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PRELIMINARY STUDIES OF THE EFFECT OF SALICYLATE AND OTHER DRUGS ON HORSE LIVER ALCOHOL DEHYDROGENASE

BY

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INTRODUCTION AND LITERATURE SURVEY

INTRODUCTION

An urgent need exists today for information regarding the mechanisms of drug interaction. The continuing increase in the use of drugs, both individually and in combination, has resulted in an alarming increase in the number of reported cases of adverse effects attributable to drug interaction.[1] This indicates that current knowledge regarding the effects of individual agents on isolated biochemical systems is inadequate; such information is required before adverse effects resulting from the simultaneous use of several medicinal agents can be predicted.

When two or more drugs are administered concurrently, there exists, in theory, a possibility for biological interaction at any of three different types of sites: the drug receptor, the enzymatic system or systems involved in biotransformation of the drug, and other biological systems which are not directly involved in the therapeutic action of the drug. Since the salicylates are among the most widely used drugs and frequently are employed during other drug therapy on an intermittant basis, further information regarding the effect of salicylates on isolated enzymic reactions would provide the foundation for the investigation of the combined effects of many drugs on such systems.

The pharmacological response which results from a given dose of drug is proportional to the amount of drug in the biophase adjacent to the receptor. Similarly, the magnitude of

many adverse drug reactions depends directly upon the drug level at other receptor sites in the body. Important factors that determine the access of drug to its site of action include absorption rate of the drug from its site of administration, distribution within body tissues, metabolic pathways and the rates involved, and the rate of elimination. One of the major determinants of blood and tissue drug levels is the rate of metabolism of the drug. When several drugs can be metabolized by a given enzymic system, the biological half-life of any of these drugs will be increased by the presence in the body of any of the other drugs. Such an increase in the half-life may result in an increased incidence of side effects or in overt toxicity at drug doses which would be safe if each drug were administered as the sole therapeutic agent. Under these conditions the basis of the adverse effect resides in drug interaction at the enzymic level.

This investigation provides a starting point for the study of drug interactions on an isolated enzyme, alcohol dehydrogenase. This enzyme is particularly suited to such work because it has broad substrate specificity and because reports in the literature suggest that enzymic activity is affected by salicylate. Salicylate also is a particularly appropriate agent to employ in a study of drug interactions since it is the most widely employed type of drug and since the salicylates cause more fatalities each year than does any other group of drugs. This investigation has the possibility of identifying an enzymic level of interaction between salicylate

and other commonly used medicinals. Any positive finding would provide a valuable increase in knowledge in the area of drug interaction.

ALCOHOL DEHYDROGENASE

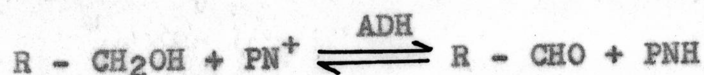
Occurrence, Physiological role and Reaction Catalyzed.

Alcohol dehydrogenase (E.C. 1.1.1.1., alcohol-NAD-oxidoreductase) activity has been found in a wide variety of species and tissues. The enzyme has been identified in *E. coli* and *Neurospora crassa* as well as many other microorganisms; it has been found in pea, corn and rice seeds in leaves of *Mentha piperita*, and in parsley, sugar beets, spinach, sunflower and tobacco.[2] Mammalian sources studied include human, hog, rabbit, lamb, horse and rat liver, frog and cattle retina,[2] and rat brain.[3] The major sources of commercial alcohol dehydrogenase (ADH) are yeast and horse-liver.

Alcohol dehydrogenase plays an important role in the visual process. It reduces to the alcohol the retinene (vitamin A aldehyde in the all-trans form), which under the influence of light, has been formed in a free state from the complex with opsin. After *cis*-isomerization and subsequent reoxidation by ADH to *cis*-retinene, a recombination with opsin produces the visual purple again. ADH is also involved in the degradation of fructose. It reduces glyceraldehyde, which is formed through cleavage of fructose-1-phosphate, to glycerol. Since significant ADH activity has been demonstrated in the soluble fraction of rat brain,[3] a cerebral mechanism that oxidizes ethanol may play a significant role in local adjustments during exposure to ethanol and in the

pathogenesis of the neural disorders associated with chronic alcohol ingestion or withdrawal. In addition, LADH has an important role in the inactivation of such foreign compounds as chloral hydrate[4] and p-hydroxyphenacetaldehyde,[5] an intermediate in the metabolism of tyramine.

The reaction catalyzed by ADH is as follows:



$$R - CH_2OH + PN^+ \overset{ADH}{\rightleftharpoons} R - CHO + PNH$$

Where PN^+ = oxidized pyridine nucleotide
PNH = reduced pyridine nucleotide

The horse liver enzyme (LADH) has a molecular weight of 84,000 and contains 2 atoms of zinc per molecule.

Substrate Specificity.

LADH possesses a very broad specificity for its substrates; possible substrates include alcohols, aldehydes, and ketones. This information is summarized by Winer[6] and is shown in part in Table 1. The enzyme reacts with a variety of primary-and sec-alcohols, both aromatic and aliphatic, but not with tert-alcohols such as tert-butanol and tert-pentanol, or with isopropanol or steroid alcohols (i. e., testosterone and hydrocortisone).[7] It reduces both aliphatic and aromatic aldehydes and also aromatic ketones, but reaction with aliphatic ketones is slow or negligible. In addition, LADH catalyzes the oxidation of vitamin A, p-nitrobenzyl alcohol, and ethylene glycol. The broad substrate specificity of LADH leaves open the possibility that other chemicals, including drugs, which are primary or secondary alcohols or aldehydes also may be metabolized by this enzyme.

Table 1. The influence of chain length and substitution on the ability of alcohols and aldehydes to serve as substrates for liver ADH.*

Alcohol**	initial velocity (M/L/min/mole ADH)	Aldehyde***	initial velocity (M/L/min/mole ADH)
n-Butanol	215	n-Butyraldehyde	510
Allyl	192	Cinnamaldehyde	350
2-Phenylethanol	184	Furfural	236
n-Hexanol	170	Isovaleraldehyde	208
Isoamyl	167	Benzaldehyde	55
Amyl	160	Acetaldehyde	30
n-Propanol	146	Formaldehyde	7
Ethanol	135	Cyclohexanone	5
Cyclohexanol	135	DL-Glyceraldehyde	2
n-Octanol	135	Glyoxal	0
Benzyl	118	Methylethylketone	0
Methylcyclohexanol	108	Acetone	0
Furfuryl	108		
3-Phenyl-1-propanol	46		
3-Hexanol	35		
Methanol	0		
tert-Butanol	0		
tert-Amyl	0		
Isopropyl	0		

* Winer, Alfred D. "A Note on the Substrate Specificity of Horse Liver Alcohol Dehydrogenase." Acta Chem. Scand. 12: 1965 (1958).
** The alcohols were tested at a concentration of 1.0×10^{-3} M; DPN, 1.20×10^{-4} M; ADH, 1.44×10^{-8} M. Glycine-NaOH buffer, 0.1 M, pH 9.50.
*** The aldehydes (or ketones) were tested at a concentration of 5.0×10^{-5} M; DPNH, 3.21×10^{-6} M; ADH, 4.84×10^{-9} M. Phosphate buffer, ionic strength 0.1, pH 6.95.

pH Profile and Km Values.

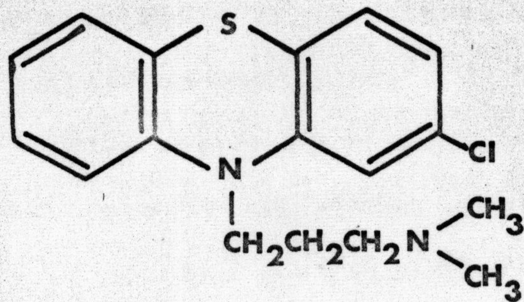
According to Theorell and Bonnichsen,[8] the pH optimum of LADH catalyzing the oxidation of ethanol is approximately pH 8.0 under conditions in which the reactants are of the following concentrations: LADH, 4 - 8 μg/ml; ETOH, 5 x 10^{-4} M; DPN, 2.5 x 10^{-4} M (180 μg/ml). However, high concentrations of ethanol (2 x 10^{-2} M and above) which far exceed the Michaelis constant (5 x 10^{-4} M - 2 x 10^{-3} M depending upon the pH) cause the reaction velocity to increase continuously from pH 6 to between pH 9 and 10. In a subsequent study by Theorell et al.[9] it was shown that the Km for the ADH catalyzed oxidation of ethanol or reduction of acetaldehyde is pH dependent and goes through a minimum between pH 7 and 8. The Km values reported by these investigators, for the ethanol-LADH reaction at pH 9.0, are 6.0 x 10^{-4} M[9] and 7.3 x 10^{-4} M.[8] Their reported Km value for the LADH-ethanol reaction atpH 9 using NAD as the variable substrate was 1.1 x 10^{-5} M.

Inhibitors of ADH.

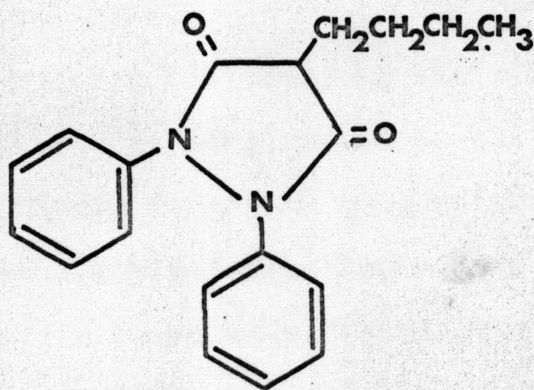
LADH is inhibited by several types of compounds: sulfhydryl binding reagents and heavy metals (Hg^{++}, Ag^{+}, Cd^{++}, Cu^{++}, Zn^{++}); organic complex forming agents such as o-phenanthroline, 9-hydroxyquinoline, EDTA; inorganic ions such as halogen ions, cyanide, perchlorate, sulfate, thio-sulfate, thiocyanate, nitrate and sulfite; organic ions such as oxalate and imidazole; fatty acids and their amides; hydroxylamine; and some drugs.[10] Drugs which have been shown to inhibit ADH include pyrazolidine drugs such as phenylbutazone or its phenolic metabolite,[11] metronidazole (Flagyl^{R}) [12]

and chlorpromazine.[13] The structures of these three drugs are shown in Figure 1.

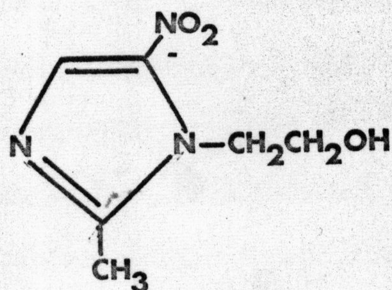
Figure 1. Drugs which have been shown to inhibit alcohol dehydrogenase activity.



CHLORPROMAZINE



PHENYLBUTAZONE



METRONIDAZOLE

SALICYLATE INHIBITION OF DEHYDROGENASES

The mechanism of salicylate inhibition of several dehydrogenases has been shown to be reversible competition with the pyridine nucleotide coenzymes.[14] This implies that any dehydrogenase enzyme requiring a pyridine nucleotide as cofactor is susceptible to inhibition by salicylate. The drug has been shown to inhibit the following dehydrogenase activities <u>in vitro</u>: glucose-6-phosphate, glyceraldehyde-3-phosphate, α-glycerophosphate, alcohol, malate, and lactate. The inhibitory action of salicylate on tissue dehydrogenases is therefore widespread.

The foundation for studying the effect of salicylate and related compounds on dehydrogenases was laid by Bryant, Smith and Hines [15] who suggested that salicylate inhibited malate and isocitrate dehydrogenase activities <u>in vitro</u> and that this inhibition could be reversed by addition of either NAD or NADP. These conclusions were reached using indirect methods to measure the effects of salicylate on the transfer of radioactivity from labelled substrates to metabolic intermediates of the TCA cycle in rat-liver mitochondria. Salicylate and resorcylate increased the incorporation of ^{14}C from 1,4 - ^{14}C-succinate into fumarate, malate, and citrate but did not alter the utilization of the 1,4 - ^{14}C-fumarate when the latter was used as the labelled substrate. This indicated that these drugs had an effect on the dehydrogenase enzymes. Resorcylate, but not salicylate, decreased the utilization of radioactive

succinate in this system. This data suggested that salicylate may be a specific inhibitor of dehydrogenases requiring pyridine nucleotides as cofactors. Support for this theory came from the observation that salicylate and resorcylate inhibited malate dehydrogenase and isocitrate dehydrogenase activities in purified <u>in vitro</u> systems by competing with either NAD or NADP. Resorcylate, but not salicylate, inhibited succinate dehydrogenase activity <u>in vitro</u>.

Subsequent to this study, Hines and Smith[16] investigated the effect of salicylates on other dehydrogenases, including glucose-6-phosphate, glyceraldehyde-3-phosphate, α-glycerophosphate, lactate, alcohol, malate and isocitrate dehydrogenase. These studies were carried out in a purified enzyme system and can be summarized as follows:

DEHYDROGENASE	COENZYME	SALICYLATE CONC. (mM)	INHIBITION (%)
Glucose-6-phosphate	$NADP^+$	15	20
Glyceraldehyde-3-phosphate	NAD^+	10	29
α-Glycerophosphate	NADH	20	34
Lactate	NAD^+, NADH	10,20	30,37
Alcohol	NAD^+	10	19
Malate	NAD^+	5	40
Isocitrate	$NADP^+$	5	20

Dawkins, <u>et al.</u>[17] studied in further detail the mechanism by which salicylate inhibits dehydrogenases. Kinetic studies were carried out using purfied lactate, alcohol, malate and isocitrate dehydrogenases, with the dual purposes of establishing the mechanism of inhibition and of determining the inhibition constants. Their results showed that salicylate

inhibits the activities of these four enzymes and that the percent of inhibition increases with increasing salicylate concentration. Dialysis experiments carried out by these investigators, in which enzyme solutions were dialyzed in either the presence or absence of salicylate, showed that complete reactivation of the inhibited enzymes occurred after dialysis. The results of their dialysis experiments thus indicated that salicylate does not have an irreversible effect on LADH. In initial velocity studies in which the concentrations of NAD and salicylate were varied in the presence of a constant concentration of ETOH, graphic representation of the data showed that salicylate had an inhibitory effect on the reaction rate of LADH which was competitive in nature. This was shown by a Woolf-plot in which NAD concentration/initial velocity was plotted against NAD concentration. According to the authors, the data excludes a mechanism involving competition of salicylate with ETOH as the substrate because the plot of 1/initial velocity versus salicylate concentration does not have a zero slope. The theoretical basis for this type of plot will be discussed later.

In a study on the inactivation of enzymes by aspirin and salicylate, Grisolia et al [18] found that alcohol dehydrogenase was inhibited to a greater degree by salicylate than by aspirin when tested over the same concentration range. Although their conclusions are not clear, the authors seem to indicate that the apparent enzyme inhibition noted may be due to protein modification or inactivation by salicylate rather

than to true reversible inhibition. Since kinetic studies were not carried out, it is difficult to evaluate the significance of their data.

More recently, Lee and Spencer[19] have studied the effect of salicylic acid and acetylsalicylic acid on enzymes involved in mucopolysaccharide synthesis. Their results showed that salicylic acid inhibits the formation of uridine-5'-diphosphoglucuronic acid (UDPGA), an intermediate step in the sequence of reactions of mucopolysaccharide synthesis. The formation of UDPGA involves a dehydrogenation reaction utilizing UDPG-dehydrogenase and NAD as the cofactor. In their investigation, it was shown that sali- acid inhibits UDPGA formation by competing with NAD. The authors have also shown that salicylic acid inhibits UDPGA formation by a noncompetitive mechanism with UDPG. This information, together with other effects of salicylate and aspirin on mucopolysaccharide synthesis, may explain the effect of aspirin on retarding wound healing in rats.

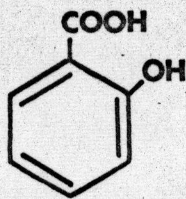
Investigations by Smith and Bryant[20] have provided information regarding the relation between chemical structure and the inhibitory action of several congeners of salicylate against malic dehydrogenase in a purified enzymic system. The structures of the salicylate congeners used by them are shown in Figure 2. Only salicylate, γ-resorcylate and 2-hydroxyphenylacetate significantly inhibited malic dehydrogenase activity *in vitro*. It was therefore concluded that a general structural requirement for inhibitory activity against the

dehydrogenase in congeners of salicylate was a phenolic hydroxyl group in the *ortho* position to a carboxyl group. The introduction of a methylene group between the benzene ring and the carboxyl group (2-hydroxyphenylacetate) did not remove the inhibitory activity. No comparable investigations have been reported for LADH.

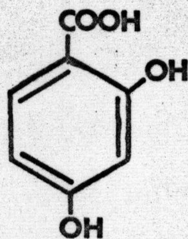
Specific Investigational Goals.

This investigation involves an isolated enzymic system using crystalline horse liver alcohol dehydrogenase (LADH). The specific aims were, first, to confirm the reported competitive inhibition of LADH by salicylate. Secondly, to gain more information regarding the structural requirements for the inhibition of LADH by chemicals structurally similar to salicylate. Thirdly to study the effect of other selected drugs on this system.

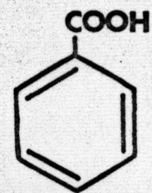
Figure 2. Congeners on salicylate which have been tested for inhibitory activity toward malic dehydrogenase.[20]



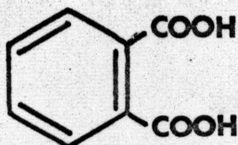
SALICYLIC ACID



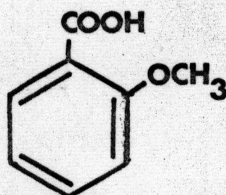
γ - RESORCYLIC ACID



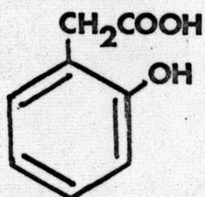
BENZOIC ACID



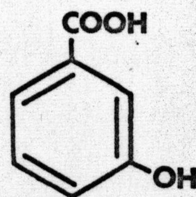
PHTHALIC ACID



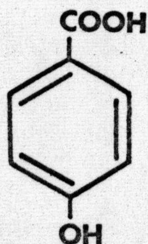
2 — METHOXYBENZOIC ACID



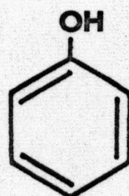
2 — HYDROXYPHENYLACETIC ACID



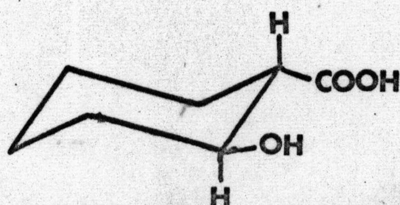
3 — HYDROXYBENZOIC ACID



4 — HYDROXYBENZOIC ACID



PHENOL



trans — HEXAHYDROSALICYLIC ACID

EXPERIMENTAL

MATERIALS

B-NAD and B-NAPH was obtained from Sigma Chemical Co. and not purified further. Horse liver alcohol dehydrogenase was also obtained from Sigma and not purified further. The specific activity of the enzyme was stated as follows: 1 mg reduces 2.4 μM NAD per minute at pH 9.6 and 25°C. Sodium salicylate, sodium benzoate, atropine sulfate and quinidine sulfate were U. S. P. grade and phenol was reagent grade. Crystallized and lyophilized bovine serum albumin was obtained from Sigma and used without further purification. Glycine was obtained from Nutritional Biochemical Co. Siliclad^R was obtained from Clay-Adams, Inc.

METHODS

All glassware was cleaned with dichromate-sulfuric acid cleaning solution and rinsed at least ten times with tap water and ten times with deionized water. The glassware was further treated with a 1 in 100 solution of Siliclad^R in order to insure against adsorption of the enzyme to the surface of the glass and to facilitate accurate volumetric measurements.

NAD stock solutions (0.008 M) to be used for assays involving ethanol as the variable substrate were prepared with deionized water and stored below 0°C in test tubes containing approximately 5 ml portions. These were thawed and used as needed for the enzyme assays.

The enzyme solutions (100 μg/ml or 200 μg/ml depending upon whether the final assay concentration was 6 μg/ml or 12 μg/ml respectively) were prepared by weighing out the appropriate amount of enzyme, transferring it to a 10 ml volumetric flask, and then dissolving the enzyme and adjusting the volume with a solution of 0.1% bovine serum albumin in deionized water.

95% ethanol was used to prepare 1 M stock solutions of ethanol in glycine buffer based on the information that the density of absolute ethanol at 20°C is 0.7893 gm/ml. Appropriate dilutions of this 1M stock solution of ethanol were made in order to prepare final stock solutions to be used for the enzyme assays.

The initial reaction velocities of the reduction of NAD were followed with a Gilford-Model 240 spectrophotometer at room temperature. Measurements of the changes in molar absorption were made at a wavelength of 340 mμ during the early studies but later were made at 365 mμ to avoid interference caused by the absorption of salicylate at the lower wavelength. The initial reaction velocities (v) were determined from the tracings obtained with an external recorder (Gilford Recorder-Model 242).

In all experiments the reactions were carried out in 1 cm cuvettes and the final reaction volume was 1 ml. The temperature of the reaction mixture was always between 21°C and 23°C. Since the reaction velocity was measured during a time interval of approximately 1 minute, no significant change in temperature

occurred as a result of the slightly higher temperature of the cell compartment of the spectrophotometer.

The compositions of typical reaction mixtures for the control reaction and for the reaction in the presence of inhibitor, using either ethanol or NAD as the variable substrate, are shown in Tables 2-5. The reactions were carried out in 0.1 M glycine buffer adjusted to pH 8.8. A typical experiment was carried out as follows: at each concentration of the variable substrate, duplicate assays were run in the order, (1) control reaction (2) reaction in the presence of 2.5 mM inhibitor (3) reaction in the presence of 5.0 mM inhibitor (4) reaction in the presence of 10.0 mM inhibitor. The ingredients of the reaction mixture were added to the cuvette in the following order:

Order	Ethanol as the variable substrate	NAD as the variable substrate
First	Buffer	Buffer
Second	Ethanol	NAD
Third	Inhibitor (when used)	Inhibitor (when used)
Fourth	Enzyme	ETOH
Fifth	NAD to initiate the reaction	Enzyme to initiate the reaction

In the later studies, where the experiments were carried out at a wavelength of 365 mμ, entire assays using both ETOH and NAD as the variable substrate were conducted on the same day using the same batch of enzyme. This was done because enzyme activity varied somewhat in each experiment.

Enzymic activity was checked at various time intervals throughout the course of a typical experiment (approximately

4 hours); there was no decrease in activity during this time interval.

Table 2. Composition of control reaction mixtures with ethanol as the variable substrate and no inhibitor present.

Ethanol conc. (M)	Vol. of ethanol stock soln. (ml)	Vol. of NAD stock soln.[c] (ml)	Vol. of LADH stock soln.[d] (ml)	Vol. of glycine buffer[e] (ml)
2.0×10^{-4}	0.02[a]	0.05	0.06	0.87
3.0×10^{-4}	0.03[a]	0.05	0.06	0.86
5.0×10^{-4}	0.05[a]	0.05	0.06	0.84
1.0×10^{-3}	0.10[a]	0.05	0.06	0.79
5.0×10^{-3}	0.05[b]	0.05	0.06	0.84
1.0×10^{-2}	0.10[b]	0.05	0.06	0.79

a - Used 0.01 M ethanol stock solution.

b - Used 0.10 M ethanol stock solution.

c - Used 0.008 M NAD stock solution to give final conc. of 4.0×10^{-4} M.

d - Conc. LADH stock solution was 100 μg/ml or 200 μg/ml depending upon whether final conc. of 6 μg/ml or 12 μg/ml respectively was desired.

e - 0.10 M; pH 8.8. NaOH-glycine buffer.

Table 3. Composition of reaction mixtures with ethanol as the variable substrate and 2.5 mM, 5.0 mM, or 10 mM inhibitor (salicylate, benzoate, or phenol) present.

Ethanol conc. (M)	Vol. of ethanol stock soln. (ml)	Vol. of NAD stock soln.[c] (ml)	Vol. of LADH stock soln.[d] (ml)	Vol. of inhibitor[e] stock soln. (ml)	Vol. of glycine buffer[f] (ml)
2.0×10^{-4}	0.02[a]	0.05	0.06	0.05	0.82
3.0×10^{-4}	0.03[a]	0.05	0.06	0.05	0.81
5.0×10^{-4}	0.05[a]	0.05	0.06	0.05	0.79
1.0×10^{-3}	0.10[a]	0.05	0.06	0.05	0.74
5.0×10^{-3}	0.05[b]	0.05	0.06	0.05	0.79
1.0×10^{-2}	0.10[b]	0.05	0.06	0.05	0.74

a - Used 0.01 M ethanol stock solution.

b - Used 0.10 M ethanol stock solution.

c - Used 0.008 M NAD stock solution to give final conc. of 4.0×10^{-4} M.

d - Concentration of LADH stock solution was 100 µg/ml or 200 µg/ml depending upon whether final conc. of 6 µg/ml or 12 µg/ml was desired.

e - The concentrations of stock solutions required to give 2.5 mM, 5.0 mM, and 10 mM final concentrations of inhibitor were 0.05 M, 0.10 M, and 0.20 M respectively.

f - 0.10 M; pH 8.8; NaOH-glycine buffer.

Table 4. Composition of control reaction mixtures with NAD as the variable substrate and no inhibitor present.

NAD conc. (M)	Vol. of NAD stock soln.[a] (ml)	Vol. of ethanol stock soln.[b] (ml)	Vol. of LADH stock soln.[c] (ml)	Vol. of glycine buffer (ml)
2.0×10^{-5}	0.02	0.05	0.06	0.87
3.0×10^{-5}	0.03	0.05	0.06	0.86
5.0×10^{-5}	0.05	0.05	0.06	0.84
1.0×10^{-4}	0.10	0.05	0.06	0.79

a - Used 0.001 M NAD stock solution.

b - Used 0.10 M ethanol stock solution to give final conc. of 5.0×10^{-3} M.

c - Conc. of LADH stock solution was 100 μg/ml or 200 μg/ml depending upon whether final conc. of 6 μg/ml or 12 μg/ml was desired.

d - 0.10 M NaOH-glycine buffer; pH 8.8.

Table 5. Composition of reaction mixtures with NAD as the variable substrate and 2.5 mM, 5.0 mM, or 10 mM inhibitor (salicylate, benzoate, or phenol) present.

NAD conc. (M)	Vol. of NAD stock soln.[a]	Vol. of ethanol stock soln.[b]	Vol. of LADH stock soln.[c]	Vol. of inhibitor[d] stock soln.	Vol. of glycine buffer[e]
2.0×10^{-5}	0.02	0.05	0.06	0.05	0.82
3.0×10^{-5}	0.03	0.05	0.06	0.05	0.81
5.0×10^{-5}	0.05	0.05	0.06	0.05	0.79
1.0×10^{-4}	0.10	0.05	0.06	0.05	0.74

a - Used 0.001 M NAD stock solution.

b - Used 0.10 M ethanol stock solution to give final conc. of 5.0×10^{-3} M.

c - Conc. of LADH stock solution was 100 μg/ml or 200 μg/ml depending upon whether final conc. of 6 μg/ml or 12 μg/ml was desired.

d - The concentrations of stock solutions required to give 2.5 mM, 5.0 mM, and 10 mM final concentrations of inhibitor were 0.05 M, 0.10 M, and 0.20 M respectively.

e - 0.10 M NaOH-glycine buffer; pH 8.8.

RESULTS

Control Studies Relating to the Spectrophotometric Assay.

Standard curves were run for NAD and NADH in which absorbance at 260 mμ or 340 mμ respectively was plotted against NAD or NADH concentration in u moles/ml. As shown in Figure 3 and Figure 4, both NAD and NADH obey Beer's Law in 0.1 M NaOH-glycine buffer, pH 8.8, the buffer system used for the assays.

In order to establish the stability of the enzyme preparation and to find a suitable enzyme concentration with which to carry out further studies the initial velocity was determined at various enzyme concentrations. The results of these determinations are shown in Figure 5. The ethanol concentrations employed were 1.0×10^{-4} M, 1.0×10^{-3} M, and 1.0×10^{-2} M, and represent the range of substrate concentrations used in subsequent assays. It can be seen that at each substrate level the initial velocity of the reaction increases linearly as the enzyme concentration increases up to 8 μg/ml.

Initial velocity studies with ethanol as the variable substrate.

Control

An initial control assay was carried out to determine Km for ethanol in the reaction catalyzed by LADH. Figure 6 shows the Lineweaver-Burke plot for this determination.

The Km value of 6.06×10^{-4} M thus obtained is in good agreement with that of Theorell _et al._[9] Although eleven different ethanol concentrations were used for this assay, the data were sufficiently reproducible so that fewer substrate levels were employed in future assays. The average Km value obtained in seven different assays was 5.61×10^{-4} M and the range was $4.20\text{-}6.45 \times 10^{-4}$ M.

Inhibition by salicylate

The effect of salicylate on initial velocity when ethanol was the variable substrate is shown in Figure 7. It can be seen that at the concentrations employed salicylate does inhibit the reaction. Although these data are not complete enough to permit any conclusions regarding the mechanism of this inhibition, the data suggest that more than one mechanism may be involved, and that the relative importance of these different mechanisms may depend upon the inhibitor concentration. A comparison of the slope of the control reaction with that in which 2.5 mM salicylate was present suggests that the inhibition may be uncompetitive or noncompetitive, while a comparison of the slope of the control reaction with those in which the salicylate concentration was 5.0 or 10.0 mM suggests that the inhibition may be mixed. The data have been graphed in another manner, as ethanol concentration/initial velocity versus ethanol concentration, in an attempt to clarify the nature of the inhibition. This information is presented in Figure 8. The slopes of the lines in this graph also suggest

that the effect of salicylate is one of producing a mixed type of inhibition at high concentrations.

Inhibition by Phenol

When tested at the same concentrations as salicylate, phenol was shown to be a more potent inhibitor of the enzymic reaction with ethanol as the variable substrate. This can be seen by a comparison of Figures 7 and 9, in which salicylate and phenol respectively serve as the inhibitor. In contrast to the situation with salicylate, phenol appears to produce a mixed type of inhibition at all levels tested. This conclusion appears justified both from the Lineweaver-Burke graph (Figure 9) and from the graph of ethanol concentration/initial velocity versus ethanol concentration (Figure 10).

Effect of benzoate

The initial reaction velocities in the presence of equivalent concentrations of benzoate were not significantly different from the control values using ethanol as the variable substrate.

Initial velocity studies using NAD as the variable substrate.

Control

Figure 11 shows the Lineweaver-Burke plot from which Km was determined for the NAD-LADH reaction. The Km value thus obtained is 2.78×10^{-5} M. The Km value obtained by Theorell et al [2] was 1.1×10^{-5} M at pH 9.0.

Inhibition by salicylate

Figure 12 shows the results obtained with salicylate as the inhibitor and NAD as the variable substrate. The results appear to confirm the competitive nature of the inhibition which has been reported by Dawkins *et al.*[17] The correlation coefficient for the points using the higher salicylate concentration was very low and the regression line is not shown in Figure 12. However, the possibility exists that the inhibition may not continue to be competitive at a salicylate concentration of 10 mM.

Inhibition by phenol

Figures 13 and 14 show the complex nature of the inhibition when phenol was tested at the same concentrations as salicylate. Again, the nature of the inhibition appears to depend upon the inhibitor concentration. It is of interest to note that the inhibition appears distinctly non-competitive at the high phenol concentration (10 mM).

Effect of quinidine and atropine

Atropine sulfate and quinidine sulfate were tested as possible substrates for LADH. The concentrations of these drugs was 1.0×10^{-3} M in the assay system consisting of 6 µg/ml LADH, 4.0×10^{-4} M NAD, and 0.10 M NaOH-glycine buffer, pH8.8. For both drugs, it was found that there was no significant change in absorption after about 15 minutes incubation. This indicates that these two drugs are not metabolized by LADH under the conditions employed.

Figure 3. Absorbance of NAD^+ 260 mμ in 0.1 M glycine buffer, pH 8.8.

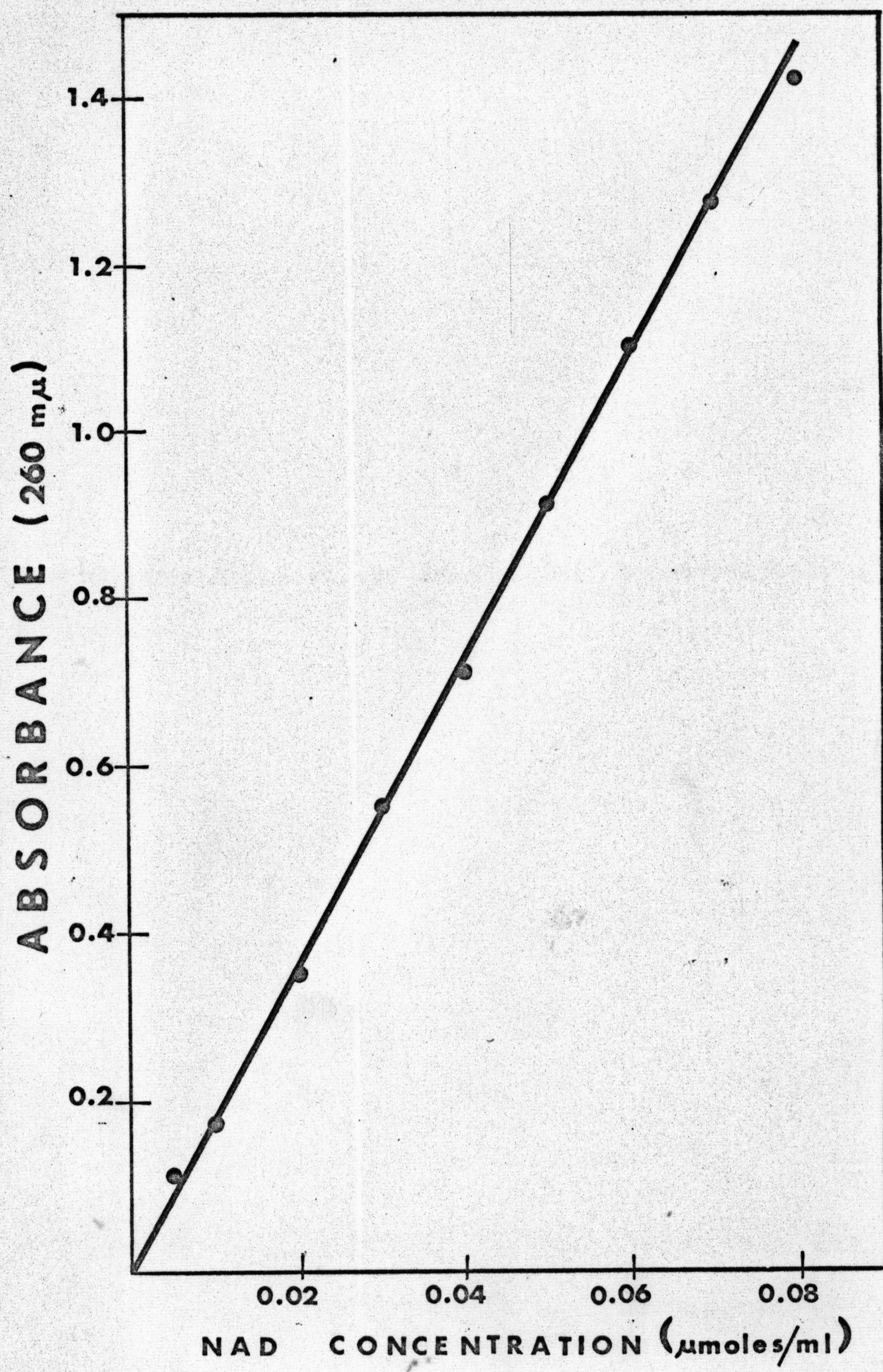

ABSORBANCE (260 mμ)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.02
0.04
0.06
0.08
NAD CONCENTRATION (μmoles/ml)

Figure 4. Absorbance of NADH at 340 mμ in 0.1 M glycine buffer, pH 8.8.

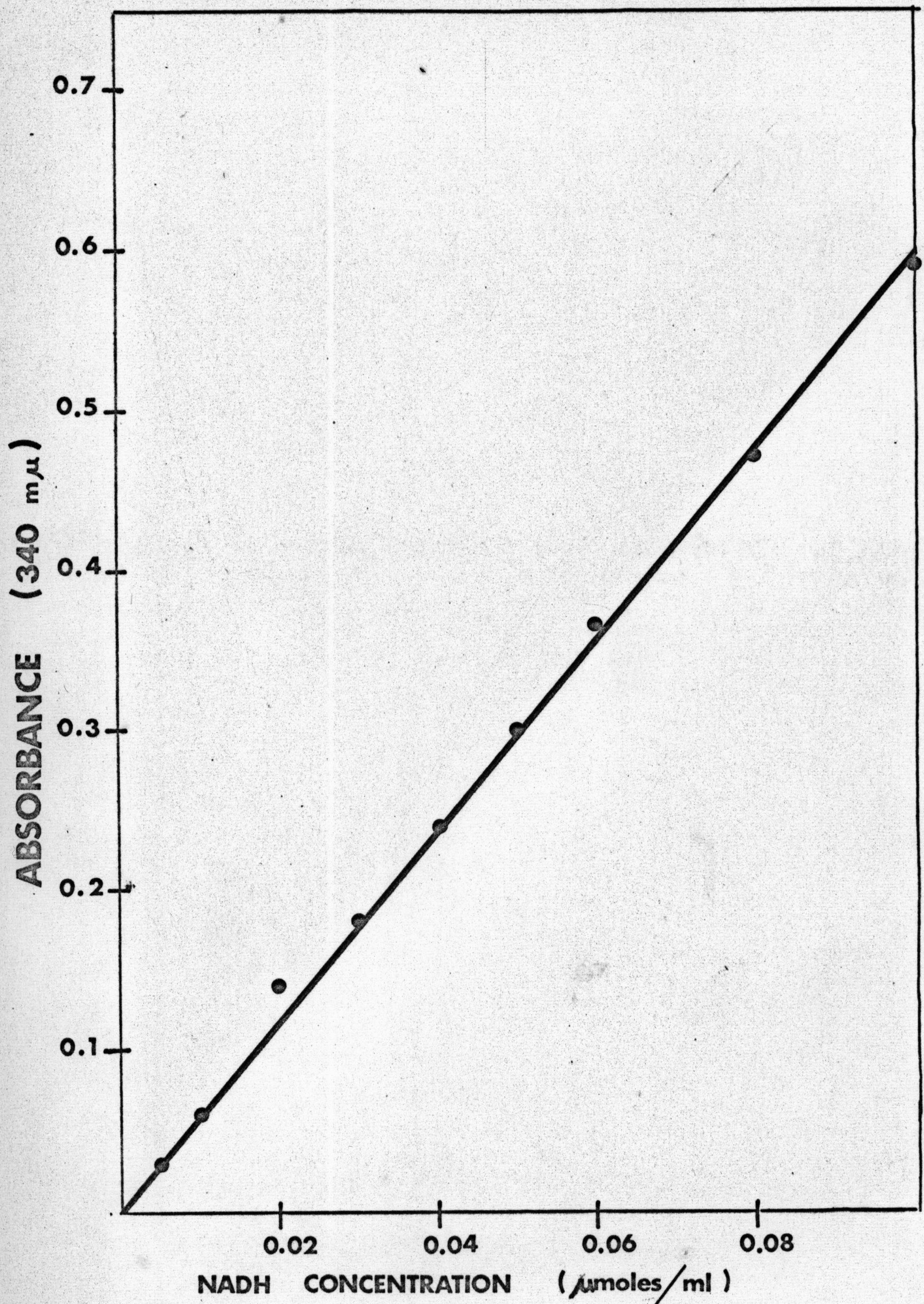
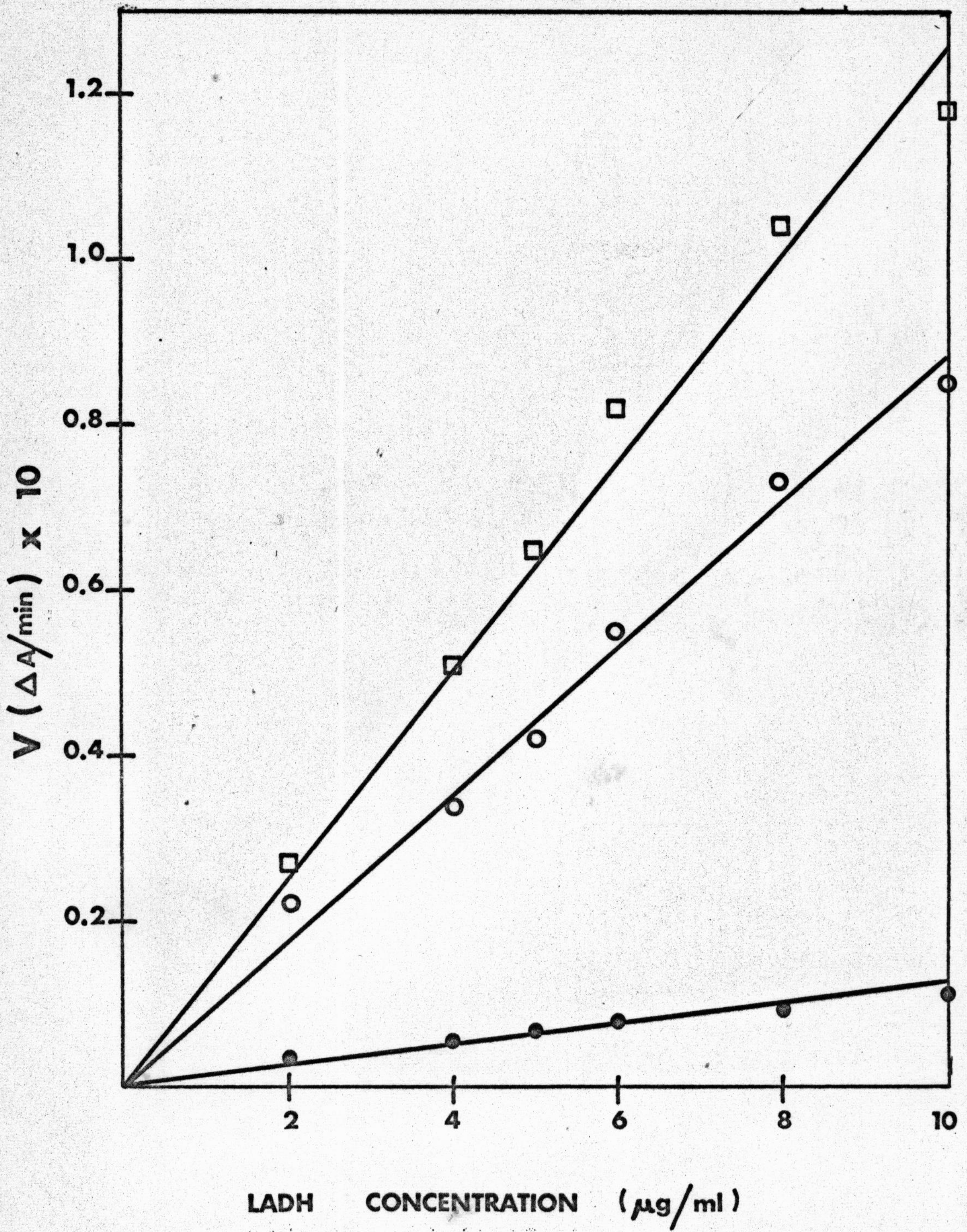
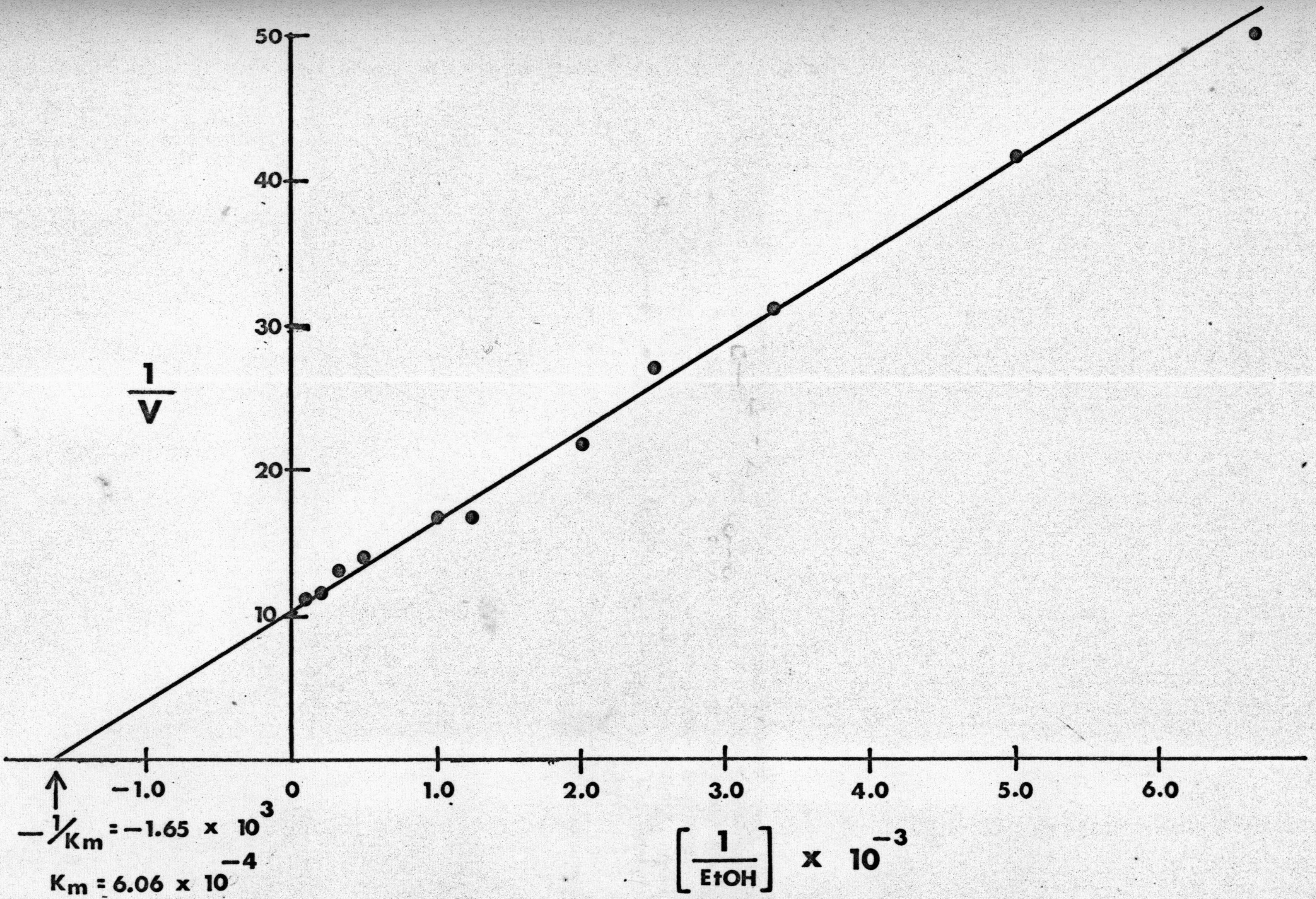

ABSORBANCE (340 mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.02
0.04
0.06
0.08
NADH CONCENTRATION (μmoles/ml)

Figure 5. The effect of LADH concentration on initial reaction velocity. Each cuvette contained: NAD, 4.0×10^{-4} M; LADH; glycine buffer, 0.1 M, pH 8.8; ethanol. The ethanol concentrations employed were: •—• , 1.0×10^{-4} M; ○—○ , 1.0×10^{-3} M; □—□ , 1.0×10^{-2} M. Initial velocity (v) was measured as the increase in absorbance at 340 mμ.



V (ΔA/min) x 10
1.2
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
LADH CONCENTRATION (μg/ml)

Figure 6. Determination of the Km for ethanol in the reaction catalyzed by LADH. The reciprocal of the initial velocity is plotted versus the reciprocal of the ethanol concentration. Each cuvette contained: NAD, 4.0×10^{-4} M; ethanol, LADH, 12 μg, glycine buffer, 0.1 M, pH 8.8. Initial velocity was measured as the increase in absorbance at 340 mμ.



50
40
30
20
10
1/V
−1.0
0
1.0
2.0
3.0
4.0
5.0
6.0
−1/Km = −1.65 x 10^3
Km = 6.06 x 10^−4
[1/EtOH] x 10^−3

Figure 7. Inhibition by salicylate of the LADH-catalyzed reaction with ethanol as the variable substrate. The reciprocal of the initial velocity is plotted versus the reciprocal of the ethanol concentration. Each cuvette contained: ethanol; NAD , 4.0×10^{-4} M; LADH, 12 μg; glycine buffer, 0.1 M, pH 8.8. Salicylate concentrations employed were: •—• none; ○—○ 2.5mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as increase in absorbance at 365 mμ.

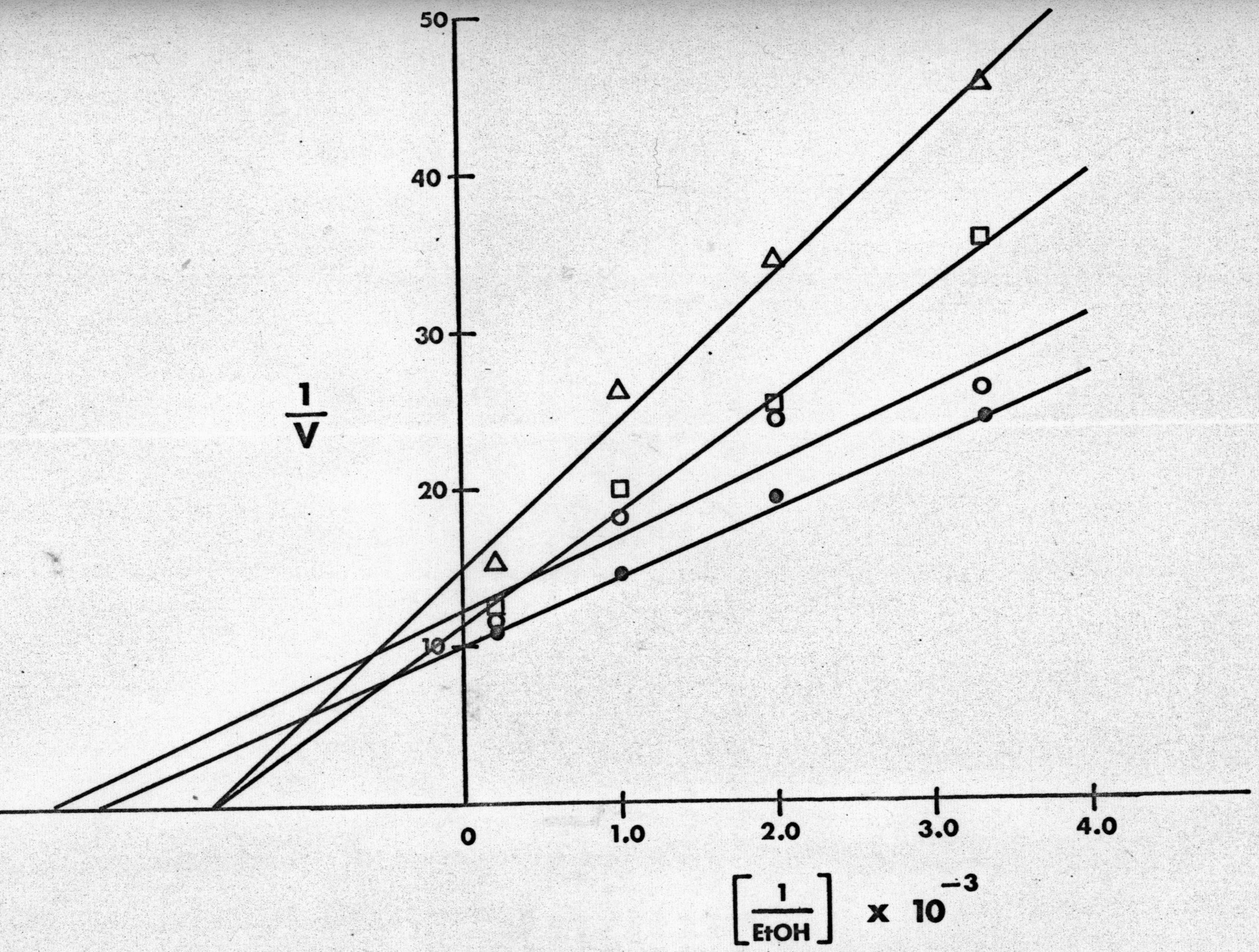

50
40
30
20
10
1/V
0
1.0
2.0
3.0
4.0
[1/EtOH] x 10^-3

Figure 8. Inhibition by salicylate of the LADH-catalyzed reaction with ethanol as the variable substrate. Ethanol concentration/initial velocity is plotted versus ethanol concentration. Each cuvette contained: ethanol; NAD, 4.0×10^{-4} M; LADH, 12 μg; glycine buffer, 0.1 M, pH 8.8. Salicylate concentrations employed were: ●—● , none; ○—○ 2.5 mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as increase in absorbance at 365 mμ.

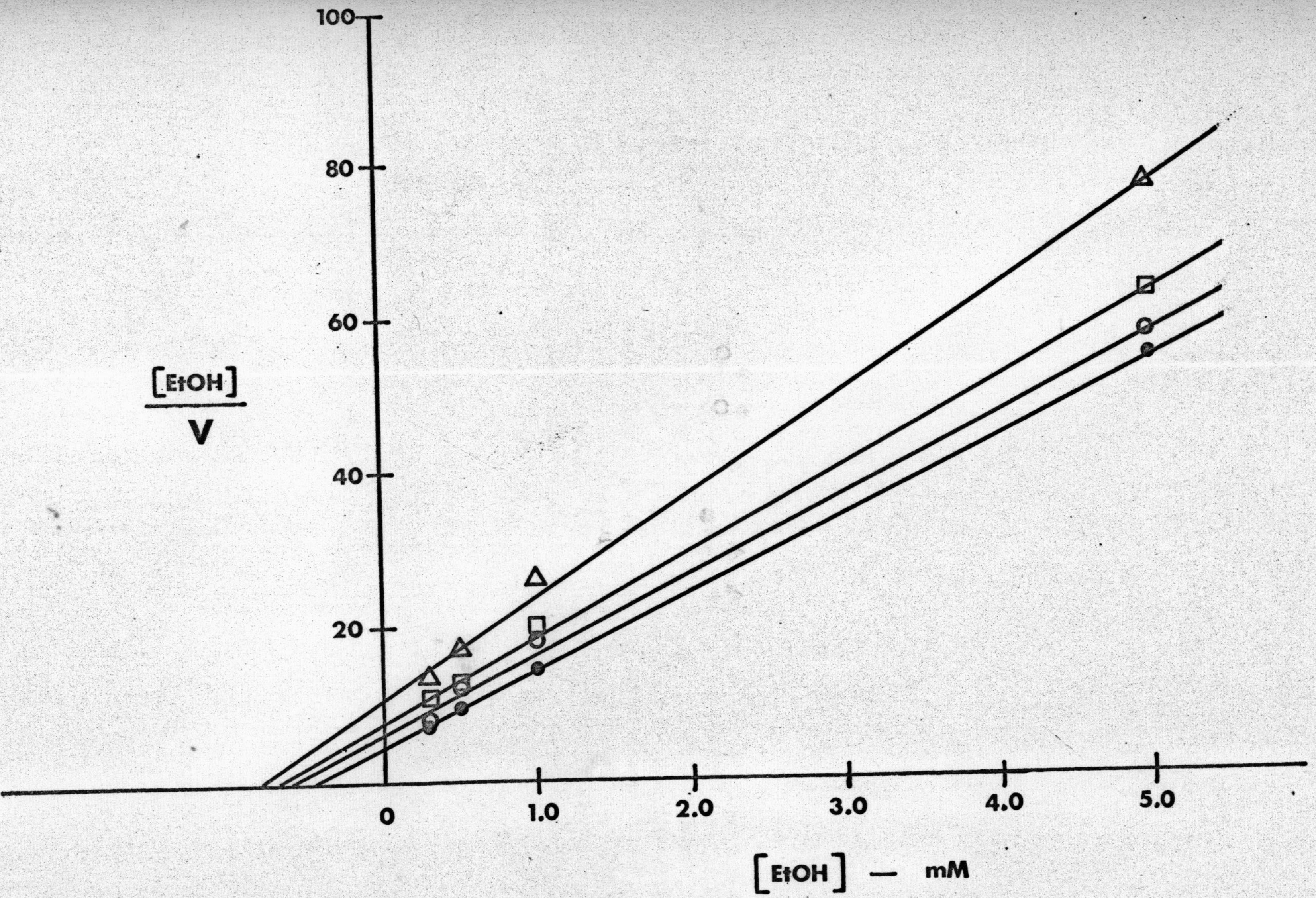

100
80
60
40
20
[EtOH]
V
0
1.0
2.0
3.0
4.0
5.0
[EtOH] — mM

Figure 9. Inhibition by phenol of the LADH-catalyzed reaction with ethanol as the variable substrate. The reciprocal of the initial velocity is plotted versus the reciprocal of the ethanol concentration. Each cuvette contained: ethanol; NAD, 4.0×10^{-4} M; LADH, 12 μg; glycine buffer, 0.1 M, pH 8.8. Phenol concentrations employed were: ●—● none; ○—○ 2.5 mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as increase in absorbance at 340 mμ.

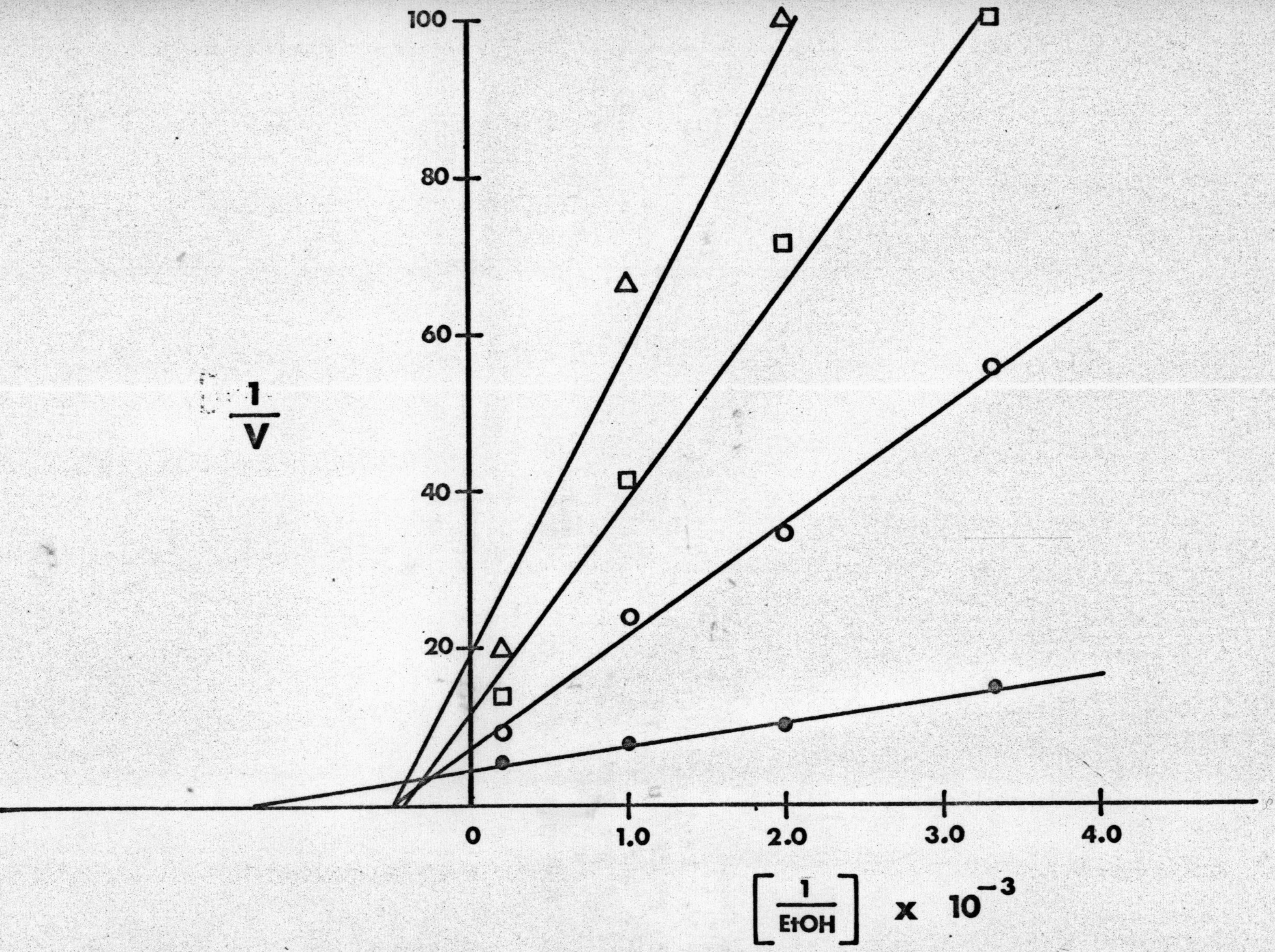

100
80
60
40
20
0
1.0
2.0
3.0
4.0
1/V
[1/EtOH] x 10^-3

Figure 10. Inhibition by phenol of the LADH-catalyzed reaction with ethanol as the variable substrate. Ethanol concentration/initial velocity is plotted versus ethanol concentration. Each cuvette contained: ethanol; NAD, 4.0×10^{-4} M; LADH, 12 µg; glycine buffer, 0.1 M, pH 8.8. Phenol concentrations employed were ●—● none; ○—○ 2.5 mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as increase in absorbance at 340 mµ.

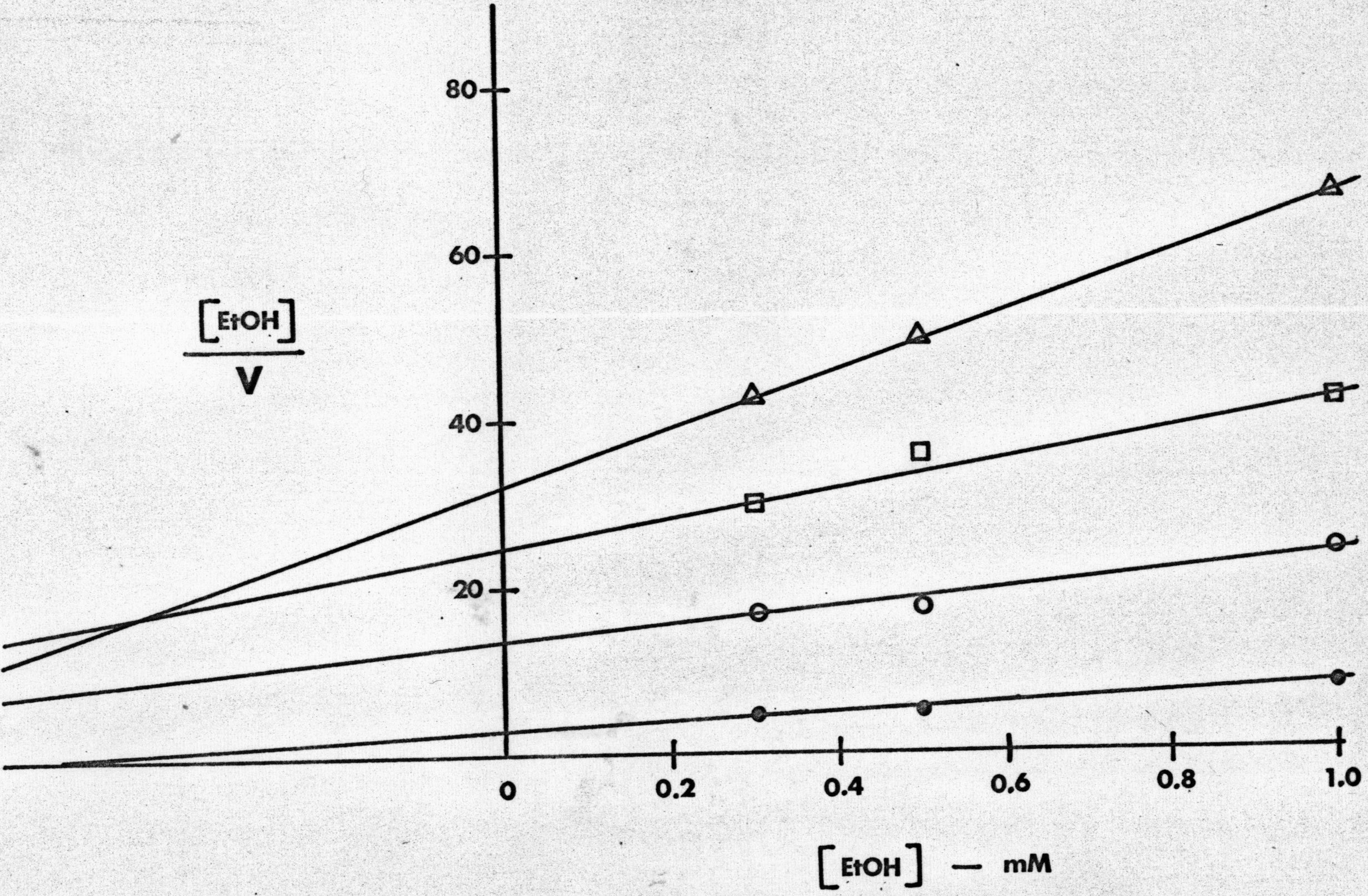
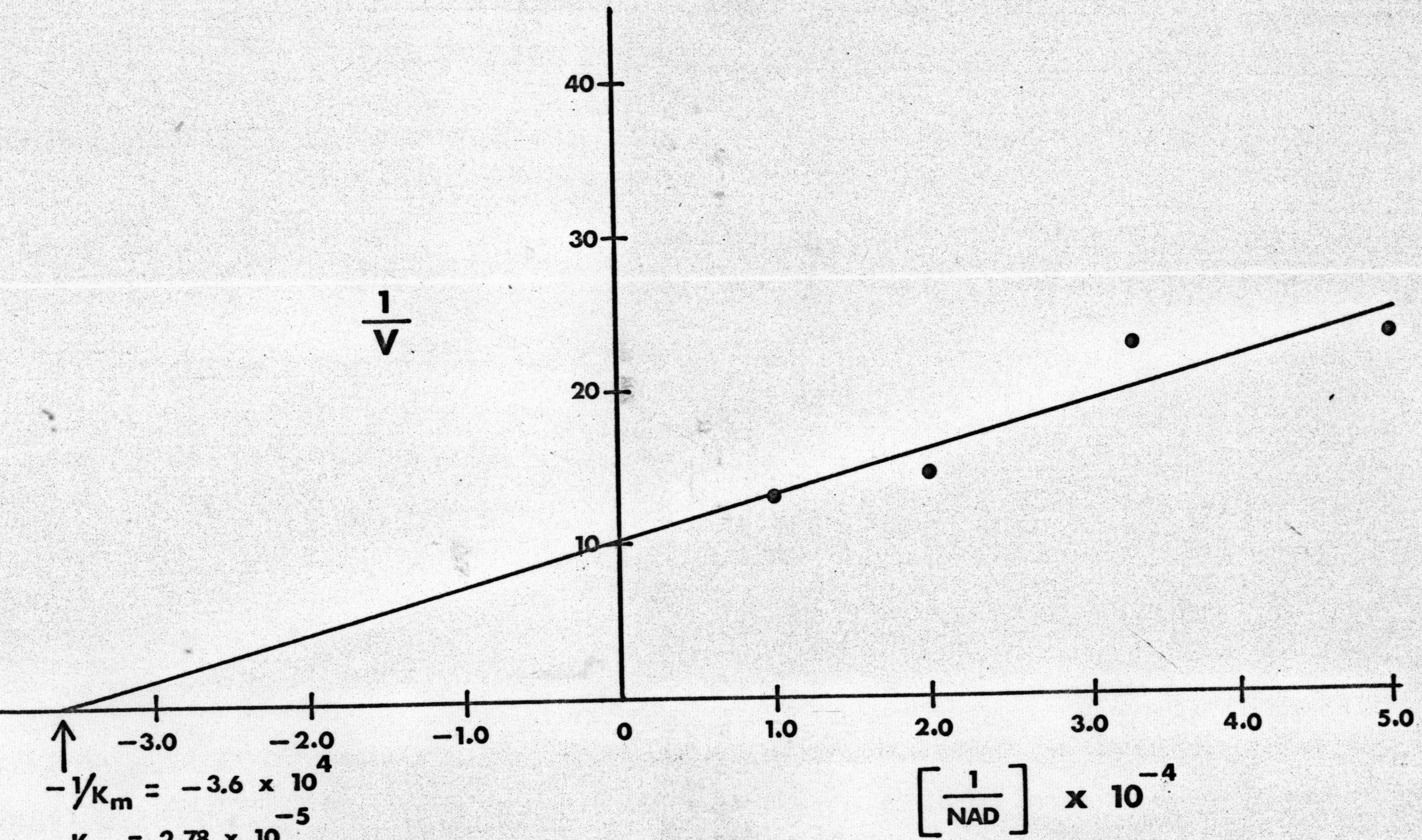

[EtOH]
V
80
60
40
20
0
0.2
0.4
0.6
0.8
1.0
[EtOH] — mM

Figure 11. Determination of the Km for NAD in the reaction catalyzed by LADH. The reciprocal of the initial velocity is plotted versus the reciprocal of the NAD concentration. Each cuvette contained: ethanol, 5.0×10^{-3} M; NAD, LADH, 12 µg; glycine buffer, 0.1 M, pH 8.8. Initial velocity was measured as the increase in absorbance at 365 mµ.



1/V
40
30
20
10
0
-3.0
-2.0
-1.0
1.0
2.0
3.0
4.0
5.0
[1/NAD] x 10^-4
-1/K_m = -3.6 x 10^4
K_m = 2.78 x 10^-5

Figure 12. Inhibition by salicylate of the LADH-catalyzed reaction with NAD as the variable substrate. The reciprocal of the initial velocity is plotted versus the reciprocal of the NAD concentration. Each cuvette contained: ethanol, 5.0×10^{-3} M; NAD; LADH, 12 μg; glycine buffer, 0.1 M, pH 8.8. Salicylate concentrations employed were: ●—● none; ○—○ 2.5 mM; □—□ 5.0 mM. Initial velocity was measured as the increase in absorbance at 365 mμ.

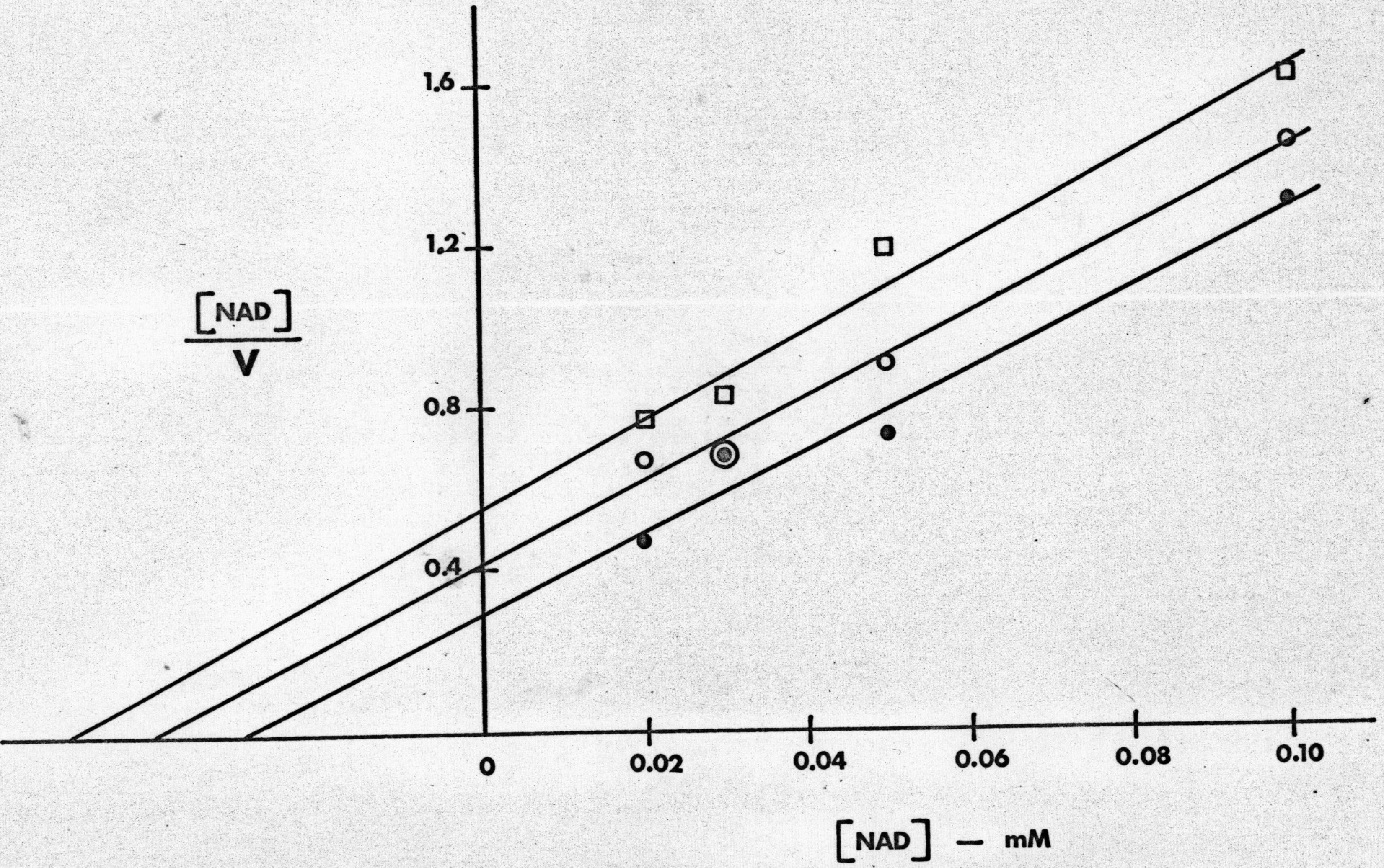
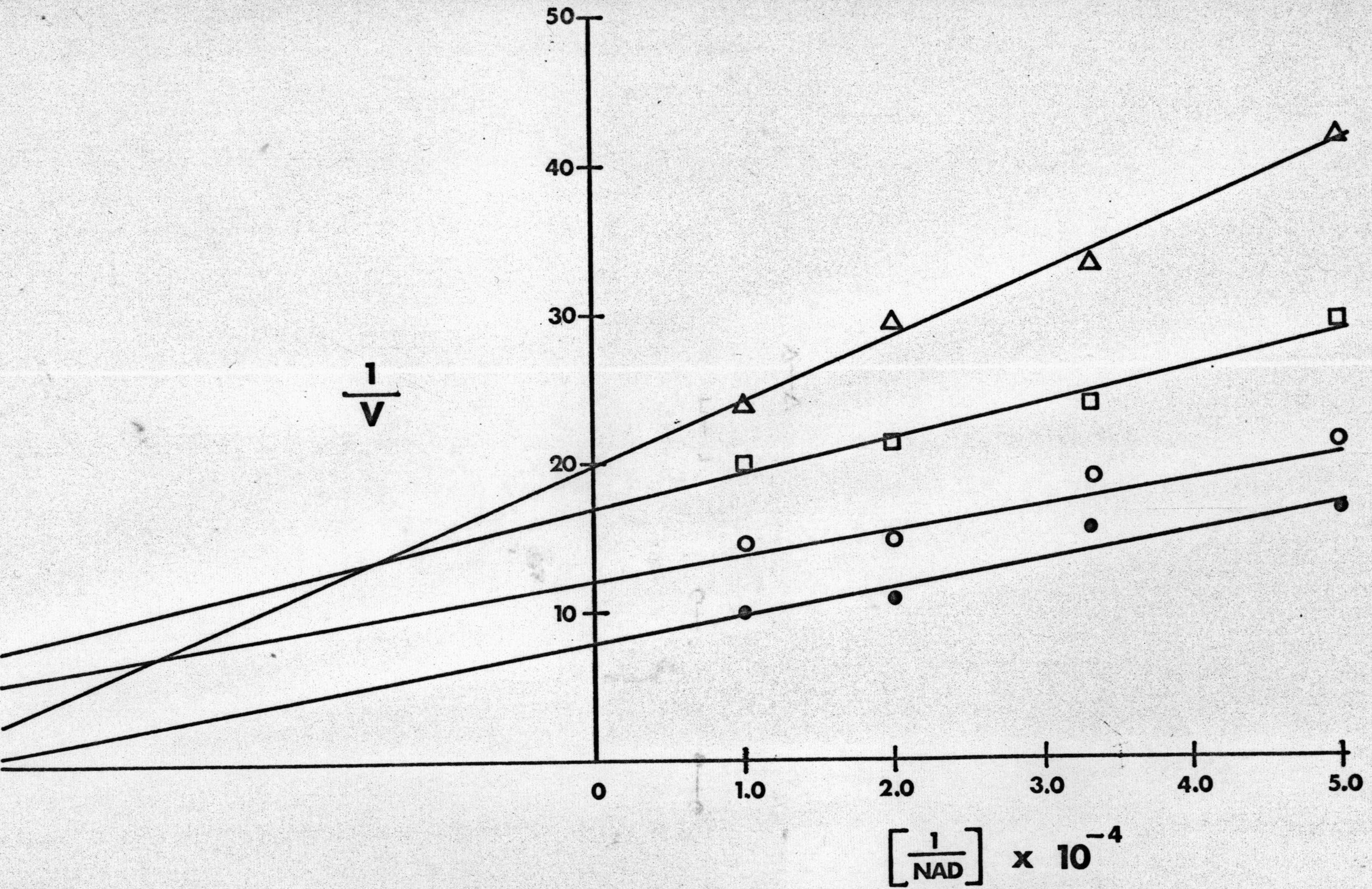
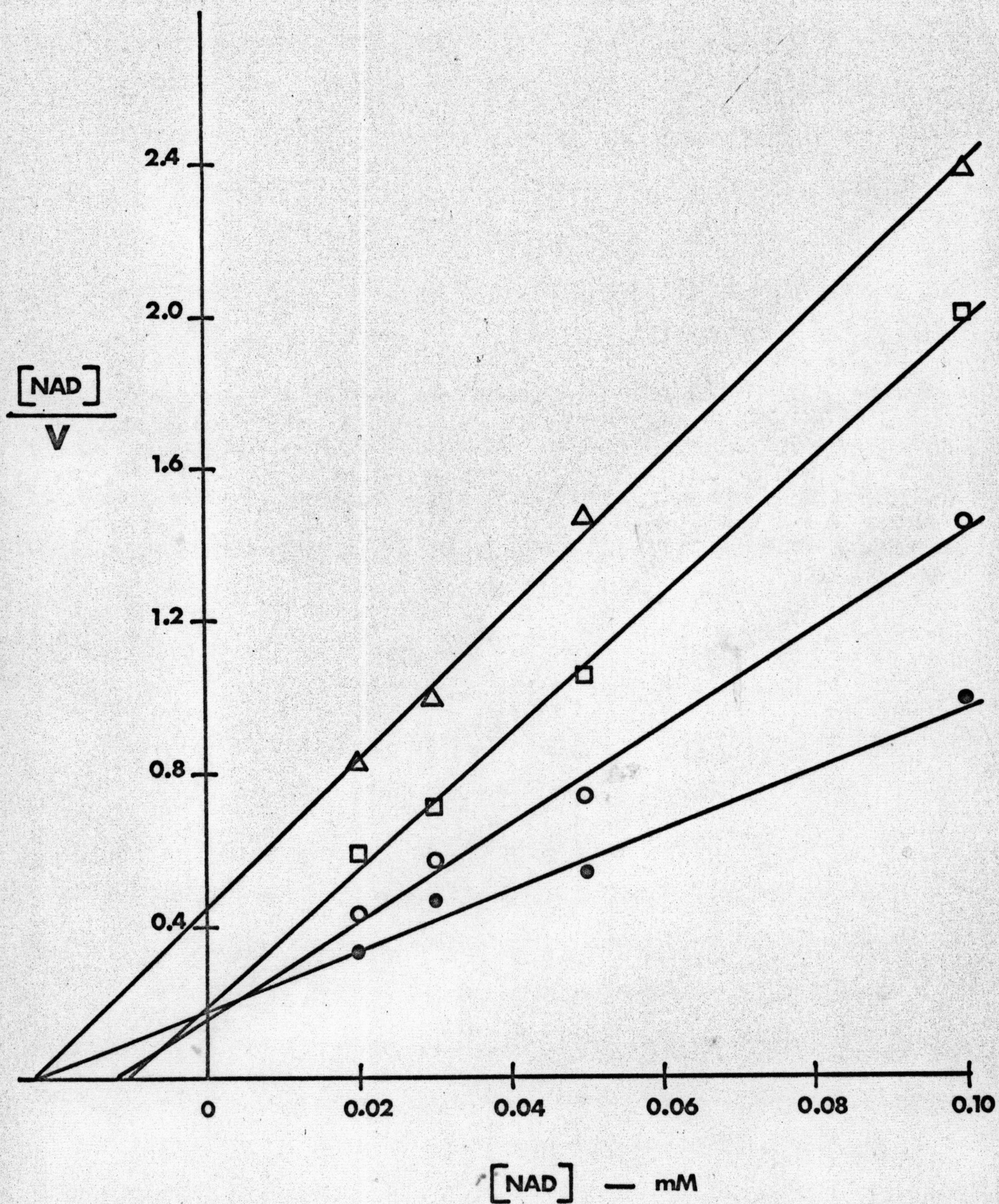

[NAD]/V
1.6
1.2
0.8
0.4
0
0.02
0.04
0.06
0.08
0.10
[NAD] — mM

Figure 13. Inhibition by phenol of the LADH-catalyzed reaction with NAD as the variable substrate. The reciprocal of the initial velocity is plotted versus the reciprocal of the NAD concentration. Each cuvette contained: ethanol, 5.0×10^{-3} M; NAD; LADH, 12 μg; glycine buffer, 0.1 M, pH 8.8. Phenol concentrations employed were: ●—● none; ○—○ 2.5 mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as the increase in absorbance at 340 mμ.



50
40
30
20
10
1/V
0
1.0
2.0
3.0
4.0
5.0
[1/NAD] x 10^-4

<u>Figure 14</u>. Inhibition by phenol of the LADH-catalyzed reaction with NAD as the variable substrate. NAD concentration/initial velocity is plotted versus NAD concentration. Each cuvette contained: ethanol, 5.0×10^{-3} M; NAD; LADH, 12 µg; glycine buffer, 0.1 M, pH 8.8. Phenol concentrations employed were: ●—● none; ○—○ 2.5 mM; □—□ 5.0 mM; △—△ 10.0 mM. Initial velocity was measured as the increase in absorbance at 340 mµ.



[NAD]
V
2.4
2.0
1.6
1.2
0.8
0.4
0
0.02
0.04
0.06
0.08
0.10
[NAD] — mM

DISCUSSION AND CONCLUSION

A drug may be defined as a chemical which interacts with some component or components of a living system to produce a therapeutic effect. This interaction takes place at that site designated as the receptor, and results in initiation of certain specific, physiological changes in the living system. In addition to these drug-induced alterations which are advantageous to the system, most drugs also produce other, less desirable alterations in function, which are designated at adverse or toxic effects, depending on the severity of the response initiated. The simultaneous administration of several therapeutic agents increases the incidence of such adverse effects since not only may each drug produce undesired actions on the body, but in addition the drugs may interact on the molecular level to result in even greater magnitude or incidence of undesirable action.

The investigations reported here represent preliminary studies aimed to elucidate a possible molecular site for drug interaction. Salicylate was chosen as a model drug for investigation since this group of analgesic-antipyretic agents is extremely widely used today. The molecular level chosen for study was the enzyme alcohol dehydrogenase from horse liver. This enzyme appears to be ideal for studies of this type because it is available from commercial sources in a highly purified form, because other investigators have reported it to be inhibited by salicylate, and because it has

an unusually broad substrate specificity. The studies carried out in this laboratory were based on the premise that the broad substrate specificity of the enzyme may indicate that other drugs also can react with it, and that these other drugs may serve either as alternative substrates or as inhibitors for the enzymic reaction.

The data presented confirm that salicylate does inhibit LADH. Although the data are not complete enough to permit determination of the mechanism of salicylate inhibition of LADH, they do suggest that the inhibition may be more complex in nature than previously reported by other investigators. The effect of salicylate on initial reaction velocity when ethanol was the variable substrate suggests that more than one mechanism may be involved and that the relative importance of these different mechanisms may depend upon the inhibitor concentration. This possibility apparently has not been considered by Dawkins _et al_.(17) Their studies were carried out at salicylate concentrations ranging from 1 mM to 5 mM, a concentration range which may not be wide enough to permit observation of the results reported here. In addition, the graphic presentation form employed by these authors is not sufficiently accurate to permit others to determine whether other interpretations of their data may be valid.

The effect of salicylate at concentrations of 2.5 and 5.0 mM on initial reaction velocity when NAD was the variable substrate appears to confirm the competitive nature of the inhibition reported by Dawkins _et al_.(17) However, in order

to make more equivalent comparisons with their data, further studies should be carried out under the same reaction conditions. The studies of Dawkins _et al_ were carried out at pH 10.0, whereas the pH at which the assays in this investigation were carried out was 8.8. It is possible that the mechanism of inhibition may be pH dependent.

The study of the structural requirement for salicylate inhibition of LADH when ethanol is the variable substrate has shown that benzoate has no inhibitory effect on the enzyme, whereas phenol is a more potent inhibitor than is salicylate. The fact that phenol is able to inhibit LADH is of interest since Smith and Bryant[20] found no inhibition of malic dehydrogenase by phenol. In contrast to the situation with salicylate, phenol appears to produce a mixed type of inhibition at all concentrations tested when ethanol was used as the variable substrate. When NAD was used as the variable substrate, the inhibition by phenol appeared to depend upon the inhibitor concentration; the inhibition appears distinctly non-competitive at the high phenol concentration (10 mM) when NAD was used as the variable substrate. Further studies are needed to determine whether this inhibition by phenol is similar to that of salicylate or whether an irreversible process such as protein denaturation is occurring.

The fact that the studies involving salicylate did not give results which indicate a distinct mechanism of inhibition may be explainable on the basis that LADH has recently been

shown to occur in multiple isoenzymic forms.[21] In these preliminary reports, these isoenzymic forms of LADH have been shown to have different substrate specificities. Difficulty in the interpretation of experimental data may arise if different commercial sources of LADH vary in the composition of the isoenzymic forms of LADH since this would produce variations in data obtained with specific substrates or inhibitors.

In conclusion, it is significant that phenol was shown to be a potent inhibitor of LADH and further investigations will be necessary to determine the mechanism of the inhibition and whether other drugs containing a phenolic hydroxyl group can be expected to inhibit LADH also. Extrapolations of these studies are to test other drugs as substrate for the LADH isozymer and then to determine the type of inhibition by salicylate or phenol. Possible drugs to test initially include chloramphenical, pyridoxal, bishydroxycoumarin, mephenesin, hydroxyzine and perphenazine. It would also be of interest to investigate the effect of some of the metabolites of salicylate such as gentisic acid and the glucuronide and glycine conjugates of salicylic acid on the activity of horse liver alcohol dehydrogenase.

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