive DNA incorporation without being treated with DNase (reversible incorporation), and the other half were assayed for radioactive DNA incorporation after DNase treatment (irreversible incorporation). The DNase-treated organisms were also assayed for frequency of transformation, and the results of the experiment appear in Table 1. The data in Table 1 show that PEA blocked

irreversible incorporation but did not block reversible incorporation. The non-competent control was included in the experiment to demonstrate that higher levels of reversible incorporation were a consequence of competence.

The data thus far presented indicate that PEA blocked transformation in *Bacillus subtilis* by rendering competent organisms incapable of irreversibly incorporating DNA. The data in Table 1 suggest that PEA affected the competent organisms rather than the transforming DNA, but the data in Fig. 1 to 4 could be explained by an effect of PEA on the DNA rather than the competent organism. Rosenkranz *et al.* (1965*a*) and Richardson & Leach (1967) were unable to demonstrate an interaction between PEA and DNA, and Leach & Richardson (1966) found that exposing transforming DNA to 0.5 % PEA for 2 hr did not affect its transforming ability. This published data confirms the implication of the data in Table 1 that PEA affects the competent organism and not transforming DNA.

Table 2. *Decrease in potential transformants compared to decrease in viable organisms and loss of incorporated ^3H DNA following treatment of potential str-r transformants with 0.3 % PEA*

Treatment	Number of transformants ($\times 10^6$)	Viable organisms ($\times 10^{-7}$)	Frequency of transformation ($\times 10^{-2}$)	Incorporated DNA (c.p.m.)
a	0.68	8.5	0.80	539
b	0.045 (6.6 %)*	6.0 (71 %)*	0.075 (9.4 %)*	489 (91 %)*

(a) 90 min. competent organisms exposed to ^3H DNA for 30 min.

(b) 90 min. competent organisms exposed to ^3H DNA for 30 min., then exposed to 0.3 % PEA for 30 min.

* Percentage of values before PEA treatment.

To determine if PEA affected the competent organism only by making it impermeable to DNA, organisms with irreversibly incorporated tritium-labelled DNA were exposed to 0.3 % PEA for 30 min. The frequency of transformation of the organism was measured before and after PEA treatment as was the amount of radioactive DNA irreversibly incorporated (Table 2). Table 2 reveals that the frequency of transformation of organisms which have already incorporated DNA was also lowered by treatment with 0.3 % PEA, indicating that simple irreversible incorporation of DNA was not the only facet of transformation affected by PEA. Examination of Table 2 reveals that under the conditions of the experiment a decrease in viable organisms was also observed. The viable count decrease was only 29 %, indicating that random loss of competent organisms could not be responsible for the 93 % reduction in number of transformants. Although there was a 93 % reduction in the number of transformants and a 29 % decrease in the number of viable organisms, there was only a 9 % decrease in the amount of DNA which had been irreversibly incorporated prior to the PEA treatment. This retention of radioactive DNA was compatible with the observation that the cell envelope of PEA-treated organisms was impermeable to DNA.

The experiments described in Fig. 2 to 4 were also performed with the *ind+* marker. The results of the *ind+* marker experiments were almost identical to the results in Fig. 2 to 4.

DISCUSSION

Phenethyl alcohol renders competent *Bacillus subtilis* organisms unable to irreversibly incorporate transforming DNA and thereby blocks transformation. In addition, organisms which have incorporated DNA prior to PEA treatment transform at a frequency that is only 9.4 % of an untreated control. Whether both consequences of PEA action are the result of a single effect on competent organisms is not clear but the information presented here offers some basis for conjecture.

At least two modes of PEA action could account for a single inhibitory effect on competent organisms. One such mode of action would be a selective killing of competent organisms by PEA. Although 0.3 % PEA is only bacteriostatic to a growing population as a whole, it could be bacteriocidal to the competent organisms in the population. The greater decrease in the number of transformants than in the viable count is consistent with the hypothesis of selective killing of competent organisms. However, the smaller decrease in frequency of transformation experienced when bacteria which had incorporated DNA were treated with PEA suggests that some process other than simple killing of competent organisms is involved. A second mode of PEA action which would explain both consequences of PEA treatment would be an impermeability of PEA-treated competent organisms to transforming DNA. Competent *Bacillus subtilis* incorporate more DNA than is immediately used to produce transformants (Bodmer & Ganesan, 1964; Pene & Romig, 1964) and prior to its integration, the irreversibly incorporated DNA is 'stored', either outside the membrane (Kammen, Wojnar & Canellakis, 1966) or in association with the membrane (Erickson & Braun, 1968; Urban 1968). It can be assumed that 'stored' DNA serves as a source of DNA for integration for the 3-5 hr during which *B. subtilis* organisms remain competent (Nester & Stocker, 1963; Nester, 1964; Kammen, Wojnar & Canellakis, 1966). If this occurs, the frequency of transformation could be lowered by merely making irreversibly incorporated, 'stored' DNA unavailable for subsequent integration. It is the making of 'stored' DNA unavailable for integration, for instance by fixing the DNA in its proposed membrane associated 'storage' site, that could be the mode of PEA action. The 'storage' of DNA is probably associated with irreversible incorporation so that an inhibition of 'storage' would probably be reflected in an inhibition of irreversible incorporation also.

Of the many possible explanations for the effect of PEA on transformation described here, the most plausible hypothesis suggests a modification of the membrane that results in impermeability to newly added DNA and the trapping of 'stored' DNA. The membrane has been involved in 'storage' of incorporated DNA, and PEA affects the cytoplasmic membrane; therefore, an effect of PEA on the cytoplasmic membrane of the competent *Bacillus subtilis* could be responsible for our observations. In our laboratory we have been unsuccessful in demonstrating DNA fixation in membranes isolated and purified from bacteria treated with PEA after irreversibly incorporating DNA; however, it is recognized that isolated cell membranes could be expected to react different from the *in vivo* situation.

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