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## THE REVERSIBLE INHIBITION OF SPORE GERMINATION BY ALCOHOLS

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**SUMMARY.** Low levels of alcohols have been found to inhibit the process of spore germination. The extent of germination is dependent upon the concentration of alcohol present in the germinating medium. This inhibition is reversible since removal of the alcohol from the spore environment allows germination to proceed.

The disinfectant action of alcohols is both bactericidal and fungicidal. Alcohols are reported to be effective only against vegetative or non-sporing cells (1). The lack of a sporicidal action for alcohols was established in early studies on the use of alcohols as disinfectants (2,3). Later studies have shown that certain alcohols, i.e., 2-phenylethanol (4), chlorocresol (5), and p-hydroxybenzoic acid esters (5), can completely inhibit the process of spore germination. The inhibition caused by these alcohols was found to be reversible since removal of the alcohols from the germinating medium allowed spore germination to proceed. The finding that such alcohols can exert an inhibitory effect on the development of bacterial spores to vegetative cells suggested that a general examination of the effect of alcohols on spore

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germination should be undertaken. This paper reports on the results of such an investigation.

#### MATERIALS AND METHODS\*

Trypticase Soy Broth (Baltimore Biological Lab.), Trypticase Soy Agar (Baltimore Biological Lab.) and Tam Sporulation Agar (DIFCO) were the nutrient media used in this investigation. The alcohols and ketone used were of analytical grade: acetone (Fisher Chem.), ethanol (Fisher Chem.), methanol (Fisher Chem.), iso-amyl alcohol (Fisher Chem.), n-propanol (Fisher Chem.), 2-propanol (Fisher Chem.), n-butanol (Mallinckrodt), phenol (Mallinckrodt), and n-octanol (Matheson, Coleman and Bell).

Spores of Bacillus subtilis var. niger and Bacillus pumilus were prepared from vegetative cells by an active culture technique. A suspension of the appropriate spore stock was heat shocked at 80°C for 15 min. and then plated on Tam agar (DIFCO). After incubation at 42°C for 24-48 hr. the plates were washed with sterile, chilled, deionized water. The resulting suspension was heat shocked, plated on Tam agar and incubated at 42°C for 4 hr. Inocula from the 4 hr. plates were swabbed onto fresh Tam agar plates and reincubated for 2.5 hr. Inocula from these plates were used to swab 30-40 Tam agar plates. The plates were incubated at 42°C for at least 24 hr. or until sporulation was complete. The spores were washed from the plates, insonated, and washed at least five times with sterile, chilled, deionized water. After each centrifugation the upper layer of vegetative debris was rinsed off and discarded. Following the final washing the spores were suspended in 95% ethanol at a concentration of  $3 \times 10^9$  spores/ml.

The germination studies were accomplished by pipetting the ethanol suspension of spores into calibrated Bausch and Lomb Spectronic 20 tubes and removing the ethanol under vacuum. Five ml. of Trypticase Soy Broth (4% w/v), containing the appropriate additive at varying concentrations, was then added to the tubes containing the spores. Insonation of these tubes for 20 seconds in an ultrasonic bath (Turco Products Inc., 20 amps, 250 volts) resulted in the complete suspension of these spores in the germinating medium. The spore suspensions were then incubated at 34°C. Periodically the cultures were shaken and optical density determinations were made using a Bausch and Lomb Spectronic 20. A spore concentration of  $9 \times 10^7$  spores/ml yielded an initial optical density (625 mμ) of 0.65. The spore concentrations used in these germination studies varied between  $8 - 11 \times 10^7$  spores/ml.

The inhibition of spore germination was shown to be reversible by removing the inhibiting additive from the spore environment via Millipore filtration (filter HA, 0.45 μ) and re-suspending the spores in germinating media free of inhibiting additive. The re-suspended spores proceeded to germinate.

## RESULTS

The process of spore germination can be followed by observing the changes in optical density for a spore suspension as a function of time (5,6). Figure 1 illustrates the data obtained when spores of Bacillus subtilis var. niger were exposed to germinating media in the absence and presence of ethanol. Increasing the level of alcohol in the germinating media caused a decrease in the extent of spore germination. Such a result was observed for all the additives studied and presented in this investigation.

Figure 1 also presents the germination data for Bacillus pumilus spores. It is of interest to note that the extent of germination for both species is nearly identical, at a given ethanol concentration, while the shape of the germination curve for each species is different. The B. pumilus spores showed a lag prior to the initiation of germination when in the presence of alcohol.

A plot of the extent of germination as a function of alcohol concentration is presented in Figure 2. From such data it was possible to obtain an extrapolated value for the level of a given alcohol required to completely inhibit germination. Figure 3 illustrates the relationship between the alcohol concentration required for the total inhibition of spore germination and the alcohol molecular weight. Analogs of n-propanol were used to study both the effect of alcohol structure and the effect of functional substituent on the extent of germination. 2-propanol was found to be a less effective inhibitor of spore germination than was n-propanol. Acetone, the ketone analog of 2-propanol, was found to be a less effective inhibitor of spore germination than 2-propanol. This data is also presented in Figure 3.

The spore suspensions containing the various alcohol concentrations, for example see Figure 1, were serially diluted and plated on Trypticase Soy Agar. The colony counts obtained from the various spore suspensions were essentially the same regardless of the alcohol, or the concentration of the alcohol, to which the spores had been exposed. Apparently, the inhibition of spore germination by alcohols could be reversed by the effective removal of the alcohol from the spore environment by the serial dilution procedure. The

reversibility of the inhibition of spore germination by alcohols is illustrated in Figure 4. The non-germinated spores were removed from the alcohol environment by Millipore filtration and re-suspended in Trypticase Soy Broth free of any inhibitory additive. The re-suspended spores showed normal germination.

#### DISCUSSION

The effect of alcohols on spores has not been extensively studied, perhaps due to the early work (2,3) which established the non-sporicidal action of alcohols. However, some studies on the effect of alcohols on the development of dormant spores to vegetative cells have been undertaken. Such studies have shown that certain alcohols, i.e., 2-phenylethanol (4), chlorocresol (5), mixed esters of p-hydroxybenzoic acid (5), ethanol (7), and n-octanol (7), can inhibit the process of spore germination. This investigation establishes that remarkably low concentrations of a wide variety of alcohols, aliphatic as well as aromatic, can totally inhibit spore germination. This inhibition appears to be completely reversible.

The activity of the alcohols in inhibiting spore germination increases as their molecular weights and chain lengths increase (Figure 3). This same relationship is observed for the action of alcohols as bacterial disinfectants (1). It is of interest that primary alcohols are more effective bacterial disinfectants than are secondary alcohols (1) and in this study a primary alcohol, n-propanol, was more effective than a secondary alcohol, 2-propanol, in inhibiting the spore germination process. Such correlations between the effect of alcohol on bacterial vegetative cells and bacterial

spores suggest that the mechanism of alcohol inactivation may be similar for both systems. A basic difference is that the inactivation of vegetative cells is non-reversible, while the inhibition of spore germination by alcohols appears to be completely reversible.

The aliphatic alcohols have been found to inhibit spore germination at remarkably low concentrations in this study. Curran and Knaysi (7) reported nearly complete inhibition of B. subtilis spore germination by ethanol at 10 percent (v/v) and partial inhibition by octyl alcohol at 0.1 percent (v/v). Complete inhibition of B. subtilis spore germination was observed at 2 percent ethanol and 0.003 percent n-octanol in this study. The differences between the work of Curran and Knaysi (7) and this investigation in defining the concentrations of alcohol required to inhibit germination are probably due to the differences in methods used to study spore germination. The concentration of n-octanol (0.003 percent) required to completely inhibit germination is an upper limit, since further study to more precisely define the level of n-octanol needed for inhibition was not undertaken. Such a study together with an investigation of even higher molecular weight alcohols could prove most interesting and may be of value to the food industry as a means of controlling the spore forming bacteria. In this regard, the observation that B. pumilus spores responded to treatment with ethanol in practically the same manner as did spores of B. subtilis (Figure 1) is of interest. The fact that two species of spores could be inhibited by alcohol suggests that the inhibition of spore germination by alcohols may have general applicability. A study to determine the capacity of alcohols to function as sporostatic agents could have very practical implications.

Speculation as to the mechanism for the inhibition of germination by alcohols must account for two observations: (1) the low levels of alcohol required for complete inhibition, and (2) the reversibility of the inhibition. Such considerations strongly suggest that the alcohols are functioning by inhibiting enzyme(s) required for germination (8). The inhibition of lytic enzymes by alcohols has been reported (9,10). Pepsin, for example, is a proteolytic enzyme which is inhibited by aliphatic alcohols (11). Both the inhibition of spore germination by alcohols and the inhibition of pepsin activity by alcohols showed that the degree of inhibition increased with the size of the alcohol molecule. Both inhibitions were completely reversible. While such a direct effect of alcohol on an enzyme mediated germination process is consistent with the level of alcohol required for inhibition and the reversibility of such inhibition, other interpretations may be postulated. For example, the alcohols may be functioning by altering the spore membrane-coat structure which, in turn, may affect the germinating enzyme(s). Such a mechanism would describe a direct effect of the alcohol on the spore membrane-coat structure, and an indirect effect on the enzyme(s) required for germination.



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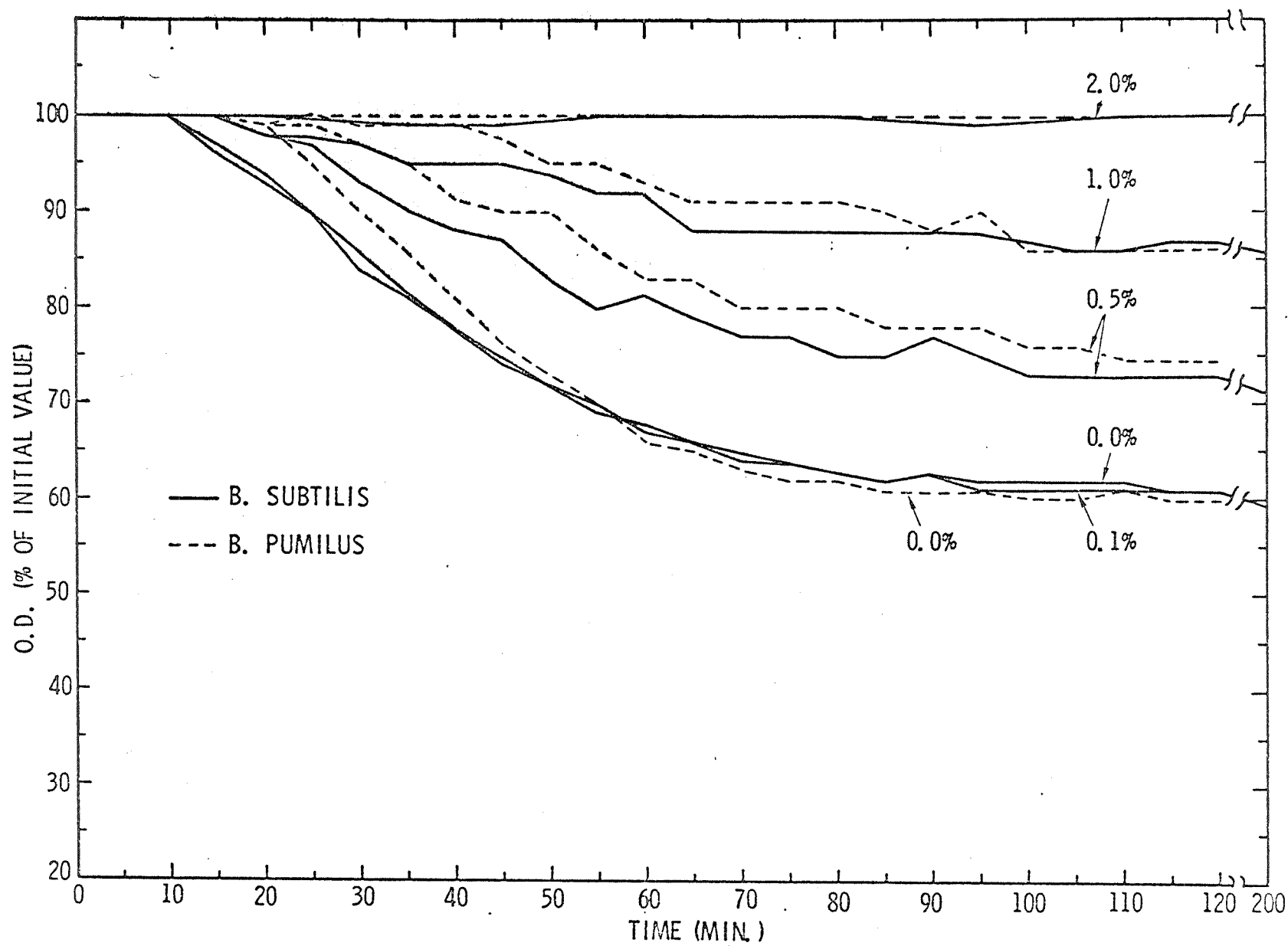
### LEGENDS FOR FIGURES

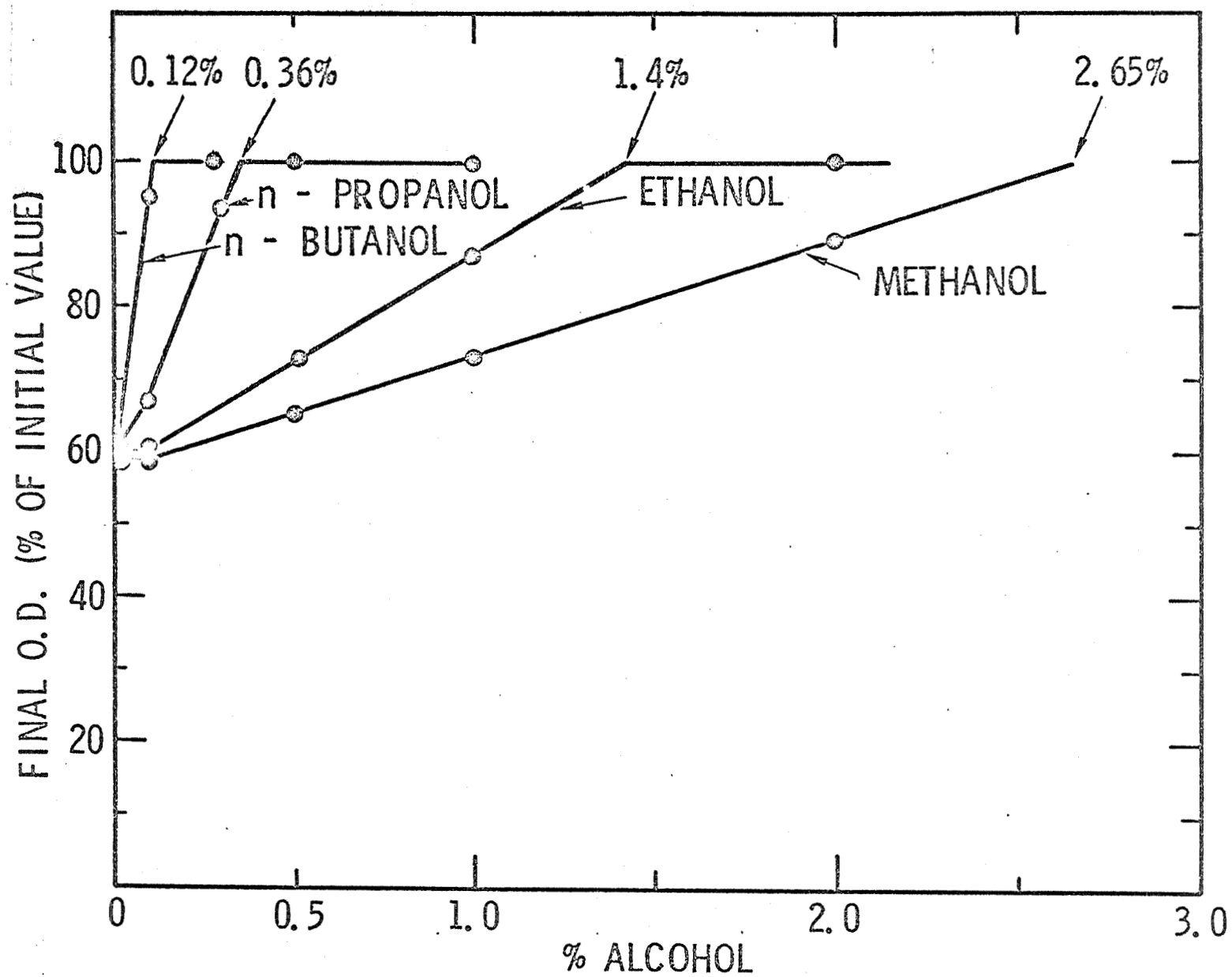
Figure 1. Effect of Various Concentrations of Ethanol on the Germination of Spore Suspension of B. subtilis and B. pumilus

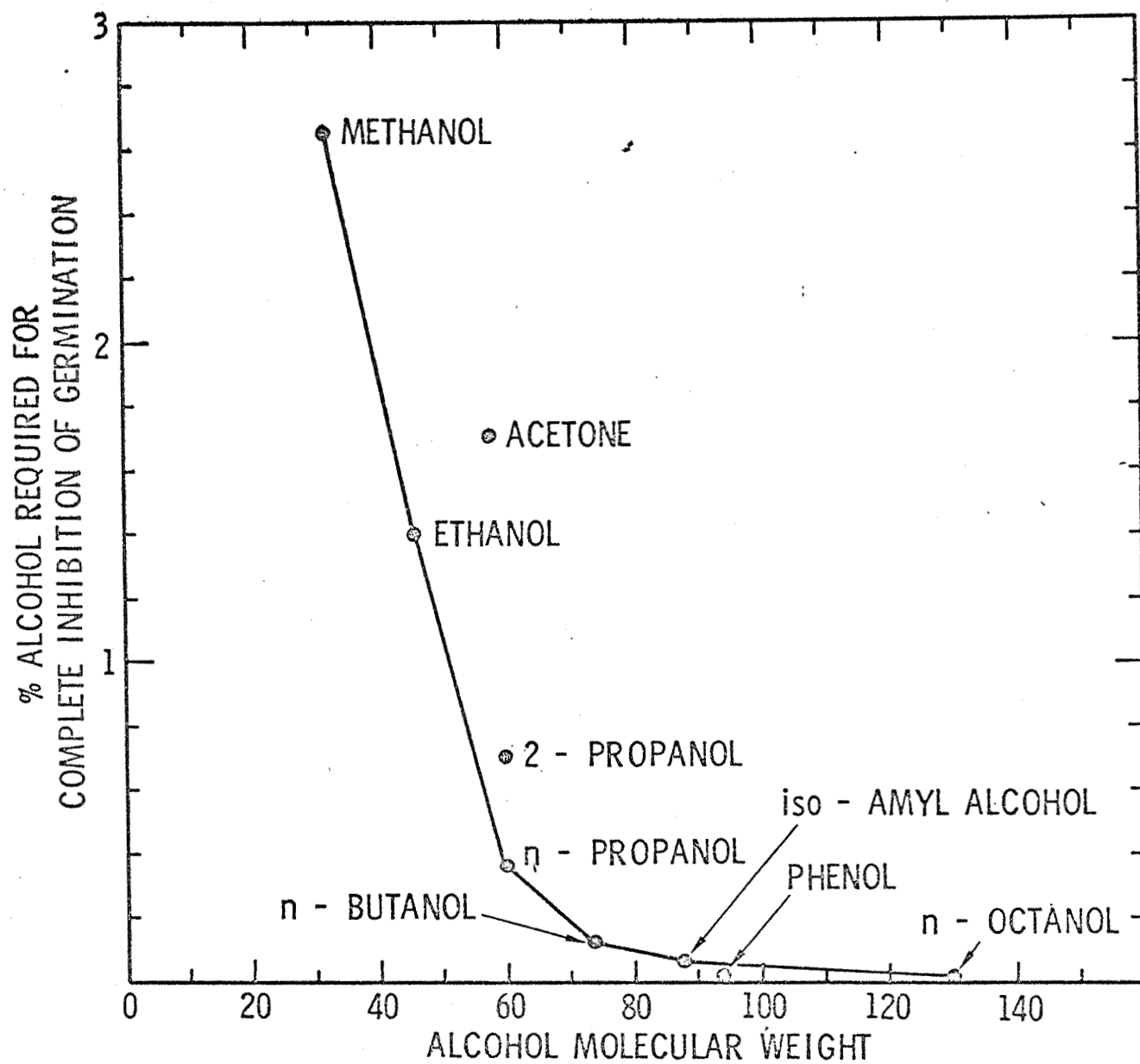
Figure 2. Inhibition of Spore Germination by Various Alcohols. The arrows indicate the extrapolated alcohol concentration required for 100% inhibition of spore germination. All additives were investigated in such a manner to determine the dependence of the inhibition of spore germination on alcohol concentration.

Figure 3. Dependence of the inhibition of spore germination on alcohol molecular weight.

Figure 4. Reversibility of the alcohol induced inhibition of spore germination. Spores were suspended in Trypticase Soy Broth with (a) no additive and (b) 0.5% n-propanol. After 70 minutes spore suspension B was passed through a Millipore filter and re-suspended in Trypticase Soy Broth containing: (c) 0.5% n-propanol and (d) no additive.







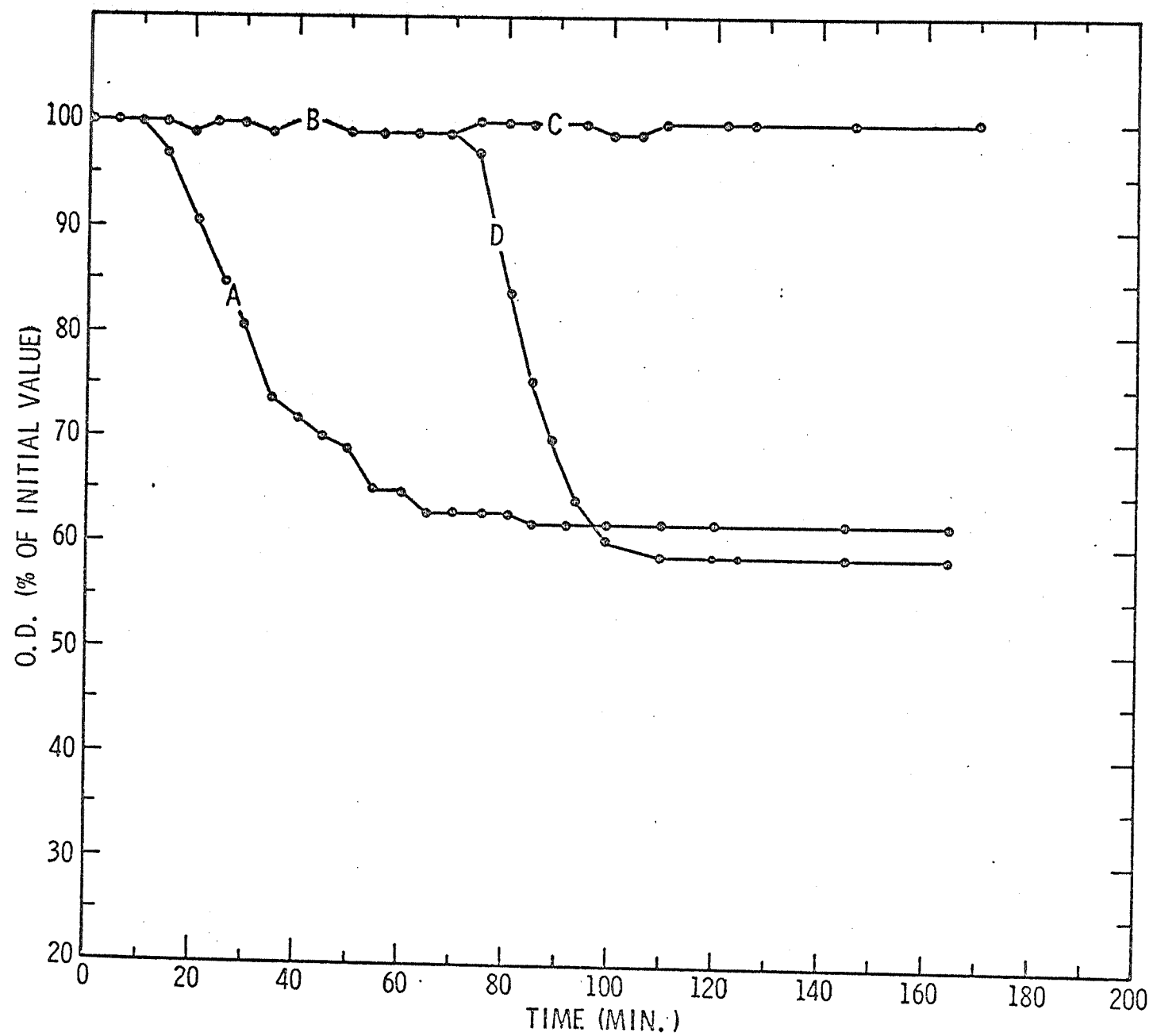


Fig. 4