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Phenethyl Alcohol Synergism with Mitomycin C, Porfiromycin, and Streptonigrin

Abstract. *Cyanide and phenethyl alcohol greatly enhance the lethal action of mitomycin C, porfiromycin, and streptonigrin on an exponentially growing culture of* Escherichia coli. *Dinitrophenol similarly enhances the lethal action of mitomycin C and porfiromycin, but only slightly that of streptonigrin. Phenethyl alcohol may be functioning in these experiments as an inhibitor of electron transport.*

The antitumor antibiotics mitomycin C and streptonigrin are similar in that both cause disruption of DNA metabolism (*1*). Mitomycin C, the more thoroughly studied of the two, causes cross-linking of the complementary strands of DNA (*2*). Both antibiotics have an amino quinone ring (*3*), and in the case of mitomycin C it appears that this ring must be reduced by the cell in order to make the molecule biologically active (*2*). We report here the synergistic effects of sodium cyanide, β-phenethyl alcohol, and 2,4-dinitrophenol on the bactericidal action of both antibiotics (*4*).

At a mitomycin C concentration of 4 μg/ml (Fig. 1), the curve showing the death rate of cells of *Escherichia coli* strain 15 Phe⁻ is biphasic, possibly because there are two populations of cells, mononucleate and binucleate (or double) cells, which are killed at different rates. Simultaneous addition of 0.01*M* cyanide with the antibiotic very rapidly kills all cells. Phenethyl alcohol (0.02*M*) and dinitrophenol (0.002*M*) are also potent synergists; sodium azide (0.01*M*) is less effective. In the absence of mitomycin C none of the synergists decreases viability (not shown). We have observed similar effects with *E. coli* strains B and W. The antibiotic porfiromycin, which differs structurally from mitomycin C only in that its aziridine nitrogen is methylated (*3*), shows the same pattern of effects when used at a concentration of 30 μg/ml.

Streptonigrin at a concentration of 10 μg/ml also gives a biphasic killing curve (Fig. 2). Cyanide and phenethyl alcohol synergize as with mitomycin, but dinitrophenol has only a slight effect (curve *B*). When phenethyl alcohol or cyanide is added to a culture which has been inhibited for 30 minutes with streptonigrin plus dinitrophenol, the cells again die very rapidly (curves *C* and *D*), showing that this antibiotic has not lost its potency and that the dinitrophenol does not antagonize the action of cyanide or phenethyl alcohol.

Because phenethyl alcohol is reported to be a specific inhibitor of DNA synthesis (*6*), this action might be the basis of its synergistic activity. If so, then cyanide and dinitrophenol might also exert their effects by stopping DNA synthesis. However, this interpretation is not correct, as is demonstrated by the following two experiments. (i) The *E. coli* strain 15 Phe⁻ was grown exponentially as described in Fig. 1. The cells were chilled, harvested, washed, and suspended in the same medium without phenylalanine for 90 minutes. Under these conditions the cells complete one round of DNA synthesis but are unable to start another (*7*), and so DNA synthesis ceases. In our culture, analysis by the diphenylamine test (*8*) showed that DNA content did not change after the first 60 minutes. After the 90-minute incubation, during which the viable cell count rose 50 percent to 10^8 cells per milliliter, mitomycin C was added, with and without phenethyl alcohol. With antibiotics alone the cells died more slowly than they did in an exponential culture (8×10^7 survivors after 15 minutes), while with phenethyl alcohol also present a potent synergism was again observed (5×10^4 survivors after 15 minutes). (ii) The thymine-requiring strain *E. coli* 15 T⁻Phe⁻ (*9*) was grown exponentially in the medium of Fig. 1, but with thymine present (4 μg/ml). This culture was harvested, washed, and resuspended at a concentration of 10^8 cells per milliliter in the same medium without thymine. Under these conditions no DNA was synthesized by the cells, and the cell count was constant for nearly an hour before "thymineless death" began. If mitomycin C at 8 μg/ml was added to the culture shortly after resuspension, 5×10^7 survivors remained after 5 minutes; but,

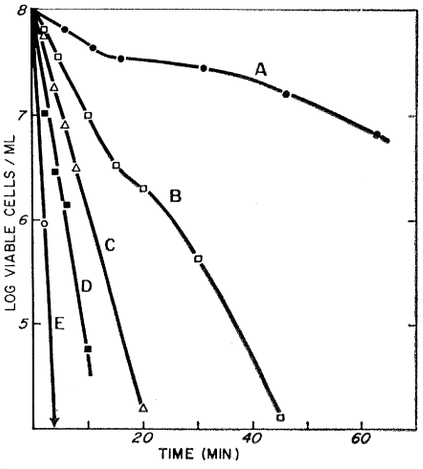

Fig. 1. A phenylalanine-requiring mutant of *E. coli* strain 15 was grown exponentially with aeration at 37°C in a tris-buffered minimal medium (*5*) containing glucose and phenylalanine, the generation time being 50 minutes. At 0 minutes there were 10^8 viable cells per milliliter, and mitomycin C (4 μg/ml) was added to all cultures. The following inhibitors were also added at 0 minutes: *A*, none; *B*, sodium azide (0.01*M*); *C*, dinitrophenol (0.002*M*); *D*, phenethyl alcohol (0.02*M*); *E*, sodium cyanide (0.01*M*).

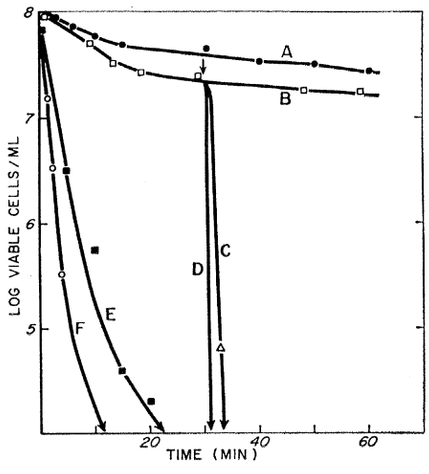

Fig. 2. Cells growing as in Fig. 1 were inhibited at 0 minutes with streptonigrin at 10 μg/ml. The following inhibitors were also added at this time: *A*, none; *B*, *C*, and *D*, dinitrophenol (0.002*M*); *E*, phenethyl alcohol (0.02*M*); *F*, sodium cyanide (0.01*M*). At 30 minutes (arrow) phenethyl alcohol (0.02*M*) was added to *C*; sodium cyanide (0.01*M*), to *D*.

if phenethyl alcohol was also added, the characteristic synergism was again observed (10^4 survivors after 5 minutes), whereas phenethyl alcohol itself offered some protection against "thymineless death."

We conclude from these experiments that the synergistic effect of phenethyl alcohol is not a consequence of inhibition of DNA synthesis. Recent reports discuss two other effects of phenethyl alcohol which cannot be consequences of the inhibition of DNA synthesis. These are the inhibition of sporulation and germination of *Bacillus megaterium* (*10*) and the inhibition of replication of a RNA bacteriophage (*11*).

The administration of cyanide to a culture of *E. coli* establishes a reducing environment within the cells by inhibiting the transfer of electrons to oxygen. Consequently, the strong synergistic action of cyanide with both streptonigrin and mitomycin C is probably due to more rapid reduction of the quinone ring in these compounds. The similarity between the effects of cyanide and phenethyl alcohol in our experiments suggests that phenethyl alcohol also may facilitate reduction, possibly by interfering with electron transport. The following results of work in our laboratory support this notion (*12*): (i) Phenethyl alcohol greatly facilitates the reduction of the tetrazolium compound MTT [3-(4,5-dimethylthiozalyl-2)-2,5-diphenylmonotetrazolium bromide] in a growing culture. (ii) The synergism of phenethyl alcohol with mitomycin C is substantially less if MTT is also added to the culture. These two facts suggest that MTT competes with the antibiotic for a source of electrons which is provided by the action of phenethyl alcohol. Although the point of interaction of MTT with the respiratory chain of *E. coli* has not been reported, in rat liver suspensions MTT is apparently reduced at two points of the respiratory chain (*13*).

The effect of dinitrophenol on streptonigrin inhibition is obviously different from the action of cyanide or phenethyl alcohol. All three synergists may facilitate the reduction of mitomycin C at various points in the system of electron transport; but, if so, dinitrophenol evidently affects a point at which streptonigrin does not readily receive electrons (*14*).

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Intracellular Perfusion of Chilean Giant Squid Axons

Abstract. *Axons of the Chilean giant squid were subjected to continuous intracellular perfusion with either potassium- or sodium-rich salt solutions. In axons immersed in natural sea water and internally perfused with potassium glutamate solution, action potentials which exceeded 160 millivolts were often observed. The resting potential did not vary appreciably with the internal concentration of potassium glutamate. With perfusing solutions containing sodium aspartate or sodium glutamate, action potentials were observed which exceeded the value calculated from the Nernst equation applied to sodium-ion activity. Nervous conduction could be maintained more than 1 hour with equal concentrations of sodium inside and outside the axon.*

South American squid, *Dosidicus gigas*, readily available at the Marine Biological Station, Montemar, Chile, usually weigh 5 to 10 kg and measure more than 1 m in body length. Each stellate nerve of these squid contains three to four giant axons ranging from 0.6 to 1.5 mm in diameter. These axons have been used for studies of the chemical properties of the axoplasm (*1*) and of the effect of intracellular injection of trypsin on nervous conduction (*2*).

In this report we describe the results of an electrophysiological study carried out at the Chilean station. Our object was to examine the nature of the resting and action potentials of the Chilean giant squid axon when subjected to continuous intracellular perfusion; the method of perfusion (*3, 4*) permitted more direct electrochemical measurements on axons than are possible by other techniques.

The technique of intracellular perfusion was described previously (*3*). The perfusing fluid was introduced through a glass pipette (0.22 to 0.25 mm in diameter) inserted at one end of the axon. The fluid was drained through a large pipette, approximately 0.5 mm in diameter, at the other end. In one stage of this investigation we used a simplified arrangement without a drainage pipette. The axoplasm in the perfused zone of the axon was removed beforehand by suction.

The length of the perfused zone was approximately 15 mm in one series of the experiments (records *A* and *B* in Fig. 1). In these instances the unperfused portion of the axon on each side of the zone under study was separated by vaseline partitions and was rendered inexcitable by external application of 0.6*M* $MgSO_4$ solution. In later experiments (record *C*), almost the entire length (35 to 40 mm) of the axon in the fluid medium was directly exposed to the internal perfusion fluid. The resting and action potentials in the perfused zone were recorded with a long glass pipette electrode (0.1 mm in diameter). Stimulating electric currents were applied to the axon through a pair of external electrodes placed near the end of the perfused zone.

Solutions used for intracellular perfusion were prepared by mixing 0.6*M* potassium aspartate (or glutamate), 0.6*M* sodium aspartate (or glutamate), and glycerol solution, 12.5 percent by