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Mechanism of Action of Phenethyl Alcohol: Breakdown of the Cellular Permeability Barrier

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ABSTRACT

Phenethyl alcohol (PEA) caused *Escherichia coli* to take up greatly increased amounts of acriflavine, a compound to which healthy growing cells are impermeable. PEA also caused an increased rate of efflux (leakage) of cellular potassium under conditions which do not greatly alter the influx of potassium via the energy-dependent potassium pump. We therefore propose that the primary effect of PEA is a limited breakdown of the cell membrane. The inhibition of deoxyribonucleic acid synthesis and other cellular functions would then be secondary consequences of the alteration in the membrane structure.

The use of phenethyl alcohol (PEA) as a bacteriostatic agent was first studied by Lilley and Brewer (15); the idea gained impetus when Berrah and Konetzka (2) reported that PEA acts by selectively inhibiting deoxyribonucleic acid (DNA) synthesis in *Escherichia coli*. Soon after, PEA was used in studies of two molecular genetic problems: the role of DNA synthesis in bacterial conjugation (3, 11, 20), and the replication and recombination of bacteriophage DNA (6, 12). In addition to its use with microorganisms, PEA has been shown to inhibit the growth of mammalian cells in tissue culture (13) and the multiplication of mammalian viruses (21).

However, there has been difficulty in several laboratories (14, 18, 22) in finding conditions for the selective inhibition of DNA synthesis while protein and ribonucleic acid (RNA) synthesis continue, and several observations have appeared which are inexplicable if PEA primarily inhibits DNA synthesis. These include reports of (i) the inhibition of the growth of an RNA phage by PEA (17), (ii) the inhibition of the germination of spores (14, 26) under conditions where DNA synthesis does not occur even in the absence of PEA, and (iii) inhibition of messenger RNA synthesis and enzyme induction (18, 22) and sporulation (19, 26) with concentrations of PEA lower than those required to affect DNA synthesis.

Treick and Konetzka (28) suggested that PEA may inhibit DNA synthesis in *E. coli* indirectly by altering the membrane site of the initiation of DNA synthesis. Lester (14) showed that PEA alters the permeability of *Neurospora crassa* to a variety of amino acids. We examined the effects of PEA on cell permeability with *E. coli* and concluded that the primary action of PEA is at the level of the cell membrane with resultant breakdown of the cellular permeability barriers. The inhibition of DNA synthesis could be due to a coupling of the initiation of DNA replication to the membrane as proposed by Treick and Konetzka (28), or it could be a secondary consequence of the initial change in cell permeability.

The membrane barrier of a cell acts in two ways: it keeps out many compounds which can only enter the cell via specific metabolic pumps, and it keeps in molecules collected by these pumps (as well as most intermediate metabolites), enabling the cell to accumulate needed materials against a concentration gradient. We studied two results of permeability breakdown. PEA caused a greatly increased uptake of acriflavine (a drug normally excluded from the cells), and an increased rate of efflux (leakiness) of cellular potassium (which is accumulated against a 100-fold concentration gradient).

MATERIALS AND METHODS

E. coli B (the usual host for bacteriophages T2 and T4) and K-12 (the usual strain in studies of bacterial conjugation) were both used.

Acriflavine uptake. Acriflavine uptake was measured according to Silver (24; *in preparation*), who has shown that the uptake of dye is passive and a measure of permeability. If the cells are permeable, the dye enters and binds strongly to the cellular nucleic acids. The present experiments provide insufficient evidence

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to prove that acriflavine uptake reflects altered permeability rather than surface binding; however, we may assume this for our present purposes. Evidence in support of this assumption includes the parallel between changes detected by acriflavine experiments and those detected by increased leakage of K^{42}. Also, toluene and chloroform, agents which are considered to destroy the cellular permeability barriers, cause similar and large changes in acriflavine binding (Silver and Spielman, *unpublished data*). The 20% of the acriflavine absorbed by the impermeable control cultures represents a noise level which is affected by bacterial growth conditions. The acriflavine (10-methyl-2,8-diamino acridine) was a purified sample with only traces of proflavine, and was a gift of the Pharmaceuticals Division of Imperial Chemical Industries, Ltd., Alderly Park, Cheshire, England. The cells were grown to about 5×10^8 per milliliter in the glucose-salts minimal medium M9 (1). Acriflavine was added at a concentration of 0.75 μg/ml, and samples were removed and centrifuged at room temperature. The amount of dye in the supernatant fluids and the resuspended pellets was measured with an Aminco fluoromicrophotometer (American Instrument Co., Silver Spring, Md.) equipped with a primary filter (Kodak-Wratten 47B) and a secondary filter (a combination of Kodak-Wratten 2A and 12 filters). The fluorescence of the supernatant fluids is expressed as percentage of the fluorescence of the 0.75 μg/ml of acriflavine in M9 medium.

K^{42} efflux and influx. Potassium flux was measured as will be described in greater detail by Silver and Spielman (*in preparation*). The K^{42} was spectrographic grade from Iso/Serve, Inc., Cambridge, Mass. *E. coli* cells were grown for several generations at 37 C in tryptone broth (8 g of Difco tryptone, 5 g of NaCl per liter of water) to a concentration of about 5×10^8 cells per milliliter. For the *efflux* experiments, 0.2 to 0.5 μc/ml of K^{42} was added 2 hr before harvesting the cells. The radioactive cells were centrifuged at 20 C, washed once with broth, and resuspended at 5×10^8 cells per milliliter. The cells were distributed in a series of small flasks, a 1-ml sample of each was filtered through membrane filters (type HA; Millipore Filter Corp., Bedford, Mass.), and then the drugs were added to each flask. The flasks were placed in a 29 C shaking water bath, and additional 1-ml samples were removed and filtered. The filters were not washed because washing did not affect appreciably the quantitative results. The filtrates were collected in small poly-vials, and both the filters and 0.5-ml samples of the filtrates were counted in a Nuclear-Chicago gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.). After corrections for geometry of the samples and decay of the K^{42}, the results are given as the percentage of the total K^{42} in the filtrates or in the cells. For the *influx* experiments, the nonradioactive cells were distributed into flasks; the drugs were added at zero-time, and K^{42} was added 1 min later. The flasks were placed in the 29 C water bath; 1-ml samples were removed from time to time, filtered, and washed twice with 5 ml each of broth at room temperature (Silver and Spielman, *in preparation*). In the influx experiments only the filters were counted. K^{42} was added in amounts between 0.5×10^{-4} and 10^{-4} M to tryptone broth, which was the medium of choice since it contains only 6×10^{-4} M potassium. In some experiments, deoxyribonuclease was added to reduce the viscosity and speed filtration.

Drugs. Phenethyl alcohol was purchased from Eastman Organic Chemical Division of Eastman Kodak Co., Rochester, N.Y. Nalidixic acid was the gift of the Sterling-Winthrop Research Institute, Rensselaer, N.Y. Phleomycin (pleomycin) was the gift of Bristol Laboratories, Syracuse, N.Y.

RESULTS

Effect of PEA on permeability of bacteria to acriflavine. Healthy, growing *E. coli* cells are generally impermeable to acridine dyes, but they can be made permeable in a variety of ways, including bacteriophage infection (24) and treatment with membrane-disrupting agents such as chloroform or toluene (Silver and Spielman, *in preparation*). In these cases, the acriflavine enters the cells and is reversibly bound, apparently by the nucleic acids. PEA caused *E. coli* cells to become permeable to, and to accumulate,

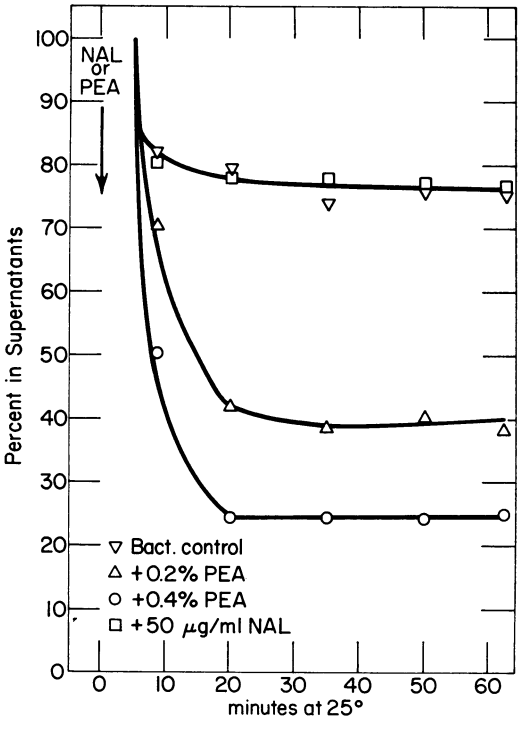

FIG. 1. *Effect of PEA on the cell permeability to acriflavine. PEA or NAL was added at time zero to 5×10^8 cells of Escherichia coli B per milliliter. Acriflavine was added at 5 min. The ordinate shows fluorescence of dye left in the supernatant fluids after centrifugation.*

acriflavine (Fig. 1). This effect was readily seen at PEA concentrations as low as 0.1% (v/v), and maximal permeability occurred with the addition of about 0.25% PEA (compare Fig. 1 and 2). However, nalidixic acid (NAL), which is being used in similar studies to those involving PEA (7, 9; Wendt and Wedel, *in preparation*), had no effect on permeability to acriflavine (Fig. 1). Similarly, pleomycin, an antibiotic which selectively inhibits DNA synthesis (27), did not affect acriflavine permeability at concentrations up to 100 μg/ml (data not shown). The effect of PEA on acriflavine permeability was more rapid than that of toluene (Fig. 2), possibly because PEA is miscible with the aqueous culture medium and toluene is not. The PEA-induced permeability to acriflavine differs strikingly from the bacteriophage-induced permeability (24) in that the PEA effects do not depend on cellular metabolism (unaffected by chloramphenicol or cyanide).

The effect of PEA on permeability to acriflavine was readily reversible (Fig. 3). In this experiment, cells were pre-exposed to PEA and were then allowed a 10-min "recovery" period. The recovery to the previous impermeable state was complete not only with pretreatment with 0.25% PEA, but also with 0.5% PEA, a higher concentration than has been used in published reports on effects of PEA. With 1.0% PEA, the permeability breakdown was irreversible, as was the toluene-induced permeability (data not shown). When the cells which bound acriflavine in the presence of PEA were centrifuged, resuspended in fresh medium with neither PEA nor acriflavine, and then centrifuged again, the originally bound dye repartitioned between the cell pellet and the second supernatant fluid in the same proportions (about 75:25) as in the first centrifugation. This again showed that the acriflavine binding was not due to a ternary complex of acriflavine, PEA, and cells.

The reversal of PEA-induced permeability may not require energy (Table 1). The cells regained their normal impermeable state even when cyanide was present during the recovery period.

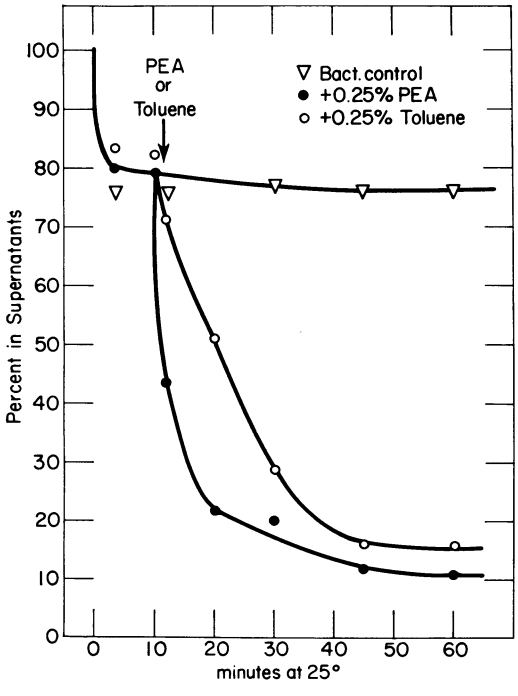

FIG. 2. *Kinetics of PEA- and toluene-produced permeability to acriflavine. Acriflavine was added at time zero to 7 × 10⁸ Escherichia coli B cells per milliliter. PEA or toluene was added at 10 min.*

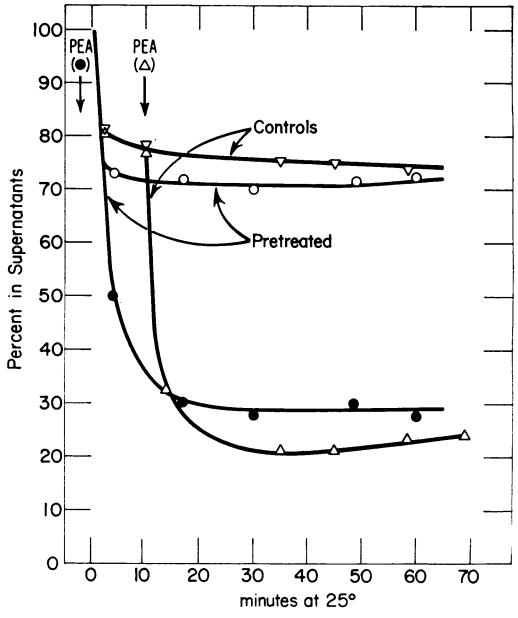

FIG. 3. *Reversibility of PEA-produced permeability. A culture of Escherichia coli B at 7 × 10⁸ cells per milliliter was divided into two samples. The "control" sample received 0.75 μg/ml of acriflavine (at time zero on the abscissa) and was again divided into two parts. To one part, 0.25% PEA was added at 10 min. The "pretreated" culture was exposed to 0.25% PEA for 10 min at room temperature, centrifuged, and resuspended in the absence of PEA. After an additional 10-min "recovery" period, during which the culture was divided into two parts and 0.25% PEA was added to one portion, acriflavine was added (at time zero on the abscissa). Acriflavine uptake was measured with the supernatant fluids after centrifugation.*

TABLE 1. *Recovery from PEA-induced permeability in the presence of cyanide*

Treatment	Per cent acriflavine in supernatant fluid [a]	
	No addition	PEA added
None (controls)	49	18
Pretreated and resuspended	55	16
Pretreated and resuspended in cyanide	50	27

[a] Average of four samples; two were centrifuged at 45 min, and two at 60 min. The experiment was conducted as described in the legend to Fig. 3, except that the cells were grown in 0.2% sodium succinate. Addition of 10^{-3} M sodium cyanide was made during PEA pretreatment, and cells were resuspended in medium with previously added cyanide.

This experiment was done with succinate-grown cells to minimize glycolysis as a possible source of energy. It was repeated several times, and the effect of cyanide plus iodoacetate on glucose-grown cells, or cyanide on broth-grown cells, was also measured. Although none of these experiments showed an energy effect on recovery from PEA-induced permeability, the possibility remained that the cells have a low level energy source which is not completely turned off by the metabolic inhibitors used.

Effect of PEA on loss of intracellular potassium. PEA caused a rapid loss of intracellular potassium (Fig. 4). The potassium leakage was dependent on concentration of PEA as were the growth-inhibitory effects and acriflavine permeability. We observed no significant differences between *E. coli* strains B and K-12 with regard to PEA-induced permeability. NAL did not affect potassium permeability, at least for the first 30 min after its addition (Fig. 4). The potassium leakage was as rapid with PEA as with toluene, but not as complete. With toluene, the cells lost over 90% of their potassium within 30 min (data not shown). The reason for this difference was that PEA, unlike toluene, did not stop the potassium pump (Fig. 5).

The inhibition of potassium influx by PEA was transient, and, after about 5 min (Fig. 5 and 6), the net rate of uptake by PEA-treated cells was similar to that for untreated cells. The reason for the recovery of net uptake rate is not known, but recovery must result from either an increased rate of influx or a decreased rate of efflux in the presence of PEA. This may reflect the ability of the cell's cation pumps to adjust the influx rate to

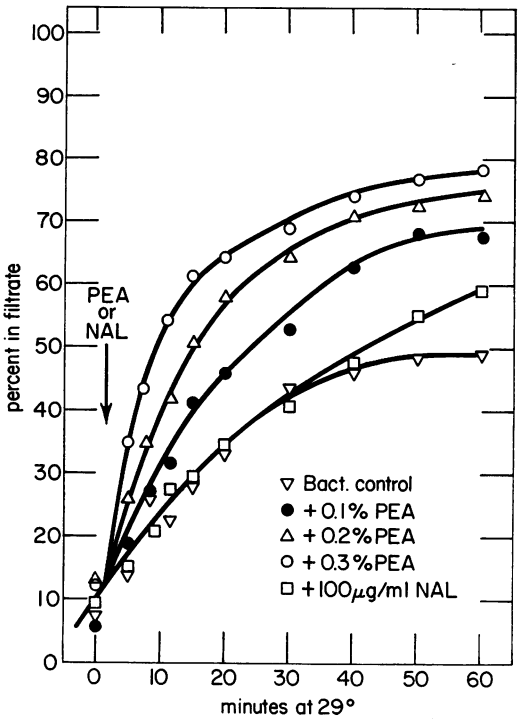

FIG. 4. *Effect of PEA on K^{42} efflux. Radioactive (K^{42}) Escherichia coli K-12 cells were centrifuged and resuspended in nonradioactive broth. Samples were taken and filtered. PEA or NAL was added at 2 min.*

maintain a constant internal potassium concentration in spite of variations in the external concentration (5) and efflux (Silver and Spielman, *in preparation*). The alternative explanation that the PEA-induced efflux is transient is less likely, since there was no apparent change in efflux rate for the first 15 min after addition of PEA (Fig. 4 and 6) and the apparent cessation of efflux in Fig. 4 and 6 is due to the establishment of equilibrium between efflux and influx (Silver and Spielman, *in preparation*).

Figure 6 shows the results of an experiment designed to measure the recovery of the normal potassium efflux rate after treatment with PEA and then the removal of PEA by centrifugation. The recovery in this case was only partial (0.3% PEA used), but it should be noted that by the design of the experiment there was no recovery period (such as in Fig. 3 and Table 2). The addition of PEA caused increased (and identical) efflux rates from the pretreated cells and from those which had not been previously exposed to PEA. In another experiment (Table 2), the cells were treated with 0.25% PEA for 25 min; the PEA was removed and the cells were "loaded"

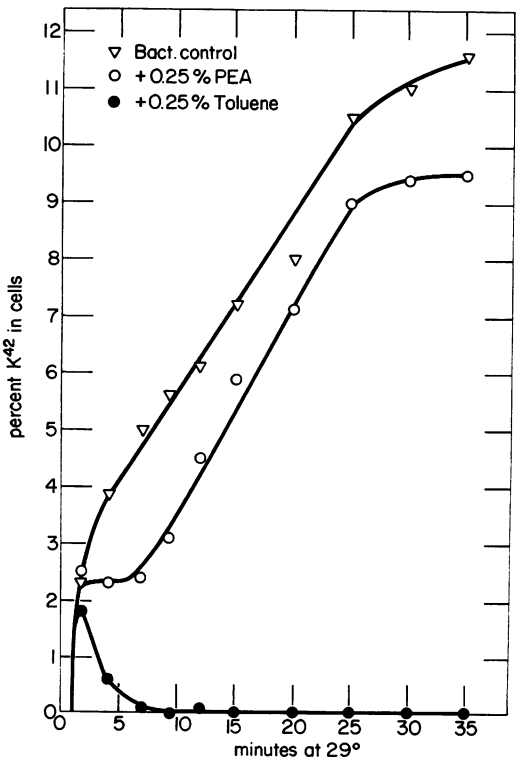

FIG. 5. *Effect of PEA on the influx of K^{42}. At time zero, 0.25% PEA or toluene was added to 5 × 10^8 Escherichia coli B cells per milliliter. K^{42} (0.5 μc/ml, 10^{-4} M) was added at 1 min. Samples were filtered and washed as indicated.*

with K^{42} for 15 min. The potassium efflux from these pretreated cells showed a 90% recovery toward the control rate.

DISCUSSION

These studies suggest that PEA causes a rapid and reversible breakdown in the permeability barriers of bacterial cells. This effect occurs over the same concentration range as the inhibitory effect of PEA on macromolecular synthesis. PEA is being compared here with toluene, which causes an equally rapid but irreversible permeability breakdown without cell lysis (10; Silver and Spielman, *unpublished data*). In fact, we think of PEA as a reversible toluene. It is reasonable to believe that the cellular permeability barrier is the primary site of action of PEA, but primacy cannot be proven experimentally. The proposed mode of action for PEA makes it easier to understand the very narrow concentration limits within which PEA is reversibly inhibitory and not lethal.

Toluene is commonly used to disrupt cellular permeability barriers and to expose internal enzymes to external substrates, but little is known about the mechanism of action of toluene (10). We have confirmed and extended the findings of Jackson and DeMoss (10). The extent and kinetics of damage of *E. coli* cells by toluene depend on the relative volume of toluene added. With less than 1% (v/v) toluene, the cells do not lyse. There is a rapid breakdown of the cellular permeability barrier (Fig. 2), and small molecules (potassium and acriflavine) can pass through the membrane. With time there is further disruption, and larger and larger molecules, such as proteins and nucleic acids, can escape from the cells (10; Silver and Spielman, *unpublished data*). Eventually, after several hours, the cells lyse and release what remains of their cytoplasm (Silver and Spielman, *unpublished data*). The similarities and differences between the effects of PEA and toluene can be considered in terms of their molecular structures (Fig. 7). Both have hydrophobic rings, but only PEA has the additional hydrophilic alcoholic group.

How can a nonspecific attack on the cell membrane by PEA account for the varied but selective inhibitions of cellular processes that have been reported? Two general types of explanation can be envisaged. The inhibition may be the result of a direct structural coupling of the process to the membrane. Bacterial conjugation, sporulation, and the germination of spores all involve growth of, or alterations in, the cell membrane. Similarly, DNA synthesis appears to take place *on* the membrane (for a review of the evidence, see 25). On the other hand, the inhibition may be more indirect and result, by leakage, from the cellular loss of necessary small molecules. A possible basis for selective but indirect inhibition of DNA synthesis is provided by the observations of Ennis and co-workers (4, 16). By use of *E. coli* mutants genetically defective in their ability to concentrate potassium, they showed that cellular DNA and protein synthesis is much more dependent on the high intracellular potassium concentration than is the synthesis of RNA.

The recovery from the PEA-induced permeability does not appear to require energy metabolism, indicating that repair synthesis is not necessary. Thus, the recovery from PEA like the initial action of PEA may be a physical rather than a physiological process. This physical effect of a small molecule (PEA) on the properties of a large macromolecule (the cell membrane) can be thought of as an example of "membrane allostery." The absorption of PEA by the membrane alters the conformation of the membrane, resulting in a breakdown of its structural integrity. When the PEA is removed, the membrane returns

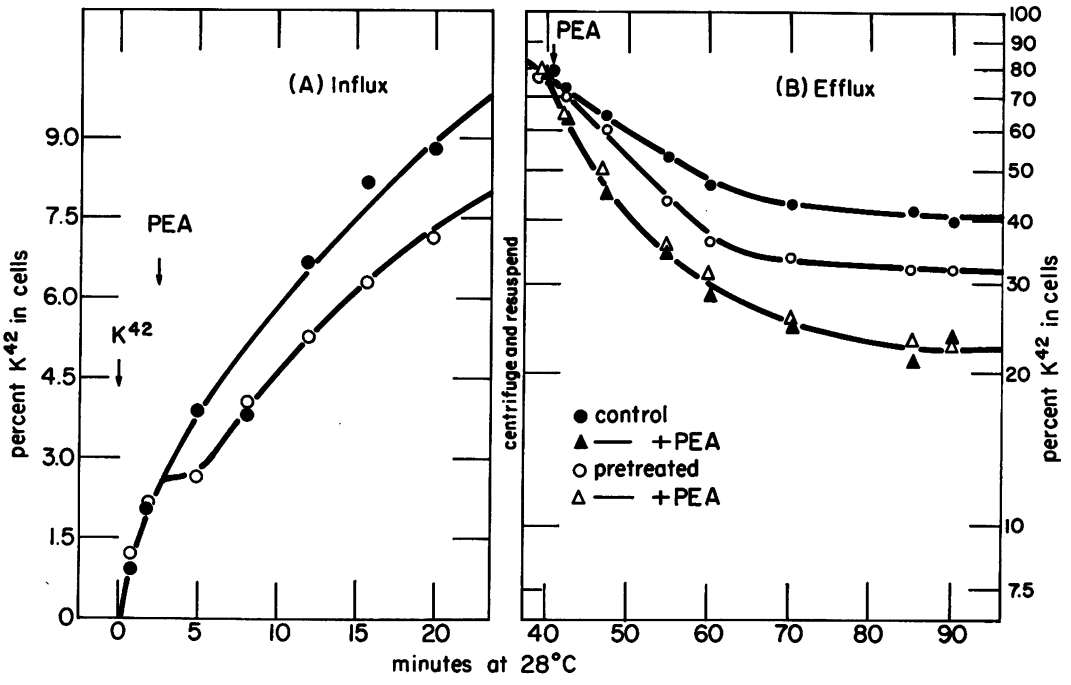

FIG. 6. *Reversibility of PEA-produced permeability to potassium. K^{42} (0.05 μc/ml, 10^{-4} M) was added to two samples of a culture of Escherichia coli B at 4 × 10^8 cells per milliliter. Samples were removed, filtered, and washed as indicated. At 2.5 min, 0.30% PEA was added to one culture. At 25 min, both samples were centrifuged at 5,000 rev/min for 10 min at 20 C. The cells were resuspended in equal volumes of fresh broth and each sample was divided in half. Samples were removed and filtered as usual. PEA (0.25%) was added to one portion each of the pretreated and control cells 40 min after the original addition of K^{42}.*

TABLE 2. *Recovery from PEA-induced permeability to K^{42}*

Culture	k^a (min^{-1})
Control	0.020
Control plus PEA	0.087
Pretreated and "recovered"	0.027
Pretreated, "recovered," plus PEA	0.087

[a] A culture of *Escherichia coli* B was exposed to 0.25% PEA for 25 min at 25 C, centrifuged, and "loaded" with K^{42} for 15 min. After an additional centrifugation to remove extracellular K^{42}, 0.25% PEA was added to a sample of this "pretreated and recovered" culture and to a parallel control culture. The k values were from the exponential part of the efflux curves and the equation: percentage of K^{42} remaining in cell = e^{-kt}. The PEA-induced efflux shows a 4.4-fold increase in rate (0.087/0.020), and the fractional recovery is 90% [(0.087 − 0.027) ÷ (0.087 − 0.020) × 100].

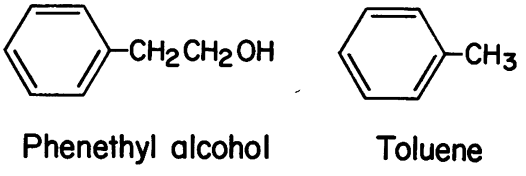

FIG. 7. *Molecular structures of PEA and toluene.*

to its original configuration. Such a reversible transition is readily imaginable within the framework of current ideas on membrane structure (8).

We suggest that previous studies in which PEA was used should be re-evaluated to consider the possibility that the primary site of action of PEA is the cell membrane.

Possible effects on the membrane of other agents which are purported to act solely on DNA synthesis should be investigated. For example, phenethyl alcohol protects against thymineless death (23), and this cannot be due to inhibition of DNA synthesis. Thymine starvation of a thymine-requiring mutant also results in increased permeability to acriflavine (Freifelder and Silver, *unpublished data*).

It is also possible that PEA might be a useful reagent for studies of other membrane and permeability phenomena, such as the structure and functioning of chloroplasts and mitochrondria.

ACKNOWLEDGMENTS

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