Inhibition of Beef Liver Hydrolytic Enzymes by Organophosphorus Pesticides

Effect of Organic Solvents on Enzyme Activation and Inhibition

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The effect of several organic solvents on the rate at which the carboxylesterases of beef liver hydrolyze *o*-nitrophenylbutyrate is reported. The organic solvents studied were acetone, methanol, ethanol, *n*-propanol, 2-propanol, glycerol, dimethyl sulfoxide (DMSO), *n*-butanol, *sec*-butanol, *tert*-butanol, isobutanol, and *n*-pentanol. These solvents also affected the inhibition of liver enzymes by the pesticide Ruelene. A preliminary study of these effects was carried out at the 2.5% solvent concentration. This was followed by a more detailed study on acetone, methanol, glycerol, and DMSO at concentrations of up to 20%. The organic solvents were classified according to their effects on enzyme activation and inhibition: those which activated and decreased inhibition, those which deactivated and decreased inhibition, those which activated and increased inhibition, and those which deactivated and increased inhibition. The concentration of the solvent was a critical factor in enzyme activation and inhibition. The effects of these solvents on the optical properties of the reaction product, *o*-nitrophenol, were also studied.

Organic solvents are used in many procedures for enzyme isolation and purification (Askonas, 1951; Connors *et al.*, 1950; Takemori *et al.*, 1967), as well as in various assay techniques (Archer and Zweig, 1959; Bergmeyer, 1965; Dixon and Webb, 1964). However, several workers have shown that these solvents can effect changes in the rates of many enzyme-catalyzed reactions. For example, many water-miscible organic solvents increased the activity of cockroach cholinesterase preparations (Colhoun, 1961). A similar activation was noted in the case of both housefly (Shatoury, 1963) and honeybee (Lewis, 1967) cholinesterase preparations.

In an earlier paper (Villeneuve and McKinley, 1968), it was observed that ethanol not only enhanced carboxylesterase activity, but also decreased the inhibitory effect of the pesticide Trithion. The purpose of this study was to determine the effects of other organic solvents on enzyme activation and inhibition and to select the most suitable organic solvent for further inhibition studies.

MATERIALS

The solutions, substrate, and inhibitor used in this study have been described previously (Villeneuve and McKinley, 1968).

The crude tissue homogenate was centrifuged at 17,500 G and 0° C. for 30 minutes. The supernatant was collected and lyophilized for storage purposes. The resulting liver powder was collected, weighed, and used as the stock enzyme source.

The working enzyme solution was prepared as follows: 75 mg. of the freeze-dried liver powder was dissolved in 0.5 ml. of distilled water, introduced onto a Sephadex G-75 column, eluted with solution A (0.1*M* sodium phosphate buffer, pH 8.0), the brown band collected in a 25-ml. volumetric flask, and made to volume with solution A. One-milliliter samples were placed in suitable storage vials and kept in the freezer for future use. The working enzyme solution was prepared by diluting the contents of one of these vials to 100 ml. with distilled water.

The inhibitor used in this study was the organophosphorus pesticide Ruelene (*O*-4-*tert*-butyl-2-chlorophenyl-*O*-methyl methylphosphoramidate), obtained as an analytical standard from Chemagro Corp. The pesticide was added in 1 ml. of the organic solvent solution. In the first experiment, a concentration of 1×10^{-6} gram (3.4×10^{-3} μmole) of Ruelene per assay tube (7 ml. final solution) was used. In subsequent experiments, the concentration of pesticide was increased to twice this amount to achieve greater inhibition.

PROCEDURE

Essentially the same procedure was used as described previously (Villeneuve and McKinley, 1968). One milliliter of the diluted organic solvent (either with or without the pesticide) was pipeted into a 15-ml. glass stoppered tube. Three milliliters of enzyme working solution were added and the mixture was pre-incubated at 25° C. and a pH of 6.30 for 15 minutes. Then 3 ml. of the *o*-nitrophenylbutyrate (ONPB) solution were added, mixed thoroughly, and incubated for 15 minutes at the same pH and temperature. The reaction was stopped after the elapsed time with the addition of one drop of diisopropyl phosphofluoridate (DFP) solution, 0.25*M* (this compound is extremely toxic and should be handled with caution). A reagent blank was prepared by first adding a drop of the DFP to the enzyme solution and then carrying out the procedure as outlined above. After the reaction was stopped, the solutions were transferred to 1-cm. silica cuvettes and the absorbance of each solution was read at 370 mμ against the reagent blank.

Effect of Enzyme Concentration on Reaction Rate. The effect of different enzyme concentrations on the reaction

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rate was determined by the change in absorbance after 15 minutes of incubation. The concentrations used ranged from 337.5 to 2700 $\times 10^{-6}$ gram of liver per 7 ml.

Effects of Various Solvents at Concentration of 2.5% on Activation and Inhibition. This preliminary study was carried out at one enzyme concentration, 1350 $\times 10^{-6}$ gram of liver per 7 ml. of final solution. The organic solvents used were: methanol, ethanol, acetone, *n*-propanol, 2-propanol, glycerol, *n*-butanol, *sec*-butanol, *tert*-butanol, isobutanol, *n*-pentanol, and dimethyl sulfoxide (DMSO). Each of these solvents was pre-incubated with the enzyme for 15 minutes at 25° C., with the final concentration of organic solvent in the incubation mixture being 2.5% (v./v.). Another set of tubes contained the pesticide (1 $\times 10^{-6}$ gram of Ruelene per 7 ml. of final solution) as well as the solvent, while a control tube without solvent or pesticide was subjected to the same procedure. At the end of 15 minutes, color development was carried out as described previously (Villeneuve and McKinley, 1968), and the absorbance of each tube read against a reagent blank. The per cent of activation and inhibition were calculated from the following equations:

$$\text{Per cent of activation or increase in enzyme activity} = \frac{\text{absorbance (solvent)} - \text{absorbance } (H_2O)}{\text{absorbance } (H_2O)} \times 100$$

$$\text{Per cent of inhibition by Ruelene in presence of solvent} = \frac{\text{absorbance (solvent)} - \text{absorbance (pesticide)}}{\text{absorbance (solvent)}} \times 100$$

Effect of Varying Solvent Concentrations on Activation and Inhibition. Four organic solvents, methanol, acetone, glycerol, and DMSO, were chosen from those used in the previous study for a more detailed investigation. The procedure was the same as for the previous study except that eight concentrations of solvent were employed, ranging from concentrations of 1.25 to 20.0% of the incubation mixture. Further, the activation and inhibition studies were carried out at two enzyme concentrations. The inhibitor was 2 $\times 10^{-6}$ gram of Ruelene per 7 ml. of final solution.

Effects of Varying Solvent Concentrations on Absorptivity of *o*-Nitrophenol. One of the possible ways organic solvents might affect this enzyme reaction would be to change the physicochemical properties of the reaction product, *o*-nitrophenol (ONP). Thus, before the results on enzyme activation could be interpreted properly, it was necessary to determine whether these solvents affected the extinction of the ONP at 370 mμ. This was accomplished as follows:

A stock solution of ONP was prepared in 0.001*N* NaOH (it was necessary to dissolve the ONP without using an organic solvent. At this concentration, the solution had no buffering capacity) at a concentration of 200 μg. per ml. From this, a working ONP solution in 0.08*M* buffer at pH 6.30 and a concentration of 40 μg. per ml. was prepared. The solution used as the blank contained 3 ml. of 0.08*M* buffer, pH 6.30, and 3 ml. of water. All other solutions contained 3 ml. of the working ONP solution and 3 ml. of the particular organic solvent at a specified concentration. The decrease in the extinction values at 370 mμ was expressed as a per cent of the solution containing water as the solvent. All absorbance measurements were made at 370 mμ with a Beckman DU spectrophotometer.

Effects of Five Solvents on Optical Properties of *o*-Nitrophenol. In addition to the data obtained from the previous study, more information was required about the effect of the organic solvents on the other optical properties of the ONP. (ONP exists, in aqueous solution, as a pH-dependent equilibrium mixture of two forms

$$NO_2 \cdot C_6H_4 \cdot OH \rightleftharpoons NO_2 \cdot C_6H_4 \cdot O^- + H^+)$$

The purpose was to study the effect of a particular organic solvent on the molar extinctions of the ionized and unionized species, the isosbestic point, the absorption spectra of both species, and the pK of the ONP. In this case, the spectra of ONP from 450 mμ to 325 mμ were determined in buffers of pH 9.37, 7.40 (near the pK value of ONP), and 5.40. Shifts in the wavelengths of the unionized maximum, ionized maximum, and isosbestic points after addition of organic solvent were noted. Also, changes in the molar extinction at both the unionized and ionized maxima were recorded. The pK values of ONP in the presence of solvent were calculated from the molar extinctions at 450 mμ. Five organic solvents—acetone, ethanol, methanol, glycerol, and DMSO—were used in this study at both the 5 and 10% concentrations.

RESULTS AND DISCUSSION

The effect of varying enzyme concentrations on reaction rate (as measured by absorbance after 15 minutes) is shown in Figure 1. The resulting curve was linear up to a concentration of 2025 $\times 10^{-6}$ gram of liver per 7 ml. and then fell off slightly. The two enzyme concentrations selected for further studies were 675 and 1350 $\times 10^{-6}$ gram of liver per 7 ml. of final solution.

The effects of 12 organic solvents at the concentration of 2.5% on activation and inhibition are shown in Table I. The greatest activation was achieved with *n*-propanol whereas *n*-pentanol had the greatest deactivating effect. (The term deactivation is used to denote a solvent effect. Inhibition refers to a decrease in absorbance as caused by the pesticide.) Acetone also gave a high activation along with methanol, ethanol, and 2-propanol. All other or-

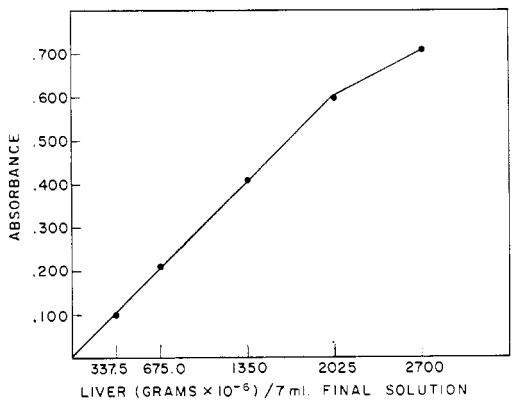

Figure 1. Effect of enzyme concentration on rate of reaction

Table I. Effect of Organic Solvents at Concentration of 2.5% on Activation and Inhibition

Solvent	Per Cent[a] Activation	Per Cent[b] Inhibition
Methanol	29.5	24.5
Ethanol	19.0	12.5
Acetone	45.0	2.5
n-Propanol	46.5	7.0
2-Propanol	12.5	10.5
Glycerol	−1.5	21.5
n-Butanol	−36.0	2.0
sec-Butanol	−14.5	4.5
tert-Butanol	−32.5	7.0
Isobutanol	−28.0	1.5
n-Pentanol	−42.0	1.5
DMSO	−24.0	12.0

[a] Determined by comparing absorptivity of reaction mixture containing solvent with that not containing solvent. [b] Determined by comparing absorptivity of reaction mixture containing solvent plus pesticide with that containing solvent.

ganic solvents had a deactivating effect. When Ruelene was present (1×10^{-6} gram of Ruelene per assay tube), methanol, glycerol, ethanol, and DMSO provided the greatest inhibition, whereas acetone, *n*-butanol, isobutanol, and *n*-pentanol were the least effective. Based on these results the organic solvents were divided into four categories: those which activated but were not effective (effective inhibition was arbitrarily considered to be any value which exceeds 5.0%) for promoting inhibition (acetone), those which activated and were effective for promoting inhibition (methanol, ethanol, *n*-propanol, 2-propanol), those solvents which deactivated and were not effective for promoting inhibition (*n*-butanol, *sec*-butanol, isobutanol, *n*-pentanol), and solvents which deactivated and were effective for promoting inhibition (glycerol, *tert*-butanol, DMSO).

In Figure 2, the effect of acetone on activation and inhibition at two enzyme concentrations is shown. There was an increase in the activation effect up to the 6.25% concentration, then a falling off with deactivation occurring at 20.0%. The same pattern was observed for both enzyme concentrations. In the presence of Ruelene, the maximum inhibition occurred at the 1.25% solvent level and gradually decreased as the solvent concentration increased.

Figure 3 illustrates the results obtained with DMSO. This solvent caused deactivation at all concentrations tested. However, the higher solvent concentrations induced a greater deactivation effect than the lower solvent concentration. In the presence of Ruelene, the inhibition was greatest at the lowest solvent concentrations, and decreased with increasing solvent concentration until 10% was reached. There was a slight increase in the inhibition at the 15 and 20% solvent concentrations as compared with 10%. Similar results were obtained for both enzyme concentrations.

The results for methanol are shown in Figure 4. The least effect on enzyme activation was at the lowest solvent concentrations and as the solvent was increased there was a corresponding increase in the activation. When the pesticide was present, the greatest inhibition was observed with 1.25% solvent. The inhibition then decreased with increasing solvent concentration and leveled off at 10% methanol. The same trends were observed at both enzyme concentrations.

The results for glycerol are shown in Figure 5. As the solvent concentration was increased, it caused a decrease in the activation and subsequently had a deactivating effect. In the presence of the pesticide the inhibition remained constant over the whole range of solvent concentrations. The same pattern applied to both enzyme concentrations.

Figure 6 illustrates the effects of various organic solvent concentrations on the absorptivity of ONP at 370 mμ. These results are expressed in terms of per cent of increase or decrease of the absorption of ONP in 0.04*M* buffer at pH 6.30. The general trend for acetone was to decrease the extinction or absorptivity of the ONP with increasing solvent concentration. However, this effect was minimal at the lower solvent concentrations and thus would not

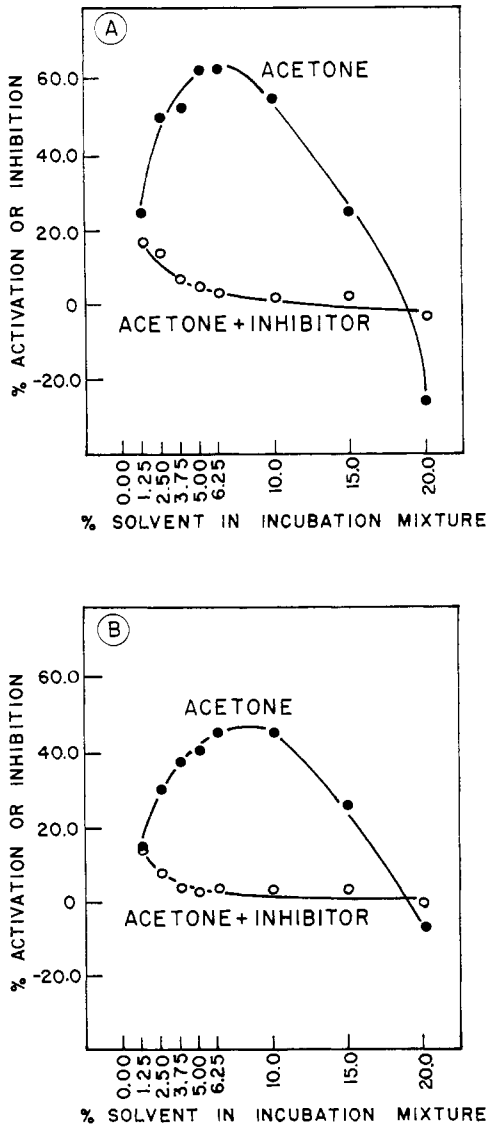

Figure 2. Effect of acetone on enzyme activation and inhibition

A. 675×10^{-6} gram of liver per 7 ml.

B. 1350×10^{-6} gram of liver per 7 ml.

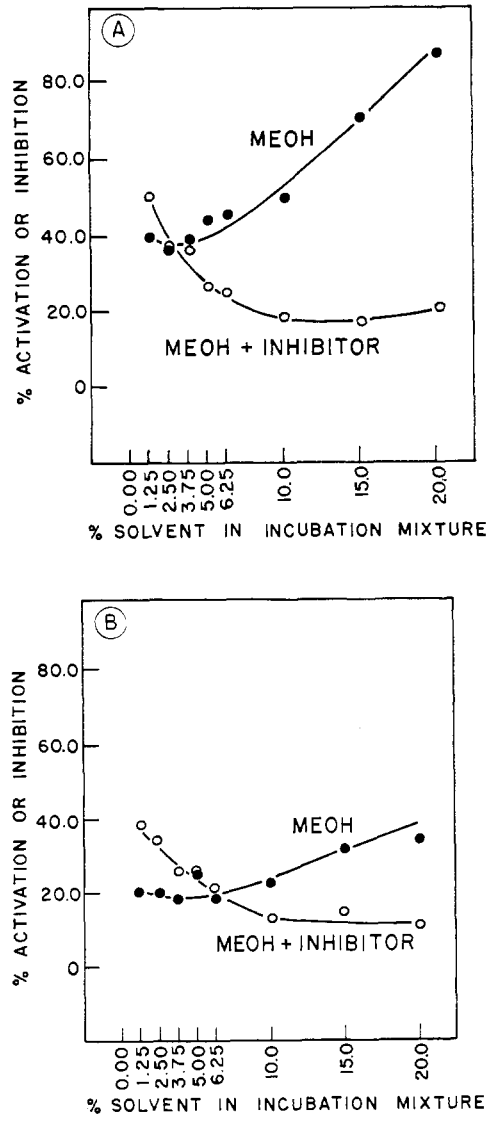

Figure 3. Effect of methanol on enzyme activation and inhibition

A. 675 × 10^{-6} gram of liver per 7 ml.

B. 1350 × 10^{-6} gram of liver per 7 ml.

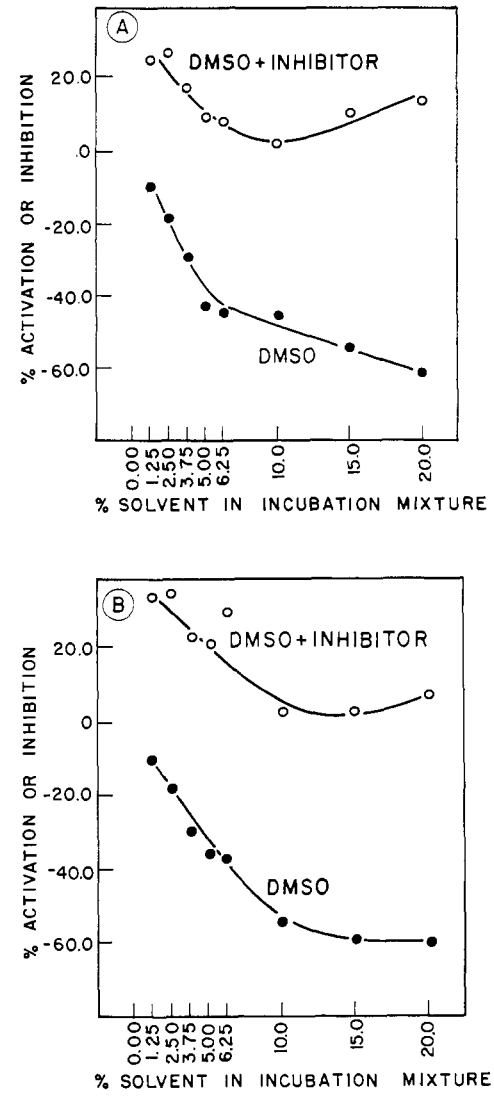

Figure 4. Effect of DMSO on enzyme activation and inhibition

A. 675 × 10^{-6} gram of liver per 7 ml.

B. 1350 × 10^{-6} gram of liver per 7 ml.

significantly influence the activation data previously shown (Figure 2). A similar trend was noted in the case of DMSO, but the effect was minimal at the lower solvent concentrations. The activation data presented in Figure 3 would not be significantly affected. Methanol had little effect on the absorptivity of ONP even at high concentrations. Thus the activation results in Figure 4 would be unaffected. With glycerol there was an increase in the absorptivity with increasing solvent concentrations and at high glycerol concentrations there was an increase in the extinction of the ONP. The activation results in Figure 5 would be affected to some degree, not so much at the lower as the higher concentrations. The net result would lead to an increased deactivation effect at higher solvent concentrations.

The effects of the five organic solvents on the molar extinction of ONP at its ionized and unionized maxima are given in Figure 7. Although most solvents at these concentrations affected the molar extinction at the unionized maximum, the change was small and negligible for the most part. However, 5% ethanol decreased the molar extinction by approximately 8%, as did 10% DMSO. With 10% methanol, there was an increase in the molar extinction of about 5% as compared with water.

In all cases at the ionized maximum, there was an increase in the molar extinction of the ONP as compared with water. Ethanol, methanol, and acetone at the 10% solvent concentration provided the greatest increase, but even this was only in the range of 5%.

The effect of these solvents on the pK of ONP (calculated at 450 mμ) is shown in Table II. There was very little effect noted with all solvents except for DMSO which altered the pK by 0.2 pH units at the 5%, and 0.3 pH units at the 10% concentration.

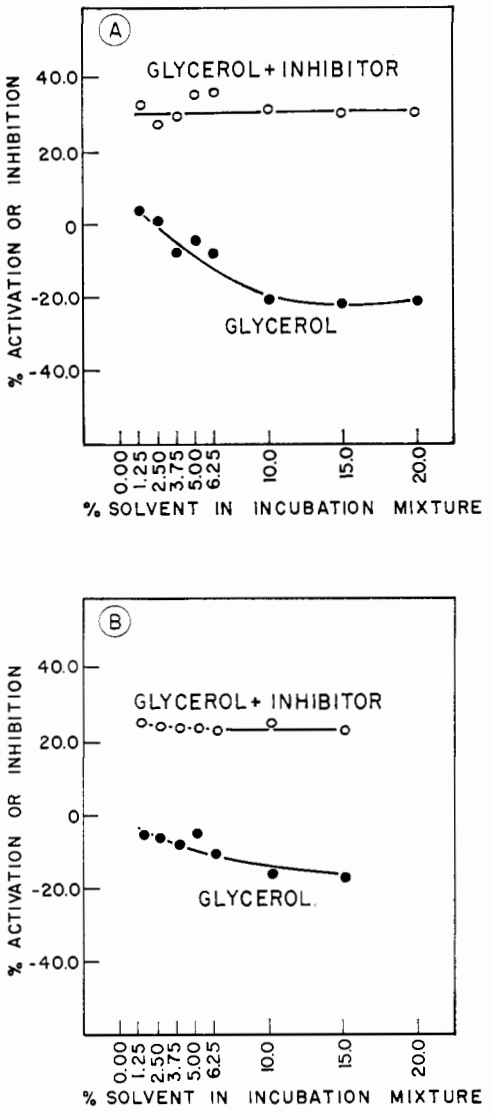

Figure 5. Effect of glycerol on enzyme activation and inhibition

A. 675 × 10^{-6} gram of liver per 7 ml.

B. 1350 × 10^{-6} gram of liver per 7 ml.

These solvents caused no perturbation of the unionized maximum wavelength or the isosbestic point. They did have some effect on the perturbation of the ionized maximum wavelength but this was only in the order of 2 to 3 mμ at the most.

These data provide evidence that the activation phenomena (or for that matter the variation in inhibition response) cannot be attributed to effects of the solvents on the optical properties of the reaction product, ONP. How these solvents affect enzyme activity is as yet unknown. The observed changes in enzyme activity could result from a variation in the dielectric constant of the medium. Changes of this type would not only affect the charge interaction of the substrate with the enzyme's active site, but also the over-all conformation of the protein (Inagami and Sturtevant, 1960). There is also the possibility that the enzyme mixture contains an impurity which serves as an

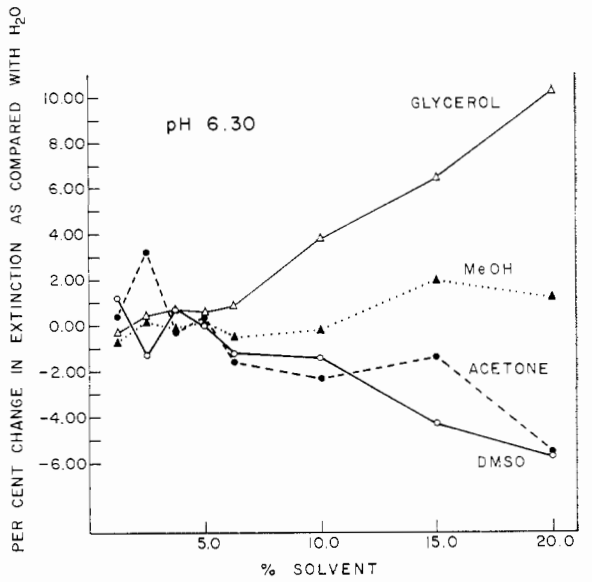

Figure 6. Effect of several organic solvents on extinction of *o*-nitrophenol at 370 mμ

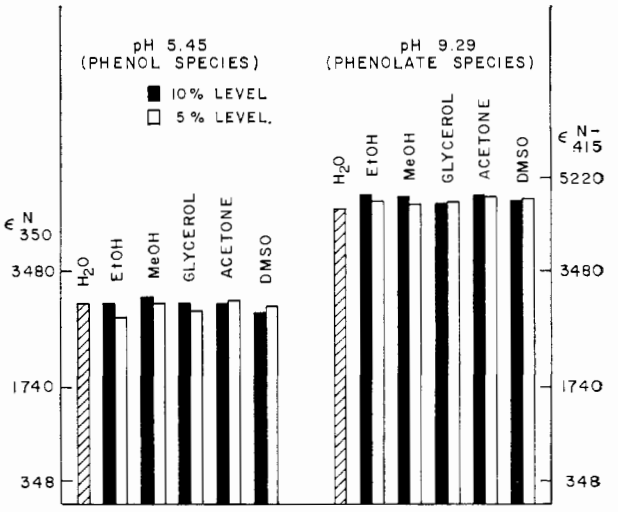

Figure 7. Influence of several organic solvents on molar extinction of *o*-nitrophenol

Table II. Effect of Organic Solvents on pK of *o*-Nitrophenol

Solvent	Calculated pK (450 mμ)
H_2O	7.162
5% Ethanol	7.045
10% Ethanol	7.050
5% Methanol	7.045
10% Methanol	7.092
5% Glycerol	7.126
10% Glycerol	7.099
5% Acetone	7.124
10% Acetone	7.092
5% DMSO	7.048
10% DMSO	6.831

inhibitor. The solvent would inactivate this inhibitor, and thus cause an increase in enzyme activity (Rammler, 1967).

Although the results do not allow any definite conclusions concerning the mechanism of activation to be drawn, it is nevertheless important to be aware of the effects that organic solvents have on enzyme reactions of the type described above.

Methanol appeared to be a suitable solvent for inhibition studies and the effect of this and other solvents on the K_m and V_{max} of the enzyme reaction is being studied. At the same time, an attempt is being made to purify the enzyme source in order to conduct these experiments on the isolated enzyme. The information obtained in these studies may provide an insight into this activation phenomenon.

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