ABSTRACT

THE ISOLATION AND CHARACTERIZATION
OF A NEW CLASS OF LACTIC DEHYDROGENASE
INHIBITORS: SUBSTITUTED PHENOLS

by Mark Chasin

In the course of studies with Krebs cycle metabolites, a potent inhibition of lactic dehydrogenase was observed in commercial malic acid solutions. This inhibitor material was isolated and purified not only to characterize its inhibition of the enzyme but also to test the hypothesis, which stemmed from Warburg's conclusions, that compounds which inhibit lactate production should inhibit cancer.

The inhibition of lactic dehydrogenase by a commercial malic acid preparation was traced to the presence of an impurity. Maple syrup, the commercial source of malic acid, was extracted with methylene chloride. This extract was dried and further extracted with water. Lyophilization of this water extract produced a Maple Syrup Fraction (MSF), which contained the inhibitory substance(s). Using thin layer and gas chromatography, as well as other chemical and instrumental analytical techniques, the following four active lactic dehydrogenase inhibitors were separated and identified from the MSF: (1) vanillin, (2) syringaldehyde, (3) p-hydroxybenzaldehyde and (4) cyclotene. These compounds, as well as those in the following section, were shown to be reversible inhibitors of lactic dehydrogenase, and to be competitive

with pyruvate and non-competitive with DPNH by Lineweaver-Burke analysis. Since it has previously been shown that only anionic substances inhibit lactic dehydrogenase, it was of particular interest that the anionic form of these substituted phenols was found to be the active inhibitory species.

In an effort to obtain a greater inhibition than that achieved with the naturally occuring inhibitors, the following series of seven analogues was tested (the K_i's, in μM, are given in parenthesis): p-hydroxybenzaldehyde (414), protocatachualdehyde (174), "methyl" vanillin (270), "ethyl" vanillin (241), "methoxy" vanillin (= vanillin) (96), "ethoxy" vanillin (108), and the bisulfite adduct of vanillin (14). The potency of the inhibitors was found to be a function of the relative negative charge on the alpha atom on the 3 position of the aldehyde ring, rather than a function of the length of the substituent, as would be predicted from purely steric considerations. The aldehyde group increased the activity of the inhibitors over and above lowering their pK, since nitro and nitroso groups cannot fully substitute for the aldehyde moiety. Vanillin, chosen as a representative inhibitor, inhibited the H_4 isozyme of lactic dehydrogenase approximately twice as much as the M_4 isozyme.

In tests of the ability of these lactic dehydrogenase inhibitors to inhibit cancer, none showed any reproducible inhibition of Walker 256 intramuscular, leukemia 1210 or sarcoma 180 carcinomas. A study of the rate and pathway of metabolism of both vanillin and its bisulfite adduct in mice

gave LD_{50}'s of 760 and 1850 mg/kg respectively. The metabolic rates were 525 and 310 mg/kg/hour respectively. Apparently the bisulfite adduct induced enzymes which promoted its metabolism, since the metabolic rate was more than doubled after a 12 day exposure (to 660 mg/kg/hour). Both compounds were mainly metabolized to vanillic acid, with minor amounts of several different conjugates also found. The rapid rate of metabolism offers a possible explanation of the lack of effect of vanillin on cancer. Moreover, despite the fact that the bisulfite adduct was both a more potent inhibitor and much less rapidly metabolized, at the doses used for carcinostatic testing, the level of even the bisulfite adduct is reduced to the base line level within 3 hours. Therefore it is not surprising that it too did not show any significant carcinostatic activity. In conclusion, several potent non-toxic lactic dehydrogenase inhibitors have been isolated, but their rapid rates of metabolism preclude any meaningful conclusions on the theory of inhibiting cancer by inhibiting lactic dehydrogenase.

During the course of this investigation, it was found that lactic dehydrogenase shows a marked inactivation when left standing at low concentrations (about 5.6 μg/ml.), but that the activity remained constant at 56 μg/ml. for up to 48 hours.

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By

Mark Chasin

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TO MY FAMILY

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Literature Review

Part I, Properties of Lactic Dehydrogenase

A) Reaction Catalyzed and Physiological Function

Lactic dehydrogenase occurs ubiquitously in animal cells and catalyzes the final reaction of glycolysis, as shown below:

$$\text{pyruvate} + \text{DPNH} + H^+ \rightleftharpoons \text{lactate} + \text{DPN}^+$$

Physiologically, its two major functions are:

(1) to convert pyruvate to lactate under energy utilizing conditions in tissues such as muscle, brain, eye and heart.

(2) to convert lactate to pyruvate in the gluconeogenic organ, liver.

B) Preparation of the Enzyme

The enzyme was first found in animal tissue by Thunberg (1920). In the early 1930's Bunga, et al. (1932), analyzed cell free extracts for activity. The first crystalline enzyme was prepared from beef heart by Straub (1940), and this has become the standard procedure for the preparation of this enzyme. The crystalline enzyme has been prepared from rat skeletal muscle (Kubowitz and Ott, 1943;

Racker, 1951; and Beisenherz, et al., 1953), rat liver (Gibson, et al., 1953), rat heart (Wieland, et al., 1959), human heart (Nisselbaum and Bodansky, 1961a), pig heart (Meister, 1952) and beef heart (Takenaka and Schwert, 1956; Schwert, Miller and Takenaka, 1962).

C) Physical Characteristics

The sedimentation coefficient of the beef heart enzyme has been reported as s = 6.36S and 6.46S for two different preparations (Meister, 1950), although the conditions of the experiment were not given. However, Neilands (1952) reported a sedimentation coefficient of $s_{20,w}$ = 7.05S, a diffusion coefficient of $D_{20,w} = 5.26 \times 10^{-7}$ cm^2/sec and a partial specific volume of 0.754 cc/g for the beef heart enzyme, but experimental details were not described. He calculated the molecular weight to be 135,000 ± 15,000. The enzyme from hog heart has a sedimentation coefficient of $s^{o}_{20,w}$ = 7.65S (Kegeles and Gutter, 1951).

Assuming a molecular weight of 135,000 (Neilands, 1952) for the native molecule, Velick (1958) estimated the minimal combining weight of the beef heart enzyme with DPNH to be 37,500.

D) Dissociation and Subunit Composition of the Native Molecule

Results of Millar (1962) suggest that electrophoreti-

cally homogeneous beef heart enzyme dissociates at concentrations below 0.2%. The sedimentation coefficient extrapolated from concentrations above 0.2% was reported as $s^{o}_{20,w} = 7.71S$. The Archibald method (1947) yielded a molecular weight of 72,000 at infinite dilution, and although the exact extrapolation seems questionable, the data do suggest that a marked decrease in molecular weight occurs. However, Appèlla (1964) reported that the molecular weight of the beef heart enzyme remained at 140,000 in dilutions to 0.04%, as measured both by light scattering and sedimentation-diffusion. Recently, Hathaway and Criddle (1966) reported the beef heart enzyme in the absence of substrate to have a sedimentation coefficient of $s_{20,w} = 5.6S$ at 3.0 μg/ml. However, they found that 10^{-3} M pyruvate could raise the sedimentation coefficient under otherwise identical conditions to $s_{20,w} = 7.7S$, and suggested that substrate can form active tetramers from inactive dimers. At present, the molecular weight of the native beef heart lactic dehydrogenase is still open to question.

Appella and Markert (1961) treated the H_4 isozyme (see below) from crystalline beef heart lactic dehydrogenase with 5M guanidine hydrochloride and 0.1M 2-mercaptoethanol. This treatment dissociated the enzyme into four inactive subunits with a molecular weight of 34,000, a sedimentation coefficient of $s^{o}_{20,w} = 1.75S$, a diffusion coefficient of $D^{o}_{20,w} = 6.74 \times 10^{-7}$ cm^2/sec., and a partial specific volume of 0.740 cc/g. These results suggesting four subunits per native molecule have recently been questioned. Steginк and Vestling (1966)

report eight amino terminal acetate residues for rat liver M_4 enzyme and 7-8 moles of acetate per mole of beef heart H_4 and MH_3 isozymes (see below). Appella (1964) reported eight amino terminal valines per mole of beef heart lactic dehydrogenase, and indicated fingerprint studies also showed eight polypeptide chains per molecule. The observation of fifteen isozyme bands rather than the predicted five (see F below) places further doubt on the four subunit models of lactic dehydrogenase.

E) Renaturation Studies

Reversible disaggregation of beef heart lactic dehydrogenase was demonstrated by Markert (1963) by freezing in 1M NaCl. Later, Epstein et al. (1964) achieved up to 60% reactivation after subjecting the enzyme to 10.5M urea and 0.12M 2-mercaptoethanol, conditions shown to cause complete dissociation of the protein into unfolded polypeptide chains. Chilson, et al. (1965a) used 7.5M guanidine hydrochloride to dissociate the chicken H_4 enzyme. The presence of 1.3×10^{-3}M DPNH in their renaturation medium yielded 30% recovery of activity, rather than the 15% observed when DPNH was not included in the reversal mixture. It had previously been shown that DPNH will protect beef heart lactic dehydrogenase from inactivation by heat, urea (Pfleiderer, et al., 1957) and sodium dodecyl sulfate (Di Sabato and Kaplan, 1964).

F) Isozymes

The procedure of Straub (1940) using heart tissue yields crystalline enzyme which exhibits two components upon electrophoresis at pH near neutrality (Meister, 1950; Neilands, 1952a and b; Pfleiderer and Jeckel, 1957). These and other workers (Vesell and Bearn, 1957 and 1958; Hill, 1958; Hess, 1958) found the several electrophoretic forms active. However, Schwert and coworkers (1962) separated the two forms on hydroxylapatite and suggested that the slower electrophoretic component was inactive, although the interpretation of their results is open to question, since their specific activity curve indicated both forms may be active. Results described in this thesis indicate both forms are active.

In 1957, Wieland and Pfleiderer reported that electrophoresis of lactic dehydrogenase from various tissues gave one to six components exhibiting enzymatic activity. Markert and Moller (1959) extended the analysis and defined the molecular basis for the observation of the several active forms. They coined the term "isozyme" to describe different molecular forms with the same enzymatic activity. Fritz and Jacobson (1963a, b, 1965) have shown twelve to fifteen enzymatically active bands by electrophoresis of mouse muscle, mouse heart and rat liver lactic dehydrogenase in 2-mercaptoethanol.

Appella and Markert (1961) found that the subunits

produced by 5M guanidine hydrochloride could be separated on the basis of charge into two different kinds and were designated A and B lactic dehydrogenase. Later, Dawson, et al. (1964) named these H and M after the tissue sources in which they predominated, namely, heart and muscle. Theoretically, combining these two types of subunits in all possible permutations of four subunits per native tetramer predicts the existence of the following five isozymes: H_4, H_3M, H_2M_2, HM_3 and M_4, and these are observed experimentally upon electrophoresis. Salthe, et al. (1965), Emerson, et al. (1964) and Chilson, et al. (1965a) have shown that when frozen together in high salt at neutral pH, a mixture of the pure M_4 and H_4 lactic dehydrogenases from different species will produce all five isozymes.

The lactic dehydrogenase isozymes have been found to differ widely in properties other than migration in an electric field, including amino acid composition (Wachsmith, et al., 1964; Fondy and Kaplan, 1965; Kaplan, 1964), immunological reactivity (Nisselbaum and Bodansky, 1961; Cahn, et al., 1962), kinetic behavior (Vesell and Bearn, 1961; Kaplan, 1964), thermal stability (Hill, 1958; Pfleiderer, et al., 1957; Wroblewski and Gregory, 1961; Zondag, 1963), subunit composition (Appella and Markert, 1961), and inhibition by lactate (Brody, 1964), pyruvate (Plagemann, et al., 1960; Stambaugh and Post, 1966), urea (Brand, et al., 1962), oxalate (Emerson, et al., 1964), sulfite (Wieland and Pfleiderer, 1957) and α-hydroxybutyrate (Elliot, et al., 1962).

Assays for directly determining the fraction of each parent type in a sample of lactic dehydrogenase have been developed by Dawson, et al., (1964) and Stanbaugh and Post (1966), based on differential inhibition by either pyruvate or lactate, and Kaplan and Cahn, (1962), based on differences in their activity toward the hypoxanthine cofactor analogue.

It is now well established (Shaw and Barto, 1963; Markert, 1963; Goodfriend and Kaplan, 1964; Fine, et al., 1963 and Cahn, Kaplan and Zwilling, 1962) that the synthesis of the two isozymes of lactic hydrogenase is under control of two separate genes. A third isozyme distinct from H or M has recently been demonstrated in sperm (Blanco and Zinkham, 1963).

G) Physiological Significance of the Isozymes

Studies by Dawson, et al. (1964), Cahn, et al. (1962), and Kaplan and Cahn (1962) indicate that the H and M forms of lactic dehydrogenase may have significantly different physiological roles. The H form is inhibited by excess pyruvate to a greater degree than the M form. In aerobic tissue pyruvate inhibits the H form and forces the cell to oxidize pyruvate by the mitochondria, yielding large amounts of energy needed by such aerobic tissue. In tissues such as striated muscle, where M is found, energy is needed in short bursts under anaerobic conditions, and can be produced despite temporarily high levels of pyruvate. Dawson, et al.

(1964) have correlated the M subunit concentration with the apparent degree of dependance of the tissues on aerobic glycolysis as an energy source. The results of Goodfriend, et al. (1966) demonstrating the differential repression of synthesis of M subunits under increasing oxygen tension support this concept of a functional role for the 2 different subunits.

H) Active Site Studies

Nygaard (1956), measured the binding of pyruvate as a function of pH and concluded that an imidazolium group could be at the binding site for pyruvate. Winer and Schwert (1958) concluded two dissociable groups were involved in binding DPNH - one with a pK of 6.8, possibly imidazolium, and the other, with a pK of 9.8, a sulfhydryl.

Measurements by fluorescence (Velick, 1958; Winer, et al., 1959), binding of DPNH analogues (van Eys, et al., 1958) and ultracentrifugation (Takenaka and Schwert, 1956) show each mole of enzyme to bind four moles of coenzyme.

I) Mechanisms for the Reaction

From the work of Alberty (1953) and their work, Hakala et al. (1956) suggest the following possible mechanisms for the action of lactic dehydrogenase:

1. Either substrate or coenzyme may be bound to the

enzyme, but the binding sites are not independent. The binding of the second reactant is influenced by the presence of the reactant which is already bound.

2. There is a compulsory order of binding of reactants. Substrate, for example, is not bound by the enzyme but only by the enzyme-coenzyme complex. Various ternary complexes may be postulated without altering the form of the equations relating initial velocity to initial concentrations of reactants.
3. There is a compulsory sequence of interaction of enzyme with reactants but in which ternary complexes are so short-lived as to be without kinetic significance - the so-called "Theorell-Chance" mechanism. (Theorell and Chance, 1951)

This third possibility is unlikely since there is a direct stereospecific transfer of hydrogen from coenzyme to substrate (Loewus and Stafford, 1960). Work with inhibitors (Anderson, et al., 1964; Novoa and Schwert, 1961), centrifugal separation experiments (Takenaka and Schwert, 1956), rapid equilibrium analysis (Silverstein and Boyer, 1964), and spectroscopic measurements (Chance and Neilands, 1952) favor a compulsory order of binding, cofactor before substrate, but there is no definite proof of mechanism as yet.

J) Equilibrium Constants and Enthalpy of Reaction

The equilibrium constant (concentrations in moles/liter) for the enzymatic reaction catalyzed by lactic dehydrogenase has been variously reported as 4.4×10^{-12} (Racker, 1950), 3.3×10^{-12} (Neilands, 1952) and 2.76×10^{-12} (Hakala, et al., 1956), all at 25.0°C. Hakala further estimated the ΔH for the oxidation of lactate by DPN^+ to be 10.3 ± 0.3 kcal/mole by direct calorimetry.

K) Substrate Specificity

Generally, multicellular organism lactic dehydrogenase is specific for L (+) lactate, with no detectable activity for D (-) lactate, nor is D (-) lactate an inhibitor. However, Dennis, et al. (1959, 1960, 1965) have reported a lactic dehydrogenase isolated from Lactobacillus plantarum specific for the D (-) lactate. Haugaard (1959) and Bennett, et al. (1966) reported the presence of both D (-) and L (+) lactic dehydrogenase in E. coli. Snoswell (1959) reported the purification of a D (-) lactate specific lactic dehydrogenase from Lactobacillus arabinosus, and Gleason, et al. (1966) found a D (-) lactic dehydrogenase in two lower fungi, Sapromyces elongatus and Mindeniella spinosora.

Meister (1950) found that a series of α,γ-diketo acids, varying in chain length from 5-11 carbons, can be reduced at about one-tenth the rate of pyruvate. Reduction

in this case occurs at the α-keto group only, oxidizing one mole of DPNH per mole substrate. Beef heart lactic dehydrogenase will also reduce α,γ-diketo-δ-methylcaproic acid and α,γ-diketo-ε-methylheptanoic acid. 2-mercaptopyruvate (Kun, 1957) and mesoxalic acid (Winer, et al., 1959) will also act as substrate but at a reduced rate.

In contrast to these, the rate of reduction of the α-keto series C_4-C_9 decreases markedly with chain length (Nisselbaum and Bodansky, 1961a). The enzyme is inactive against trichloroacetic acid, 3-phosphoglyceric acid (Frank and Holz, 1959), acetaldehyde, acetone, methyl ethyl ketone, 3,6-dimethyl-2,5-p-dioxane, methyl lactate and glycolic acid (Neilands, 1954).

It should be noted that both Steginк and Vestling (1966) and Dabich (1960) have found that although rat liver lactic dehydrogenase is resistant to hydrolysis by leucine aminopeptidase, up to 20% of the molecule can be digested by carboxypeptidase without effecting the catalytic activity of the enzyme.

L) Cofactor Specificity

As has been found for most dehydrogenases, lactic dehydrogenase requires DPNH with a beta nicotinamide ribosidic linkage and shows no activity toward the alpha isomer (Kaplan, et al., 1955). The enzyme is also specific for the alpha hydrogen in the para position of the nicotinamide ring, as

shown by Loewus and his coworkers, (1953, 1960). See Noller (1957) for nomenclature system.

Although TPNH will serve as cofactor for the reaction, DPNH is 100-380 times as effective (Meister, 1950). Anderson and Kaplan (1959) determined the ability of a series of DPN^+ analogues to function as cofactor for the reaction. Using beef heart enzyme, substitution of the 3-amide group in the pyridine ring with a hydroxamide acid, hydrazide, formaldoxime, thioamide or isobutyryl gave an active cofactor, but the activity was generally less than that of DPN^+. Substitution of benzoyl, amino, acetamide or 2-butenylamide groups gave an inactive analogue. It should be noted that Lactobacillus arabinosus has been shown to contain a DPNH independent lactic dehydrogenase (Snoswell, 1959).

M) Inhibitors of the Reaction

1) Substrate analogues

For reasons described below, inhibitors of lactic dehydrogenase have been sought for many years. The following is a key observation for the thesis work presented here on the development of a lactic dehydrogenic inhibitor. Summarizing past conclusions, Winer and Schwert (1959) state "The information which is available on inhibitors for the lactic dehydrogenase system indicates that only anionic substances function as inhibitors. The simplest interpreta-

tion of this observation is that there is a cationic site on the enzyme surface which functions as a binding site for the negatively charged portion of the substrates and of inhibitors." This cationic site may be an ε-amino of lysine.

Of these anionic inhibitors, the most widely studied have been oxamate (Novoa, et al., 1959; Colowick and coworkers, 1961a and b, 1965a and b), malonate and tartronate (Ottolenghi and Denstedt, 1958). Oxamate is competitive with pyruvate and noncompetitive with lactate, while malonate, tartronate and oxalate are competitive with lactate and noncompetitive with pyruvate. As was noted by Ottolenghi and Denstedt (1958), most substrate analogue inhibitors of lactic dehydrogenase contain both an anionic moiety and a α-keto group. For instance, oxalic acid, HOOC-COOH, is a good inhibitor, while malonic acid, HOOC-CH_2-COOH is a less powerful inhibitor and succinic acid, HOOC-CH_2-CH_2-COOH is devoid of any inhibitory effect. Thus, as the α-keto group is removed from the anionic moiety, activity diminishes. One exception to this general rule is phenoxyacetic acid, Ph-O-CH_2-COOH, and these authors suggest that the oxygen atom involved in the ether linkage is sufficiently negative to bind to the enzyme much as the α-keto group of malonic acid is able to bind.

Fluoropyruvate has been shown to be a powerful, noncompetitive, irreversible inhibitor of lactic dehydrogenase (Busch and Nair, 1957). These authors also list some thirty-nine other compounds which have been tested and found to be

relatively ineffective as inhibitors of lactic dehydrogenase.

It should be noted here that almost without exception, these substrate analogue inhibitors of lactic dehydrogenase are not specific for this one enzyme, but are rather general inhibitors of the other dehydrogenases to a significant degree as well (Ciaccio, 1966; Martin, et al., 1958; Busch and Nair, 1957; Busch, 1962; Goldberg, et al., 1965; Quastel and Wooldridge, 1928; Webb, 1963).

2) Cofactor Analogues

Karnes et al. (1960) found several analogues of DPN^+ which were rather potent inhibitors of the enzyme, including ethyl nicotinate DPN^+, the isonicotinic hydrazide analogue of DPN^+, ADP-ribose, and diadenylic acid. AMP, ADP and IMP were moderate inhibitors, and nicotinamide mononucleotide and nicotinamide were very weak inhibitors. The enzyme is inhibited by the 3-acetyl pyridine analogue (Anderson and Kaplan, 1959; Ciaccio, 1966). The benzoyl pyridine and thionicotinamide analogues have also been found to inhibit lactic dehydrogenase (Anderson and Kaplan, 1959), but do not inhibit yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase or rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. In spite of this apparent specificity, no further use appears to have been made of these inhibitors as cancer chemotheraputic agents (see Part II below).

3) Inhibitors Which Are Not Substrate or Cofactor Analogues

Several compounds not related to either substrate or cofactor inhibit lactic dehydrogenase, including bisulfite (van Eys, et al., 1958), the bisulfite adduct of acetaldehyde (Zelitch, 1957), and p-mercuribenzoate (Neilands, 1954; Takenaka and Schwert, 1956). P-mercuribenzoate presumably acts by inactivating the sulfhydryl group shown to be involved in the active site (Winer and Schwert, 1958; Takenaka and Schwert, 1956). In addition, two isozyme specific peptide inhibitors of lactic dehydrogenase have recently been isolated and purified (Wacker and Schoenenberger, 1966a, b), and are the most potent inhibitors of lactic dehydrogenase yet found, inhibiting 100% between 10^{-8} and 10^{-9} M.

N) A Model for Enzymatic Action and Inhibition

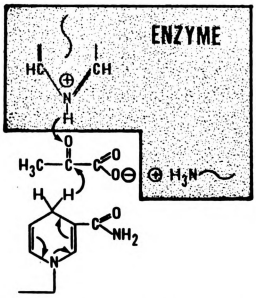
The evidence presented above favors the following mechanism of enzymatic activity for lactic dehydrogenase, as depicted diagramatically in figure 1. The enzyme-DPNH complex is probably formed first (Takenaka and Schwert, 1956; Chance and Neilands, 1952; Anderson, et al., 1964; for other references see section I, I above), through interactions of the pyridine nucleotide with an sulfhydryl group (Winer and Schwert, 1958; Takenaka and Schwert, 1956) and an imidazolium group of histidine (Winer and Schwert,

1958). This binary complex is then able to bind pyruvate (Takenaka and Schwert, 1956) by means of an ionic bond between the negative carboxyl group of pyruvate and the positive ε-amino group of a lysine residue of the enzyme at the active site. An electronegative moiety such as an α-keto or α-ether group alpha to the anionic portion of the substrate acts as a further aid to substrate binding (Ottolenghi and Denstedt, 1958; Meister, 1950). It should be noted that direct evidence for this ternary complex has not been demonstrated, but is merely assumed from the work with inhibitors described below. Although Ottolenghi and Denstedt (1958) suggested separate binding sites for lactate and pyruvate, present evidence favors one site altered allosterically to accomodate both reactants (Novoa, et al., 1959). For the mechanism described below, it will be assumed that the ternary complex of enzyme-DPNH-substrate is the active species, and further that there is one binding site for both lactate and pyruvate, as is depicted in figure 1.

The reduction of pyruvate to lactate requires two protons one of which is directly transferred from the alpha hydrogen of DPNH (Loewus and Stafford, 1960) and one of which is presumably donated by a histidine residue at the active site (Nygaard, 1956; Winer and Schwert, 1959). These hydrogen transfers result in the oxidation of DPNH to DPN^+, deprotonation of the histidine residue, followed by release of lactate and DPN^+ from the enzyme surface. It has been shown (Anderson and Kaplan, 1959) that the rate of reaction is

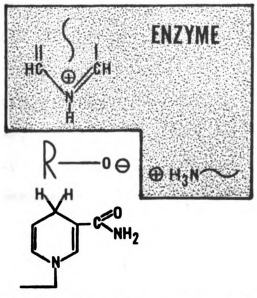
Figure 1. Enzyme-DPNH-Substrate complex with subsequent reaction mechanism depicted by the arrows. Shaded portion represents the enzyme surface.

Figure 2. Enzyme-DPNH-Inhibitor complex as described in the text. Shaded portion represents the enzyme surface.



ENZYME–DPNH–SUBSTRATE COMPLEX

Figure 1



ENZYME–DPNH–INHIBITOR COMPLEX

Figure 2

lowered by substitutions in the 3 position of the pyridine ring which lower the reducing potential of the DPNH.

Figure 2 represents a schematic diagram of the enzyme-DPNH-inhibitor ternary complex which has been shown to exist for several different inhibitors of the reaction (van Eys, et al., 1958; Novoa, et al., 1959; Winer and Schwert, 1959). Although substrate will not bind to the enzyme alone, it has been shown that the inhibitor will bind to the enzyme alone as well as to the enzyme-DPNH complex (Novoa, et al., 1959).

There are three major possible classes of lactic dehydrogenase inhibitors, namely:

1) those which inhibit the binding of substrate
2) those which inhibit the binding of cofactor
3) nonspecific inhibitors

Since the major emphasis of this thesis will be on substrate analogue inhibitors of lactic dehydrogenase, the discussion below will concentrate on the first class of inhibitors.

The inhibitor pictured in figure 2 is schematically represented as R-O, where the R group can vary from HOOC-CO- to C_6H_5-O-CH_2-CO-. Although all previous work on inhibitors of lactic dehydrogenase has concentrated on carboxylic acid derivatives, it is pertinent to this thesis to point out that other organic anions, such as ionized phenols, could and do also function as inhibitors, as will be shown. As was mentioned above, an electronegative group alpha to the anionic moiety apparently facilitates binding of inhibitor to enzyme (Ottolenghi and Denstedt, 1958). Once bound, the inhibitor

presumably acts by preventing binding of substrate. It is reasonable to suppose that an inhibitor could interfere with both substrate and DPNH, since as can be seen in figures 1 and 2, DPNH is bound immediately adjacent to the substrate binding site. Such a "dual-function" inhibitor would presumably be much more potent than one which only displaced substrate.

It should be emphasized that compounds which inhibit an enzyme catalyzed reaction in one direction may not necessarily inhibit the reverse reaction. This is because the three dimensional structure of the substrate binding site is considerably altered during the reaction (Novoa, et al., 1959). As has been described above (section I, M, 1), this is indeed the case for lactic dehydrogenase, since several inhibitors are specific either for pyruvate or lactate only.

Part II, Lactic Dehydrogenase and Cancer

A) Introduction

A normal aerobic cell will produce much of its energy by cycling pyruvate through the citric acid cycle rather than by converting it to lactic acid. Only under the anaerobic conditions of sudden, intensive or prolonged physical stress will lactate be the major product.

Warburg (1924) found that one of the most general and distinctive features of malignant tissues was the formation of unusually large amounts of lactic acid in the presence of an ample supply of oxygen. He state, "The respiration of the carcinoma tissue is too small in comparison with its glycolytic power." This phenomenon of large lactic acid production under aerobic conditions has come to be called aerobic glycolysis. It should be noted for clarity that aerobic glycolysis is defined as glycolysis (production of lactate) under aerobic conditions; this is not to suggest that glycolysis itself functions aerobically, since this would be a contradiction in terms.

From his observations, Warburg (1924) developed a theory of carcinogenesis (1956) based upon an irreversible injury to the respiratory chain of a cell as the cause of cancer. He postulated that such an injury caused the cell to increase glycolysis to compensate for the energy loss due

to diminished oxidative phosphorylation. He further postulated that this shift in metabolism from an oxidative to a glycolytic pathway was accompanied by a dedifferentiation, reasoning that the glycolytic cell no longer needed the complex cellular apparatus and highly structured cell. In this way he explained the familiar dedifferentiation so characteristic of neoplasms.

The observation of a large production of lactate under aerobic conditions very early stimulated investigations into the biochemical relationship between lactic dehydrogenase and cancer. Burk and his coworkers (1956) found that the higher the malignancy of a tumor, the greater the glycolysis and the smaller the respiration. In addition, they found that in normal cells undergoing very rapid division, the introduction of oxygen caused a complete halt in lactate production, whereas such addition to a cancer cell merely slowed the rate slightly, even in a low malignancy tumor.

Burk and Schade have written (1956), there is "... overwhelming evidence for the occurence of various forms of respiratory impairment in neoplastic cells, available in some one thousand experimental papers ..." However, Warburg's theory could not be placed on a firm experimental basis until the discovery of this impairment, the lack of α-glycerolphosphate dehydrogenase, described below. The importance of glycolysis to neoplastic cells could now be experimentally investigated. (see G below).

Many other theories of carcinogenesis and its treat-

ment have been developed, and one of the most recent and intriguing has been developed by Szent-Györgyi and his coworkers (1965, 1966, 1967). They found 2 α-ketoaldehydes, retine and promine, which can control cellular division, presumably through reaction with the cells' free sulfhydral groups, shown to be required for cellular division (Hammett, 1929; Rapkine, 1931).

B) Intracellular Location of Cofactors

The current biochemical explanation for the observations of Warburg place great emphasis upon the coenzymes DPN^+ and DPNH as well as the mechanism by which DPN^+ is regenerated. For this reason, it is important to know the basic facts about location, levels, and transport of the pyridine nucleotides.

The enzymes and cofactors necessary for glycolysis are present in the extramitochondrial portion of the cell in mammalian cells (Wu and Racker, 1959; Lynen, 1958; Chance and Hess, 1959 a b; LePage and Schneider, 1948; Kennedy and Lehninger, 1949). For instance, DPN^+ and DPNH are present in only microgram/gram quantities in rat liver (Jacobson and Kaplan, 1957), and thus must be recycled. It has also been shown that DPNH is unable to pass through the mitochondrial membrane (Lehninger, 1951). Since the DPN^+ cannot be regenerated directly by diffusion into the mitochondria, some mechanism must exist to transfer the hydrogens from DPNH into

the mitochondrial oxidative system to regenerate DPN^+. The following scheme provides such a mechanism.

C) The α-glycerolphosphate Shunt as a Mechanism for Regeneration of DPN^+

The only oxidative reaction occuring in glycolysis is that catalyzed by glyceraldehyde-3-phosphate dehydrogenase, requiring DPN^+ as a cofactor. The DPNH produced in this reaction has two major possible routes of reoxidation to DPN^+, depicted in figure 3. Normally, the action of α-glycerolphosphate dehydrogenase oxidizes DPNH to DPN^+, producing α-glycerolphosphate, to which the mitochondrial membrane is permeable (Sacktor and Cochran, 1958; Estabrook and Sacktor, 1958). Mitochondrial enzymes oxidize α-glycerolphosphate using the phosphorylating electron transport chain (Ringler and Singer, 1958). Previously only an FAD linked mitochondrial α-glycerolphosphate dehydrogenase had been reported and the inefficiency of getting only two ATP equivalents per mole of DPN made somewhat questionable the physiological significance of this mechanism. However, Tomita and Helling (1965) have recently reported a DPN^+ linked mitochondrial α-glycerolphosphate dehydrogenase.

Summing the reactions involved:

fructose-1,6-diphosphate ⟶ dihydroxyacetone phosphate + glyceraldehyde-3-phosphate (1)

$DPN^+ + P_i$ + glyceraldehyde-3-phosphate ⟶ 1,3-diphosphoglycerate + DPNH (2)

Figure 3. Alternate pathways of DPNH oxidation serving to regenerate DPN^+ for glycolysis.

ALTERNATE PATHWAYS OF DPNH OXIDATION

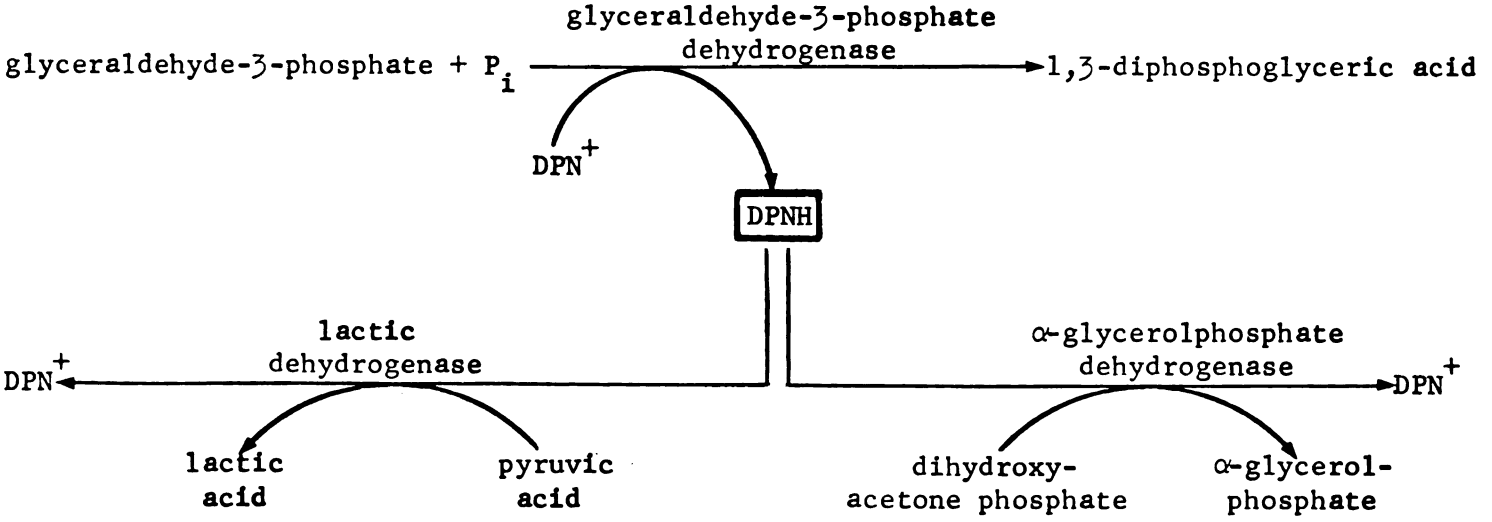


Figure 3

DPNH + dihydroxyacetone phosphate ⟶ α-glycerolphosphate + DPN^+ (3)

O_2 + α-glycerolphosphate ⟶ energy via the phosphorylating electron transport chain (4)

SUM: O_2 + fructose-1,6-diphosphate ⟶ 1,3-diphosphoglyceric acid + energy

The only obvious alternative scheme for regeneration of DPN^+ from DPNH is by reduction of pyruvate to lactate using lactic dehydrogenase. The lactic acid then produced must then be transported to the liver prior to any further metabolism.

This scheme leads to the prediction that under anaerobic conditions, the levels of both lactate and α-glycerolphosphate would increase and this has been observed by Ciaccio, et al. (1960) in a number of tissues. That α-glycerolphosphate dehydrogenase may be indispensible to the efficient growth of a cell, namely through maximum oxidative pathways, is thus evident.

D) The β-hydroxybutyrate Shunt

An alternative mitochondrial shuttle similar to that given above utilizes acetoacetate and β-hydroxybutyrate dehydrogenase and has been described by Boxer and Devlin (1961). Present evidence is insufficient to evaluate its existance and operation as a physiologically significant process.

E) Absence of α-glycerolphosphate Dehydrogenase in Tumors

In most malignant tissues, α-glycerolphosphate dehydrogenase activity is either missing or very low (Holzer, et al., 1958; Boxer and Shonk, 1960; Delbruck, et al., 1959; Sacktor and Dick, 1960; Foster and Taylor, 1965). This is not due to an inhibitor, but rather to a decreased amount of enzyme present (Boxer and Shonk, 1960). This lack of α-glycerolphosphate dehydrogenase results in an almost complete halt in production of α-glycerolphosphate (Ciaccio, et al., 1960).

The only exceptions to this rule are the Ehrlich-Lettre tumor of the mouse (Boxer and Devlin, 1961) and the Morris hepatoma 5123 of the rat (Morris, et al., 1960). Even though it has normal levels of α-glycerolphosphate dehydrogenase activity, the Ehrlich-Lettre tumor does not produce α-glycerolphosphate during glycolysis, for some as yet unknown reason. Both of these tumors completely lack the acetacetate shunt (Boxer and Devlin, 1961).

F) Results of a Non-Operative Shunt in Tumors

If shuttles other than the α-glycerolphosphate dehydrogenase shuttle are known or assumed to be absent, this leads inexorably to the conclusion that a cell lacking α-glycerolphosphate dehydrogenase will be absolutely dependent upon lactic dehydrogenase to regenerate the DPN^+ necessary for

glycolysis. Results by Busch (1955), showing that 2-C^{14} pyruvate injected into tumors was primarily converted to lactic acid, support this conclusion. All energy, therefore, comes from glycolysis, even under aerobic conditions, and the absence of α-glycerolphosphate dehydrogenase provides a biochemical basis for an explanation of Warburg's earlier observations (1924).

Recently, a detailed study by Goldman, et al. (1964) of a number of different human neoplasms showed that tumors generally have a higher concentration of lactic dehydrogenase M isozyme than surrounding noninvolved tissue. Since the M isozyme is more suitable for high rates of lactic acid production, this observation fits very well the total picture of neoplastic glycolysis. The results have been confirmed and extended by Peznanska-Linde, et al. (1966).

Further evidence for the absolute requirement of tumors for lactic dehydrogenase has been furnished by Gregory, et al. (1966) and Ng and Gregory (1966). By preparing specific antilactic dehydrogenase antibodies and testing them both against liver and tumor homogenates and tumor cell culture, they demonstrated complete abolition of aerobic glycolysis in tumors, and almost complete inhibition of tumor cell reproduction, while normal cells were almost unaffected by these antibodies.

The above picture of neoplastic glycolysis makes one fact very clear - lactic dehydrogenase is an absolutely indispensible enzyme for tumor survival. Without it, the

glycolytic pathway would soon be inoperative through a lack of DPN^+, and the cell's energy supply would be fatally depleted. Since the normal tissue has α-glycerolphosphate dehydrogenase and thus has no such dependence, this suggests an approach to the chemotherapy of cancer with a fundamental biochemical basis. In the words of Busch (1961), this conclusion that

> "... the carbohydrate metabolism of neoplastic tissues was deranged ... was one of the most important which has been developed in the cancer field, and has served as the focal point of a very large number of important experiments which have been relevant both to the field of cancer research and general biochemistry; it still remains one of the most important differences between many tumor and non-tumor tissues."

Selective inhibition of lactic dehydrogenase may then kill a cancer cell without harming normal cells.

G) Inhibitor Studies

Based upon the above conclusions, inhibitors of lactic dehydrogenase have been tested for activity against cancer. Several inhibitors, including oxamate (Novoa, et al., 1959; Colowick and coworkers, 1961a,b, 1965a,b), oxalate (Busch and Nair, 1957; Novoa, et al., 1959), tartronate (Ottolenghi and Denstedt, 1958), and fluoropyruvate (Busch and Nair, 1957) have been found. Fluoropyruvate, tested on the Walker 256 tumor (Davis and Busch, 1958), was shown to inhibit both the reduction and oxidation of pyruvate and to

have central nervous system toxicity. Tartronate, tested on the Yoshida ascites hepatoma (Fiume, 1960), had very little effect on tumor growth and was shown not to be specific for lactic dehydrogenase (Fiume, 1959). Hydroxamic acid, a more potent inhibitor of lactic dehydrogenase, also proved to be ineffective against the Yoshida hepatoma.

Colowick and coworkers (1961a,b, 1965a,b) have intensely studied both oxamate and oxalate in both Ehrlich ascites tumor and in HeLa S3 cell culture. They have found that oxamate can inhibit both glycolysis and tumor growth, and have demonstrated the site of action as being lactic dehydrogenase. To get these results they had to use an energy supply high in glucose to force the use of the glycolytic pathway by repressing other pathways. They found that oxamate exhibits nonspecific toxicity which eliminated it from further consideration as an antitumor chemotherapeutic agent.

Many of these inhibitors have been toxic and this has discouraged and slowed the efforts in this direction almost to a complete halt. As a result, the theory has not yet been tested in an unequivocal manner because of the lack of a good non-toxic lactic dehydrogenase inhibitor system. A major objective of this thesis was to provide such a system and test this hypothesis.

Enzyme Preparations

I) Rabbit Muscle Pyruvate Kinase

Rabbit muscle pyruvate kinase was prepared by a slight modification of the method of Tietz and Ochoa (1958). The enzyme was kept concentrated during the ethanol fractionation ,, rather than being diluted to 20 mg/ml. Following the final heat step, an ammonium sulfate fractionation was carried out. The enzyme finally used in these studies was the 40-55% ammonium sulfate fraction. It was stored in the cold at a concentration of 90-100 mg/ml, in 0.02M imidazole buffer, pH 7.00, containing 0.001M EDTA.

II) Lactic Dehydrogenase

Beef heart lactic dehydrogenase was purchased from Worthington Biochemicals, lot BH-LDH 6010, as a crystalline ammonium sulfate suspension. It was found to be completely free of pyruvate kinase activity in the range of the assay concentrations. The rabbit muscle lactic dehydrogenase used in the isozyme studies was a crystalline ammonium sulfate suspension from Sigma.

III) Various Other Glycolytic Enzymes

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase,

phosphoglyceric acid kinase, phosphoglucomutase and glucose-6-phosphate dehydrogenase were purchased from Sigma. AMP deaminase, prepared by the method of Smiley, et al. (1967), and UDPG pyrophosphorylase, prepared by the method of Albrecht, et al. (1966), were kindly donated by the respective authors.

IV) Polyphenol Oxidase

The mushroom polyphenol oxidase used in these studies was of two kinds - the first, lot TY-602, was purchased from Worthington Biochemicals, and the second, prepared as described below, was kindly donated by Dr. S. Constantinides. In a typical preparation, each gram of Agaricus campestris was gently crushed with 5 ml of 0.05M phosphate buffer, pH 7.20, using a mortar and pestle. The resulting suspension was centrifuged at 300 x g to remove the cellular debris, then at 10,000 x g for 15 minutes to remove the mitochondrial fraction, and finally at 100,000 x g for one hour to remove the microsomes. The supernatant fraction from this final centrifugation was used for the studies described below.

Materials

The pyruvic acid used in the assays was twice distilled from Matheson, Coleman and Bell lot PX-2125. The ether, methylene chloride, acetone and lactic acid were Baker analyzed reagents.

Eastman Organic Chemical products included vanillin, p-hydroxybenzaldehyde, protocatachuic acid, p-hydroxybenzoic acid, gallic acid, pyrogallic acid, o-, m- and p-methoxyphenol, syringic acid, o-ethylphenol and coumarin. Chemicals purchased from Aldrich Chemicals included o-cresol, 3-ethoxy-4-hydroxybenzaldehyde ("ethoxy" vanillin), 2,6-dimethylphenol, 2,6-dimethoxyphenol, 2-methyl-4-nitrosophenol, 2-chloro-4-nitrophenol, guaiacol, 2-methylcyclohexanone, a duplicate sample of vanillin to check the Eastman sample, and 2-hydroxy-1-methyl-1-cyclopentene-3-one hydrate (cyclotene). A separate sample of the latter was kindly provided by Dr. Riley of Dow Chemicals.

K and K Labs products included syringaldehyde, vanillic acid, ferulic acid, 3,5-dihydroxy-benzoic acid, salicyl alcohol, 2-methylcyclopentanone, protocatachualdehyde, p-hydroxyphenylacetic acid, p-nitrophenol, p-coumaric acid, colchicine, colchiceine and 3-methyl-1,2-cyclopentanediol. Quinic acid and L-malic acid were purchased from Nutritional Biochemicals Corporation. Caffeic acid was donated by Dr. H. M. Sell of this department, to whom it had been

provided by the California Foundation for Biochemical Research.

The following chemicals were obtained from Sigma; disodium β-DPNH·$3H_2O$, lot 43B-608, phosphoenolpyruvate, tricyclohexylammonium salt, lot 94B-5200, α-ketoglutaric acid, lot K42B-217, DL-isocitric acid, lot I107-87, fumaric acid, lot 15B-0760, and adenosine diphosphate, lot 45B-7000.

Chemicals obtained from Calbiochem included cis-aconitic acid, lot 30074. Mallinckrodt succinic acid and Fisher citric acid and methylethyl ketone were also used.

The maple syrup used for the preparation of the Maple Syrup Fraction was obtained from several areas of the country, and differed both in appearance and in yield of the MSF produced per liter. Michigan syrup gave the highest yield and appeared darkest. The Vermont and New York syrups gave the lowest yield, and appeared thin and light. All syrup used was 100% grade A or grade Fancy maple syrup. The Vermont and New York syrup was supplied through the services of Sugarbush Industries, Lansing, Michigan in bulk. The Michigan syrup was purchased locally, mostly from Carl Gearhart in Charlotte, Michigan, in five gallon lots.

I) Assays

A) Pyruvate Kinase - Spectrophotometric and Potentiometric

The coupled spectrophotometric assay with lactic dehydrogenase measured the decrease in absorbance at 340 mμ as DPNH was converted to DPN^+. The assay contained 0.01M $MgCl_2$, 0.10M KCl, 0.02M adenosine diphosphate, 3.4×10^{-4}M phosphoenolpyruvate, 5.6 μg beef heart lactic dehydrogenase, 0.10M Tris-HCl, pH 7.50, and 1.8×10^{-4}M DPNH, at 25.0°C in a total volume of 0.40 ml. The conditions were found to give maximum velocities and the velocities were proportional to enzyme concentrations. The enzyme and compound to be tested were each added in 5 μL volumes. The specific activity was measured in units of μM of phosphoenolpyruvate reacted/min/mg of pyruvate kinase. All assays were performed on a Gilford spectrophotometer equipped with an automatic cuvette positioner. Protein concentrations were measured by absorbance at 280 mμ on the Gilford spectrophotometer, using an extinction coefficient for pyruvate kinase of 0.54 OD/mg/cm after Bucher and Pfleiderer (1955). The potentiometric assay involved measuring the rate of uptake of 1.035×10^{-3}M HCl in a Radiometer automatic pH stat, model TTT1. The assay vessel contained 0.008M $MgCl_2$, 0.002M adenosine diphosphate,

0.001M phosphoenolpyruvate and 0.10M KCl, pH 7.50 at 25.0°C. Pyruvate kinase was added in one 5 µL sample, as was the sample to be tested.

B) Lactic Dehydrogenase

For the spectrophotometric assay of lactic dehydrogenase, the decrease in DPNH concentration was followed spectrophotometrically at 340 mµ. The assay contained 3.3×10^{-4}M sodium pyruvate, 0.03M potassium phosphate buffer, 6.7×10^{-5}M DPNH, 3.3×10^{-3}M NaCl and was at pH 7.40. The compound to be tested and the lactic dehydrogenase (0.1 µg) were each added in 5 µL volumes, bringing the final assay volume to 0.30 ml. Only initial rates were measured. These conditions were found to give accurate and reproducible results, in which the velocities were proportional to enzyme concentration. The specific activity was measured in units of µM of pyruvate reacted/min/mg of enzyme. For this assay, the change in optical density per minute,, when multiplied by the factor 1090.0, yields the specific activity. All assays were performed on a Gilford spectrophotometer equipped with a constant temperature water bath at 25.0°C and an automatic cuvette positioner. Protein concentrations were measured by absorbance at 280 mµ, using an extinction coefficient for lactic dehydrogenase of 0.698 OD/mg/cm (Schwert and Winer, 1963).

As described under results, the assay was changed

after finding that in this assay the enzymatic activity decreased with time, and the following assay was used for all of the kinetic work described under results. For the new assay, the enzyme was kept at enzyme stock concentration of 56 μg/ml. and the cuvette contained the following: 6.9×10^{-4} M NaCl, 1.4×10^{-4} M sodium pyruvate, 0.10M phosphate buffer, pH 7.40 and 2.6×10^{-5} M DPNH. All other conditions were as above.

C) Polyphenol Oxidase

The assay of polyphenol oxidase contained pH 6.50, 0.167M sodium phosphate buffer, 3.3×10^{-4} M tyrosine and approximately 0.025 mg enzyme in a final volume of 3.0 ml. The enzyme was added by layering it on a close-fitting plunger with small holes in it. The plunger was rapidly moved up and down in the cuvette, serving both to aerate and mix the contents. Although the rate of this reaction can be measured by the rate of increase in optical density at 280 mμ, for the reasons outlined under results, the increasing optical density was more routinely measured at 460 mμ. All assays were run as is described above for pyruvate kinase.

II) Spectra - Ultraviolet, Infrared, Nuclear Magnetic Resonance and Mass Spectrometry Analysis

The ultraviolet absorbance spectra were run on a

Beckman DB recording spectrophotometer. Infrared spectra were taken in KBr pellets in the Beckman 5 infrared spectrophotometer. The mass spectrum was measured in a Consolidated Electrodynamics Corporation model 21-1030 mass spectrometer. NMR spectra were taken in D_2O using a Varian A-60-A nuclear magnetic resonance spectrophotometer.

III) Titrations and pK Determinations

Titrations were performed automatically using the Radiometer Corporation model TTTlc automatic titrator, with 1.0 ml. samples. The inflection points of the pK determinations were calculated as follows:

1) The linear portions of the curve on either side of the inflection point were extended.
2) The slope line for the linear region at the inflection point was drawn to intersect the above two lines.
3) The inflection point falls exactly halfway between the two intersections plotted above.

The hydrochloric acid was standardized against 0.9761M NaOH, which was standardized against potassium acid phthalate. A solution was prepared which was exactly 1.0000M HCl, and the acid for titration was prepared from dilutions of this stock, so that quantitative titrations could be performed. For the pyruvate kinase assay, 0.001M HCl was used, and for the pK determinations, 0.10M HCl was most generally employed.

IV) Chromatographic and Electrophoretic Techniques

A) Gas Chromatography of Maple Syrup Fraction (MSF)

Initially, the gas chromatography was performed on the Aerograph 660, varying the column temperature but keeping the injector port at 215°C. An ionizing flame detector was used, and the collector port:detector port ratio was kept at 9:1 throughout the experiments. In later gas chromatography work, a model 400 F and M gas chromatograph with a 5% carbowax on chromosorb W column measuring six feet by one-quarter inch was used. The oven was maintained at 250°C, the flash heater at 300°C and the flame detector at 360°C.

B) Column Chromatography of MSF

Sephadex G-10, G-200, LH-20, Dowex 50 and Amberlite CG-50 were all prepared and used according to the instructions provided by the supplier. Silicic acid columns were prepared using Mallinckrodt 100 mesh silicic acid well washed with both distilled water and the elution solvent. With the methylethyl ketone solvent, the silicic acid turned yellow at first, but after many consecutive washings appeared completely white.

C) Thin Layer Chromatography of Both MSF and the Compounds to be Tested for Inhibitory Activity

Since many solvent systems were used, they will be described under results. The solid phase in all cases was silicic acid, 250 μ thick for analytical work and 1 or 2 mm thick for preparative procedures. Two types of plates were routinely prepared using the DeSaga-Brinkmann apparatus; one measuring 2 inches by 8 inches, and the second 8 by 8. By using a fluorescent binder in the silicic acid, it was possible to visualize all spots present after chromatography by a masking of the fluorescence of the binder everywhere any material was present. Ascending chromatography was used in all cases, and the chromatograms were run until the solvent front was one-half inch from the top of the plate. The samples to be chromatographed were spoted one inch from the bottom of the plate, using either water, ether or acetone solutions, depending upon the solubility of the material to be run.

D) Paper Chromatography and Electrophoresis of MSF

The techniques and equipment used for these studies were reported in detail by Katz and co-workers (1959). Whatman number 1 paper, 18¼ inches by 22½ inches, was used for both chromatography and electrophoresis. Desending chromatography utilized n-butanol:acetic acid:water, 4:1:5,

while the electrophoresis was conducted in a pyridine:acetic acid:water buffer, 1:10:289, pH 3.70.

E) Polyacrylamide Gel Electrophoresis

The polyacrylamide gel electrophoresis was performed according to the method of Ornstein (1964) and Davis (1964), using 7% polyacrylamide gels prepared using chemicals purchased from Canalco and a Tris-glycine buffer at pH 8.6.

The bands were visualized after electrophoresis in two ways. The first was by staining with Amido-Schwartz black and destaining either electrophoretically or by soaking in 7½% acetic acid. The second was by using an activity assay in the gels. For polyphenol oxidase, the gels were soaked in a solution of substrate, and the bands of enzyme converted the colorless substrate into colored product. Although the MSF itself was colored, its enzymatic product could easily be distinguished from the background color. For lactic dehydrogenase, the gel activity assay of Markert and Ursprung (1962) was used, omitting the hydrazine trapping agent to achieve a colorless background (Kaplan and Cahn, 1962).

Results

I. Study of the Effect of Krebs Cycle Metabolites Upon Pyruvate Kinase and Discovery of a Potent Lactic Dehydrogenase Inhibitor Present as an Impurity in Commercial Malic Acid

A) Introduction

> "A living cell consists in large part of a concentrated mixture of hundreds of different enzymes, each a highly effective catalyst for one or more chemical reactions involving other components of the cell. The paradox of intense and highly diverse chemical activity on the one hand and strongly poised chemical stability (biological homeostasis) on the other is one of the most challenging problems of biology." (Atkinson, 1965)

The original purpose of this research was to gain some insight into the systems of controls in the glycolytic pathway utilized by the higher vertebrates to enable them to more efficiently respond to the energy requirements placed upon them in their environments. This research led to the discovery of a natural inhibitor of lactic dehydrogenase from maple syrup. Although the major emphasis in this thesis is on the lactic dehydrogenase inhibitor, the studies on the control of pyruvate kinase serve as a good introduction to the discovery of these inhibitors.

In controlled pathways, regulation has generally been found to be accomplished by a simple feedback mechanism, in which the end product of the system controls the entire pathway through inhibition of the first enzyme involved. Pathways involving threonine deaminase (Umbarger and Brown, 1957) and aspartate transcarbamylase (Yates and Pardee, 1957) are two well studied examples of this type of control.

Glycolysis, on the other hand, does not seem to possess such a simple control system. It is clear that a very highly complex system of controls exists to determine the state of the system. In an attempt to simplify the problem of defining the control points, Pye and Eddy (1965) studied the levels of the glycolytic intermediates and suggested four probably controlled steps. These were glucose entry and glycogen degradation, 3-phosphoglyceric acid kinase, phosphofructokinase and pyruvate kinase. Of these, only phosphofructokinase and phosphorylase have been studied in any detail.

Phosphorylase has been shown to be activated by AMP (Cori, Colowick and Cori, 1938; Brown and Cori, 1961; Krebs and Fischer, 1962), and inhibited by ATP, glucose-6-phosphate (Morgan and Parmeggiani, 1964) and UDPG (Madsen, 1963).

The citrate inhibition of phosphofructokinase has been very well documented (Parmeggiani and Bowman, 1963; Passonneau and Lowry, 1963; Vinuela, et al., 1963; Mansour, 1963; Garland, et al., 1963; and Williamson, et al., 1964) for several systems under a variety of conditions, as has

its activation by AMP (Mansour, 1963; Passonneau and Lowry, 1962; Ranaiah, Hathaway and Atkinson, 1964).

Pyravate kinase is known to be noncompetitively inhibited by diethylstilbestrol (Kimberg and Yielding, 1962) and ATP (Boyer, 1963), but to date there has been no work done on the effect of the tricarboxylic acid cycle intermediates upon the enzyme. Results, section I, C describes a systematic study of the control of pyruvate kinase by the Krebs cycle substrates.

B) Purity of the Pyruvate Kinase

The pyruvate kinase used for the studies described below was first physically characterized to insure that it was essentially pure and homogeneous. It was found to be homogeneous in sedimentation velocity experiments in the Spinco model E analytical ultracentrifuge. Chromatography on Sephadex G-200 yielded activity and protein curves which coincided. Polyacrylamide gel electrophoresis revealed one major band and only trace contamination. The pattern was similar to that which had been reported for pure pyruvate kinase previously (Tietz and Ochoa, 1958; Warner, 1958).

C) Lack of Inhibition of Pyruvate Kinase by Krebs Cycle Metabolites

The pyruvate kinase was tested for inhibition by the

Krebs cycle intermediates, including citrate, cis-aconitate, isocitrate, α-ketoglutarate, succinate, fumarate and malate. As shown in figure 4, malate, citrate and isocitrate gave the only apparent significant inhibitions at low concentrations of metabolites; cis-aconitate, α-ketoglutarate, succinate and fumarate gave no significant inhibition. Oxaloacetate spontaneously decarboxylates in aqueous solution to pyruvate, the substrate for the lactic dehydrogenase in the coupled assay employed. This eliminated consideration of this compound. Due to solubility problems in the assay system, fumarate was tested at a maximum concentration of 1.5×10^{-3} M.

Citrate and isocitrate gave appreciable inhibition at higher concentrations than malate. To test the possibility that this apparent inhibition might be due to chelating effects, experiments were run in which excess $MgCl_2$ was added to the assay medium along with citrate or isocitrate. As shown in figure 5, the apparent inhibition caused by the citrate and isocitrate was essentially abolished, indicating that a chelating effect had caused the apparent inhibition. The malate curve was totally unaffected by such addition.

D) Impurity of the Malic Acid

Since malic acid was the only Krebs cycle substrate which gave significant inhibition not ascribable to a chelating effect, the purity of this sample was checked by

Figure 4. Activity of pyruvate kinase as a function of the concentration of the Krebs cycle metabolites. Spectrophotometric assays as described under Methods. A note on the inhibition by fumarate appears in the text.

Figure 5. Effect of $MgCl_2$ on the inhibition of pyruvate kinase by citrate and isocitrate. $MgCl_2$ was added in concentrations equal to the compound being tested. Spectrophotometric assays as described under Methods.

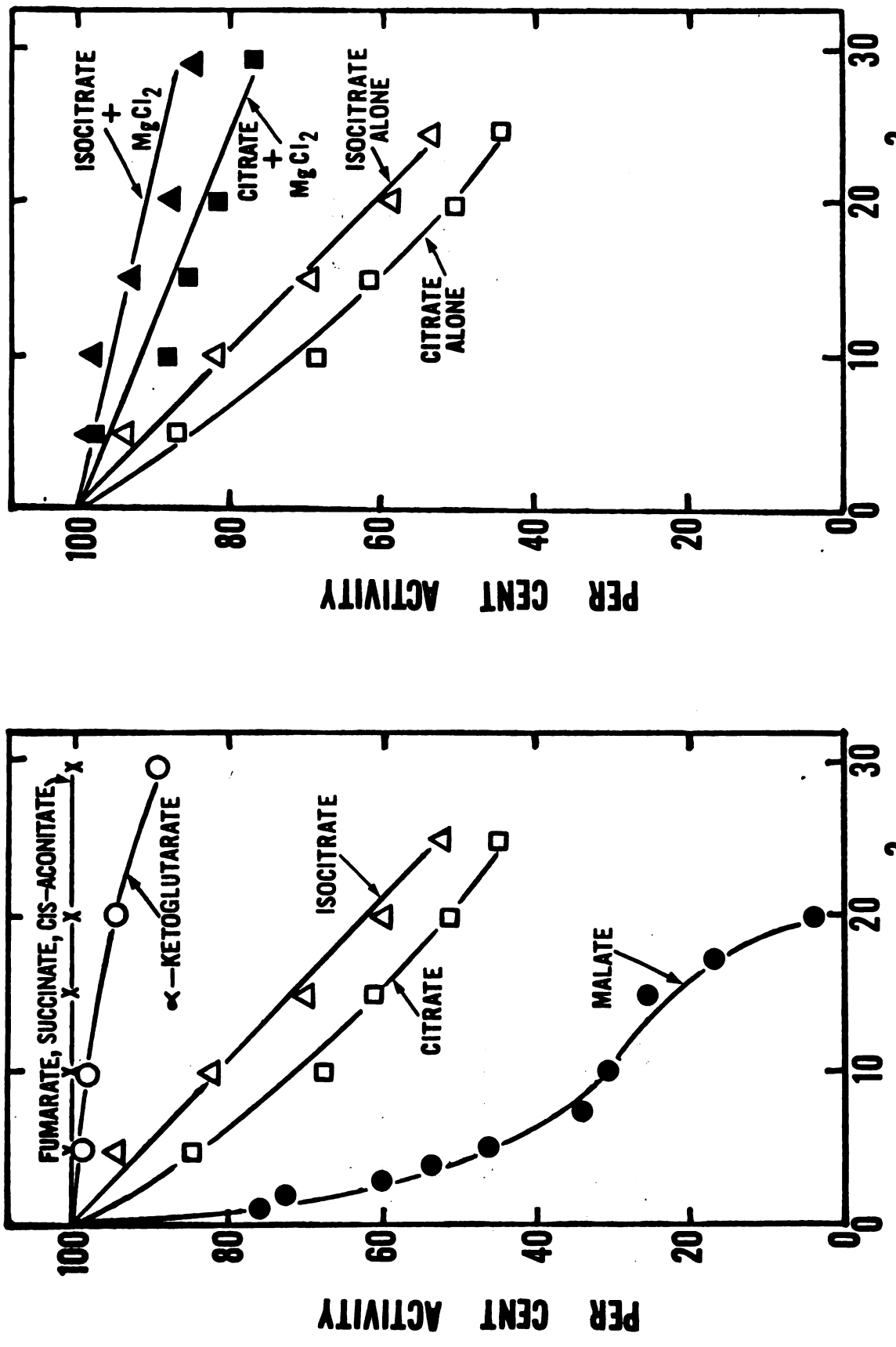


Figure 5

Figure 4

infrared spectrophotometry to determine whether an impurity might be present and account for the observed enzyme inhibition. The spectrum gave no evidence of any impurities. However, to check this further, Sigma lot M81B-98 L-malic acid was tested in the same assay system previously described. No inhibition was observed. Therefore, it was concluded that an impurity in the Nutritional Biochemicals sample of malic acid was responsible for the observed inhibition, although an infrared spectrum of the Sigma malic acid was identical in all respects to the Nutritional Biochemicals sample, indicating that the impurity had to be a very minor component in the malate preparation.

Extraction of an aqueous solution of the Nutritional malic acid with ether, followed by drying the ether phase and dissolving it in water, gave a sample which had significantly greater inhibition in the pyruvate kinase assay than did the malic acid alone, showing the inhibitor to be soluble in ether.

Since a coupled assay was used, there was no way of knowing which enzyme was being inhibited. Therefore, the pH stat assay was used to measure pyruvate kinase activity independent of lactic dehydrogenase and no inhibition of pyruvate kinase was found with either the Sigma or Nutritional malic acid, or with the above ether extract. This suggested that the impurity might be a rather potent inhibitor of lactic dehydrogenase, and warranted further investigation, as described in the remainder of this thesis.

II. The Isolation, Purification and Identification of a Class of Potent Lactic Dehydrogenase Inhibitors Including Vanillin, Syringaldehyde, p-hydroxybenzaldehyde and Cyclotene from Maple Syrup, the Commercial Source of Malic Acid

A) Introduction

As discussed in the literature review, lactic dehydrogenase appears to be an indispensible enzyme to a cancer cell, while a normal cell has no such requirement for this enzyme. Treatment of an organism containing both normal and malignant cells with a potent specific lactic dehydrogenase inhibitor might then selectively kill the cancer cells without adversely affecting the normal cells. For this reason, the discovery of an inhibitor of lactic dehydrogenase as an impurity in commercial malic acid led to the investigation of its structure, specificity, mode of action and other pertinent facts concerning its possible application toward cancer chemotherapy.

In the process of devising a plan to isolate the inhibitor, it was discovered that commercial malic acid is prepared from maple syrup by precipitation as the free acid during the concentration of the syrup from the sap. Thus, maple syrup was used as a source for the inhibitor. A fraction active as a lactic dehydrogenase inhibitor was

isolated and named "Maple Syrup Fraction" (MSF) after its source. Its preparation is described in the following section.

B) The Maple Syrup Fraction, MSF

1) Preparation of the MSF from Maple Syrup

Since it had been found that the active constituents of the malic acid (section I, D above) were extractable into ether, ether was originally chosen as the extracting solvent. A typical preparation of MSF consisted of extracting one liter portions of pure maple syrup with 3 consecutive 200 ml. portions of ether. The ether extracts were pooled and evaporated to dryness on a rotary evaporator kept at 35.0°C. To this ether extract, now very yellow in color, three consecutive small (2-5 ml) portions of distilled, deionized water were added, and the tube was swirled very gently. These three portions were combined and lyophilized to dryness. This procedure was repeated twice more on the lyophilized product. The third water extract was the sample used for testing in the initial stages of the problem.

In an effort to improve the yield, the solubility of the above product was tested in a variety of organic solvents, and the results are shown in table 1. It should be noted that with the exception of nitrobenzene, the solubility

Solvent	B.P., °C	Dielectric Constant	Solubility in H_2O, g%	Solubility of MSF, mg/ml
ether	35	4.3	7.5	2.6
CH_2Cl_2	40	9.1	2.0	5.8
n-hexanol	157	13.3	0.6	5.0
acetophenone	202	17.4	1	6.7
nitrobenzene	211	34.8	0.2	4
water	100	78.0	∞	66.7

Table 1. Solubility of Crude MSF in various organic solvents. Solvent physical properties taken from Hodgman, et al., (1959).

of the MSF was proportional to the dielectric constant of the solvent, indicating that the MSF was most probably polar in nature. The following principles guided the final selection of an extraction solvent:

(1) low solubility in water to facilitate extraction from aqueous maple syrup

(2) low boiling point to facilitate removal of the solvent after extraction

(3) high dielectric constant to increase the MSF extraction yield.

The solvent best filling these criteria was methylene chloride. It was subsequently used for all extractions.

It was found that water would precipitate an inactive fraction from an acetone solution of the crude MSF, and so this step was incorporated into the procedure for the preparation of the MSF, to yield the following final procedure.

Each 1200 ml. portion of 100 per cent maple syrup was extracted for 20 minutes with 400 ml. of CH_2Cl_2. This extract was then evaporated to dryness as above. The dry, yellow extract was dissolved in a minimal volume of acetone, and five times this volume of deionized, distilled water was added, which precipitated an oily organic residue. The resulting suspension was centrifuged at 18,000 rpm for 30 minutes at $4^{\circ}C$, and the supernatent solution was withdrawn and lyophilized. Two further water extractions followed by lyophilization were performed as above, and the final dry, paracrystalline, yellow material was used for testing. The yield was approximately 25 mg/liter of maple syrup, as compared to the yield using ether of only about 10 mg/liter.

2) Physical Properties of MSF

The MSF prepared as described above and used in these studies was a yellow, paracrystalline compound with a strong maple aroma. It was extremely hygroscopic, absorbing water even in a high vacuum over P_2O_5. This made a melting point determination impossible, since the compound was molten even at room temperature. It was seen as a crystalline-like compound only after prolonged lyophilization at a vacuum of 1-2 μ. When removed from this vacuum, it could be seen to absorb atmospheric water vapor, and appear to melt. It should be noted that for calculations of concentrations, an arbitrary molecular weight of 184 was assigned to MSF on

the basis of some very preliminary mass spectral analysis performed on crude MSF. This turned out by chance to be quite close to the molecular weights of the actual active species, as will be discussed in section II J 4.

3) Stability of the MSF

To test the stability of MSF under various conditions, samples were stored at 10 mg/ml for forty days at 3 pHs at -10°C, 5°C, and 25°C. Another sample was frozen and thawed at intervals. The initial inhibition of all samples was 51%. As seen in Table 2, the MSF seems to be more stable in acid under all conditions. For oral admin-

	% Inhibition by 9.0×10^{-4} M MSF		
Temperature	pH 2.0	pH 5.0	pH 9.5
25°C	19	16	10
5°C	32	0	0
-10°C	63	27	31
refrozen	13	19	17

Table 2. Stability of MSF under various conditions.

istration of a drug to be feasible, it must be stable at pH 1 for at least 3 hours at 37°C, conditions approximating those in the digestive tract. MSF is stable under these

conditions, giving the same inhibition as a sample kept near neutrality. MSF in the dry state is stable for a period of months.

C) Degree of Inhibition of Lactic Dehydrogenase by MSF and Demonstration of the Reversibility of the Inhibition

A typical preparation of MSF at 5.0×10^{-4} M inhibited lactic dehydrogenase 35%. The best preparation inhibited 67% at 4.5×10^{-4} M.

The inhibition of lactic dehydrogenase by MSF is reversible. Lactic dehydrogenase was incubated for one hour at 0°C with 5 mg/ml MSF, in 0.01M NaCl. Under these conditions, the enzyme is inhibited 100%. When portions of this solution are dialyzed for 48 hours at either pH 5.0 or 9.0 full activity of lactic dehydrogenase is observed. The reactivated enzyme is still sensitive to MSF inhibition.

D) Lack of Effect of MSF on Other Enzymes

As discussed in the introduction to this section, to have application as a chemotherapeutic agent for cancer, a lactic dehydrogenase inhibitor must be specific for only this one enzyme and not affect others. At a concentration of 2×10^{-3} M, MSF is completely free of inhibitory activity for all of the animal enzymes tested, including glucose-6-phosphate dehydrogenase, phosphoglucomutase, UDPG pyrophos-

phorylase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglyceric acid kinase and AMP deaminase.

E) Initial Chromatography Which Failed to Show the Heterogeneity of the MSF

Many methods were originally tried in an effort to separate the components of the MSF. Among these were paper chromatography with both n-butanol:acetic acid:water, 4:1:5 and n-butanol:ethanol:water, 52:32:16, preliminary gas chromatography using the Aerograph model 660, column chromatography using Sephadex G-10, Sephadex LH-20, Dowex 50 and Amberlite CG-50, high voltage paper electrophoresis, and thin layer chromatography using fourteen solvent systems, several of which are listed below:

1) n-butanol:acetic acid:water, 4:1:5
2) n-butanol:acetic acid:water, 8:2:2
3) n-butanol:ethanol:water, 52:32:16
4) acetic acid:n-butanol:ethyl acetate:water, 1:1:1:1
5) n-propanol:1N acetic acid, 3:1
6) acetic acid:pyridine:water, 10:1:289
7) benzene:proprionic acid:water, 2:2:1
8) benzene:acetic acid:water, 2:2:1

When none of these methods provided any significant resolution of the MSF, it was decided that more would have to be known about its chemical structure before it could be resolved. Therefore, both instrumental and chemical

analysis were used in an attempt to determine the structure of the MSF or its components.

F) Preliminary Instrumental Analysis of MSF

The initial structural tests on MSF consisted of instrumental analysis using infrared, nuclear magnetic resonance, ultraviolet and mass spectrophotometry, and titration. Since, as was discovered later, the MSF was a mixture of many compounds, the results were, in general, quite ambiguous. However, several functional groups were found to be present. Strong infrared absorption near 6 μ indicated the presence of a carbonyl, most likely an aldehyde or ketone since the spectrum was featureless in the region of hydrogen-bonded carboxylic acids. Strong infrared absorption at 2.8 μ indicated the presence of one or more hydroxyl groups, as did the nuclear magnetic resonance spectrum. Evidence for some aromaticity was provided by infrared absorption at 14.3 μ, ultraviolet absorption near 280 mμ, and the nuclear magnetic resonance spectrum. Strong peaks at masses 15, 31, and 45 in the mass spectrum indicated the presence of one or more methyl or methoxy substituients.

The titration, as described under Methods, indicated that the MSF had a pK of 7.04, and the shift in the ultraviolet absorption maximum with pH indicated that the ionizing moiety was most likely part of the aromatic system.

With some indication provided by this instrumental

analysis of MSF of the types of substituents likely to be found, chemical analysis for these and related groups was undertaken to further characterize the MSF.

G) Chemical Analysis of MSF Suggesting a Phenol

A detailed chemical analysis was performed and the results are summarized in table 3. Note that neither the semicarbazide hydrochloride nor the 3,5-dinitrobenzoate derivatives could be made. This suggested that the carbonyl and alcohol groups indicated by the other tests might be too sterically hindered to undergo addition of these bulky groups.

The carbonyl indicated here and by the instrumental analysis above seemed to be an aldehyde, which backs up the previous conclusions. The nitrogen analysis indicated that the imidizole ring could not be present, but the diazotized sulfanilic acid test was positive. This apparent contradiction is explained by the fact that this test is positive with a variety of activated ring structures, such as are found in phenols, (Bolling, 1949); all tests of MSF for phenols were positive. The question mark by the ceric nitrate indicates that no conclusions were possible because a heavy precipitate formed during the reaction. The listed reference mentions that such an interference reaction is often caused by the presence of phenols.

On the basis of all of the analytical data presented,

Table 3. Summary of the chemical tests performed to elucidate the structure of the MSF. Tests were conducted as described in the indicated reference as follows:

*I refers to Feigl (1965)

*II refers to Shriner (1964)

Functional Group	(+),(-)	Method	Reference*
carbonyl	+	$NaHSO_3$-starch-I_2-KI	I 191
carbonyl	-	semicarbazide HCl	II 253
ester	-	hydroxylamine HCl	I 214
carboxylic acid	-	ferric hydroxamate	I 212
aldehyde	+	indole condensation	I 199
nitrogen	-	elemental analysis	- - - - -
nitro	-	$FeSO_4$-alcoholic KOH	II 128
amino	-	ninhydrin	Block(1958) p. 123
unsaturation	+	$KMnO_4$	II 149
unsaturation	+	Br_2 in CCl_4	II 121
sulfer	-	elemental analysis	- - - - -
chlorine	-	elemental analysis	- - - - -
bromine	-	elemental analysis	- - - - -
iodine	-	elemental analysis	- - - - -
alcohol	+	vanadium oxinate	I 174
alcohol	?	ceric nitrate	I 175
alcohol	-	3,5-dinitrobenzoate	II 247
phenol	+	$FeCl_3$	II 127
phenol	+	α-nitroso-β-naphthol	Block(1958) p. 139
phenol	+	nitration-dilute HNO_3	Noller(1957) p. 507
phenol	+	bromine water	II 123
imidazole (see text)	+	diazotized sulfanilic acid	Bolling (1949)

Table 3

both instrumental and chemical, the following conclusions were reached:

1) The MSF contains a carbonyl group, probably an aromatic aldehyde.
2) It probably contains a phenolic ring.
3) Both the phenol and the aldehyde may be sterically hindered.
4) The MSF contains no halogens, sulfur or nitrogen.
5) The sample is most likely not pure, and one of the impurities may be hydrocarbon in nature.

H) Test of the Phenolic Character of MSF by Its Action as a Polyphenol Oxidase Substrate

1) Introduction

Since the chemical and instrumental data presented above suggested that the MSF contained phenolic material, it was thought that it might serve as a substrate for polyphenol oxidase. Polyphenol oxidase catalyzes both the hydroxylation of a number of monophenols and the oxidation of o-diphenols. The general reactions can be written as follows, after Mason (1957):

$$\text{monophenol} + 2e^- + O_2 \longrightarrow \text{o-diphenol} + O^=$$

$$2\ \text{o-diphenol} + O_2 \longrightarrow \text{o-quinone} + 2H_2O$$

The enzyme then catalyzes the formation of colored products such as dopachrome and melanin from the o-quinone.

Dressler and Dawson (1960) report that the oxidation of monophenols does not proceed through a diphenol intermediate.

The mushroom enzyme exists in multiple forms (Constantinides, 1966). These forms, separated by polyacrylamide gel electrophoresis, have been found to exhibit differences in substrate specifity.

The phenolic nature of the MSF indicated by the analytical data presented above could be confirmed if the MSF could serve as a substrate for polyphenol oxidase. Furthermore, by determining the specificity of the polyphenol oxidase, structural features of such phenolic compounds might be revealed. Therefore, studies were undertaken to determine both the specificity of the mushroom polyphenol oxidase and its ability to utilize MSF as a substrate.

2) Polyphenol Oxidase Assay

The activity of polyphenol oxidase is usually measured by following the increase in optical density at 280 mμ as the o-quinone is produced. This was not possible with the MSF because it had such a strong ultraviolet absorption that it was impossible to zero the spectrophotometer at this wavelength.

Since polyphenol oxidase catalyzes the further reaction of o-quinones to highly colored products, it seemed likely that the assay might be conducted in the visible

region of the spectrum to avoid the previous difficulties. To test this a product was made in the following way. Two samples of MSF were mixed with buffer and incubated at 25°C, one with Worthington polyphenol oxidase and one without. After fifteen minutes, the sample containing the enzyme was considerably darker than the control. A difference spectrum of these two samples was run on a Beckman DB spectrophotometer to determine the exact wavelength at which the product of the reaction absorbed. This difference spectrum peaked at 460 mμ. Therefore, this wavelength was selected for the assays of the polyphenol oxidase. A control assay comparison of the tyrosine reaction at 280 mμ and 460 mμ showed the rates to be identical, within experimental error. The major difference was longer lag in the assay at 460 mμ; this is to be expected from the reaction mechanism described in Section II H 1.

3) Relation of Structure of Phenols to Their Activity as Polyphenol Oxidase Substrates

The relative reaction rate of several phenol-like compounds were measured and compared to tyrosine which was arbitrarily assigned a rate of 100. Two milligrams of each substrate were present in each assay and the Worthington enzyme was used. The results are summarized in table 4 along with the structures of each of the tested substrates. The MSF had a relative rate of 85, indicating a high content

Table 4. Structure of some phenol-like compounds and their relative rates as substrates for polyphenol oxidase. See text for discussion and details.

* Tyrosine arbitrarily set to a rate of 100 and the other rates are relative to this as a standard.

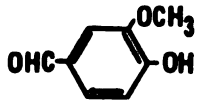
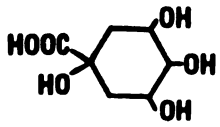
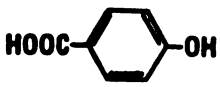
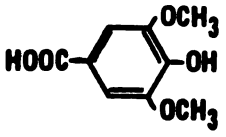
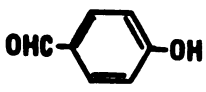
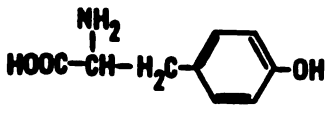
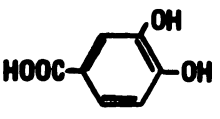
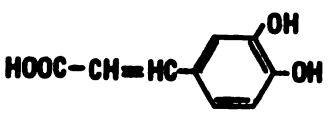
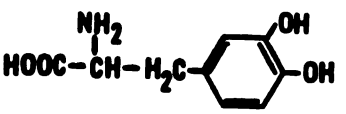
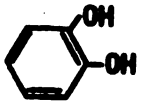
COMPOUND	STRUCTURE	RELATIVE RATE *
vanillin		0
quinic acid		0
p-hydroxy-benzoic acid		0
syringic acid		1
p-hydroxy-benzaldehyde		4
tyrosine		100*
protocatachuic acid		189
caffeic acid		692
dihydroxy-phenylalanine		1733
catechol		7600

Table 4

of phenolic material, and confirming the previous analytical results. However, the most active inhibitory fraction (see Figure 6) had a relative rate of only 28. This surprising observation is discussed in the following section.

4) Reaction of MSF with the Multiple Forms of Polyphenol Oxidase

A member of this laboratory worked on the separation and specificities of the multiple forms of polyphenol oxidase from mushroom (Constantinides, 1966). He found that although 20-25 protein bands were seen when the gels were stained with amido-Schwartz black using the mushroom enzyme (see Enzyme Preparations IV), only a few of these bands were shown to be active as polyphenol oxidase. Using MSF as substrate for the color reaction (see Methods, IV E) three bands could be seen. DOPA gave 7 bands, tyrosine 2, catechol 5, catechine 2, caffeic acid 2 and chlorogenic acid gave only 1. Most significant was the fact that the three bands seen with MSF were the same bands generally seen with the other substrates. That is, the MSF reacted only with the most nonspecific isozymes of polyphenol oxidase.

The data presented above and in table 4 show that the most rapid rates of oxidation by polyphenol oxidase were achieved with the o-dihydroxyphenols, although a small, but measurable, rate was seen with several monohydroxyphenols. The lack of reaction with quinic acid suggested

that substrates for polyphenol oxidase must be aromatic. That the crude MSF had a high relative rate of 85 may indicate the presence of one or more dihydroxyphenols. However, since the most active inhibitory fraction had a relative rate of only one-quarter that of the crude MSF, it would seem that the inhibitory component or components are not o-dihydroxyphenols. Indeed, if they are phenols at all, the possibility exists that the position ortho to the hydroxy group may be blocked, as in vanillin or syringic acid, thus accounting for their reduced activity with polyphenol oxidase.

I) Separation of MSF into Several Components Based on its Phenolic Structure

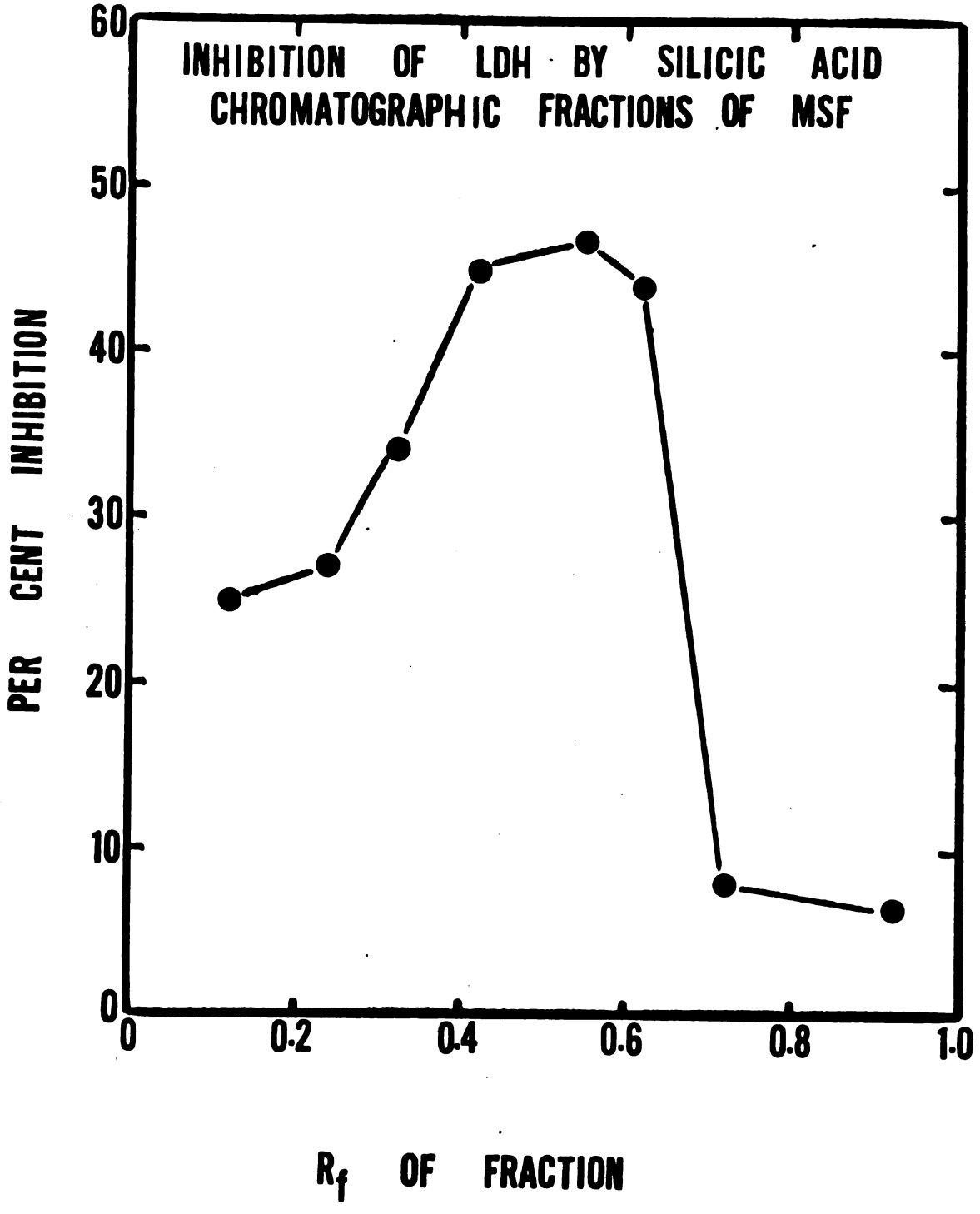
1) Thin Layer Chromatography

Now that the basic chemical nature of the MSF had been determined, it was possible to resolve it into its components. Block (1958) describes two solvent systems designed to separate mixtures of phenols; (1) 20% KCl in water and (2) methyl ethyl ketone:2N ammonium hydroxide, 2:1, pH 11. Thin layer chromatography on silicic acid using the 20% KCl solvent system gave only two spots. However, the methyl ethyl ketone system resolved the MSF into about twenty spots, several of which fluoresced when excited by ultraviolet light. The most prominent of these

was a spot which fluoresced a very bright blue. It had an R_f of about 0.3, but this varied greatly depending upon the concentration of material applied to the plate. This spot served as an excellent marker in later work, since by virtue of its intense fluorescence, it was detectable at very low concentrations.

The next problem was to determine which of the components was active. Preparative thin layer chromatography was carried out to obtain enough of each of the components to assay. The fractions were eluted from the silicic acid using acetone. This procedure yielded fractions which were not completely soluble in water, probably due to some residue from the acetone or from the chromatography solvent. Therefore, as much as possible of each fraction was first dissolved in a small volume of water. It was then filtered through a millipore filter, lyophilized to dryness, weighed and redissolved in water to a known concentration of 5 mg/ml and assayed at an assay concentration of 80 µg/ml. A plot of per cent inhibition of lactic dehydrogenase versus R_f in the methyl ethyl ketone solvent is shown in figure 6. Conveniently for later detection, the maximum inhibition centered on the bright blue fluorescent band. Although the spots using this solvent system are rather distinct, the inhibition is still spread out over quite a large area of the chromotograph. This probably indicates the presence of more than one active species, although this fact was not appreciated for quite some time; initially, the simplest

Figure 6. Inhibition of lactic dehydrogenase by silicic acid chromatographic fractions of MSF. The fractions were eluded from thin layer plates developed in the methyl ethyl ketone solvent system described in the text. Spectrophotometric assay for lactic dehydrogenase activity is described under Methods.


INHIBITION OF LDH BY SILICIC ACID
CHROMATOGRAPHIC FRACTIONS OF MSF
PER CENT INHIBITION
60
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
R_f OF FRACTION

explanation, suspected breakdown of a single active species, was thought to account for the spreading.

Since the inhibitory activity peak was centered on the blue fluorescent band, attempts were made to purify this band by repeated chromatography in the methyl ethyl ketone solvent system. After six consecutive thin layer chromatographs, a sample was obtained which, upon further chromatography in the same solvent system, showed only the blue fluorescent band. An assay of this sample proved it to be inactive as a lactic dehydrogenase inhibitor. This suggested that the active species probably chromatographed just ahead or just behind this blue spot. The fluorescent material probably has a very complex structure, since an ultraviolet spectrum in ether shows peaks at 338, 296, 284, 258 and 223 mμ. An infrared spectrum indicated the presence of hydroxyl and carbonyl groups with a prominent CH stretch. No significant infrared long wavelength absorption was seen. Comparison of the infrared spectrum using the Sadtler index produced no positive identification of the fluorescent material.

Attempts to purify the spots on either side of the fluorescent band by repeated chromatography failed. Upon each rechromatography, a larger and larger fraction of the material eluted from the middle of the chromatograph moved near the solvent front. This result indicated that the material was probably decomposing during the time needed for several consecutive chromatographs. An assay of the material moving near the solvent front showed it to be

devoid of inhibitory activity toward lactic dehydrogenase.

2) Silicic Acid Column Chromatography

In an attempt to collect a larger amount of the active chromatographic region in a shorter period of time, a silicic acid column was run using the methyl ethyl ketone solvent to elute the fractions of MSF. By examining one microliter portions of consecutive fractions of the eluant for fluorescence by spotting them on filter paper and exposing the spots to ultraviolet light, it could easily be determined when the fluorescent band was eluted. The lactic dehydrogenase inhibitory activity was centered mainly about this region, as shown above. However, a good separation on a column required a very slow flow rate. This resulted in conversion of the active fraction moving near the center to the inactive fraction moving near the solvent front. This was shown by immediate rechromatography of the column fractions on thin layer using the usual system. This solvent system was therefore abandoned.

From the previous experiments one positive result was obtained. This was evidence for more than one active inhibitor. While rechromatographing the column fractions on thin layer, it was seen that the active fractions had several compounds that the inactive fractions did not. These compounds chromatographed directly above and below the fluorescent one, right where the presence of the inhibitor was

strongly suspected. In addition, a slightly active fraction eluted from the column well ahead of the bright blue fluorescent material contained a third spot which was not present in any other fraction, having an R_f of 0.71, within the same limitations noted for R_f's in this thin layer system. The evidence for there being more than one active inhibitory species continued to mount.

J) Identification of the Components of MSF

1) Compounds Likely to be Found in MSF

The problem now was having a large number of compounds with the possibility that several might be active. Since the commercial value of maple syrup has stimulated considerable chemical research upon its components, this was turned to as a source of information. Also, since maple syrup, the source of MSF, is a wood product, a search was made in the literature for compounds known to be present in wood and sap. One such list which proved to be very helpful was found in Pearl and McCoy (1960). In addition to the list of twenty compounds, their R_f's in two different solvent systems and the color reactions of each compound with ten different stable diazo spray reagents were detailed. From the work of Pearl and Dickey, (1951), five common color reactions for wood derived phenolic compounds were obtained. About twenty other compounds were subsequently added to this

list from other sources, resulting in a list too long for it to be feasible to assay all compounds for inhibitory activity against lactic acid dehydrogenase.

A series of papers by Underwood and coworkers (1961, 1963, 1964, 1965) described the isolation and identification of some of the components of a chloroform extract of maple syrup. This chloroform extract had properties considerably different from the CH_2Cl_2 extract, MSF, but it was thought that their results might form a starting point for identification of the components of MSF. After organic fractionation of the chloroform extract, they identified by gas chromatography the following components of maple syrup (see Underwood and coworkers, 1961, 1963, 1964, 1965):

1) ethyl acetate
2) ethyl alcohol
3) acetoin
4) acetol
5) cyclotene
6) phenol
7) diacetin
8) a long chain fatty acid
9) vanillin
10) ethyl vanillate
11) syringaldehyde
12) dihydroconiferyl alcohol
13) syringoyl methyl ketone
14) guaiacol
15) 2,6-dimethoxyphenol
16) coumarin
17) coniferyl aldehyde
18) 2,6-dimethoxybenzoquinone
19) lignin

Since lignin is a very common constituent of plant extracts, its presence was sought in MSF.

2) Identification of Lignin in the MSF

The classic phloroglucinol reaction for lignin, treatment of the sample with a saturated solution of phloroglucinol in concentrated HCl, was performed on the MSF, and it produced a strongly positive reaction. Lignin is insoluble in diethyl ether, and when the MSF sample was repeatedly treated with ether until no further precipitation occured, the ether soluble fraction gave no phloroglucinol reaction, while the precipitate still gave a strongly positive reaction. This was taken as conclusive proof of the presence of lignin in the MSF, and lignin became the first component to be identified in the MSF.

Lignin samples from the MSF were assayed for inhibitory activity against lactic dehydrogenase in the usual assay system. Its activity increased with time after isolation from MSF, although it never approached the activity of the MSF preparations. Lignin is known to oxidize in air and undergo decomposition into many products. From the above data, it seemed possible that the active components in the MSF might be degradation products of the lignin known to be present. Such products are generally aromatic structures with many methoxy substituents, which would be consistent with previous structural data.

3) Comparison Thin Layer Chromatography of Standards

Since the R_f of the active inhibitory region had been

well defined, thin layer chromatography was performed on a series of compounds related to wood, wood products and lignin degradation products from the above lists in an attempt to reduce the number of compounds to assay. The following compounds had an R_f near zero in the methyl ethyl ketone solvent system, and so were eliminated from consideration:

1) syringic acid
2) protocatechuic acid
3) caffeic acid
4) quinic acid
5) p-hydroxybenzoic acid
6) p-hydroxyphenylacetic acid
7) coumaric acid
8) 3,5-dihydroxybenzoic acid
9) vanillic acid
10) gallic acid
11) DPN^+
12) DPNH

These last two were tested since they are involved in the activity of the enzyme, but their R_f's eliminate them from consideration as causing the observed inhibition.

Since lactic acid can inhibit lactic dehydrogenase by product inhibition, a control to test the ability of lactic acid to inhibit this system was run. At a lactic acid concentration of 1.0×10^{-3} M, the enzyme had 100% of the control activity. Therefore, lactic acid cannot be responsible for the observed inhibition by MSF. It should be noted that oxamate, a well known potent inhibitor of lactic dehydrogenase, cannot be the active factor either, since it exhibits a positive ninhydrin reaction, while the MSF reaction is negative (see Table 3).

Table 5 lists the compounds which did move in the above solvent system and their R_f's, with the bright blue

fluorescent band included as a standard R_f marker.

Compound	R_f
syringaldehyde	0.27
blue band	0.35
vanillin	0.50
cyclotene	0.64
p-hydroxybenzaldehyde	0.67
guaiacol	0.74
coumarin	0.83

Table 5. The R_f's of several migrating species in the methyl ethyl ketone solvent system described under Methods.

Two things should be noted about this table. First, vanillin (moved just ahead of) and syringaldehyde (moved just behind) are the two compounds moving closest to the blue fluorescent band which acted as a marker for the inhibitory region. Second, the R_f values for guaiacol, cyclotene and p-hydroxybenzaldehyde are near 0.7; this is approximately the R_f value of the faster moving inhibitory region seen in the silicic acid column chromatography.

4) Identification of the Active and Inactive Components by Assay

Now that the large list of possible compounds had been narrowed down to a more workable size, it was feasible to

assay them for inhibitory activity against lactic dehydrogenase. The six compounds in Table 5 were tested first at a concentration of 4.5×10^{-4} M in the usual assay system. The results are shown in Table 6.

Compound	% inhibition
coumarin	5
guaiacol	13
cyclotene	39
syringaldehyde	62
p-hydroxybenzaldehyde	71
vanillin	100

Table 6. Inhibitory activity of several compounds found in MSF. % inhibition is expressed as per cent inhibition of the standard assay of lactic dehydrogenase by a 4.5×10^{-4} M solution of material.

The results of 13% inhibition by guaiacol and 5% by coumarin were not considered significant, being almost within experimental error. The other four gave very significant inhibition at this concentration. No combination of them in pairs gave any indication of synergistic inhibition.

Proof of the actual presence of these four good inhibitor compounds in the MSF was next undertaken. The first method of proof consisted essentially of running thin layer chromatograms of the MSF and the known compounds for comparison. Thin layer chromatography was carried out with

vanillin and syringaldehyde on one plate along with MSF. After development in the usual solvent, the plate was sprayed with a saturated solution of 2,4-dinitrophenylhydrazine, in 2N HCl, after Pearl and Dickey (1951). Vanillin gave a yellow-orange spot corresponding in color and position to a spot in the MSF sample. The syringaldehyde gave a red-brown spot which likewise had a similar spot in the MSF sample. No color reaction could be found for cyclotene; the region near where p-hydroxybenzaldehyde moved gave a very dense color with all of the sprays tested, obviating confirmation of their presence by this method.

5) Organic Fractionation for Gas Chromatography

The second method used for proof of the presence of the active inhibitors in MSF was gas chromatography. In Underwood's (1961) gas chromatography scheme, the crude extract was subjected to a simple organic fractionation before gas chromatography was attempted. A similar but far more detailed organic fractionation was attempted on the MSF. However, when the fractions produced by such fractionation were subjected to thin layer chromatography, it could be seen that the separation achieved left much to be desired. For instance, the fraction supposedly containing only the strong acids present in the MSF proved to contain at least 13 compounds. These were shown by thin layer

chromatography in the methyl ethyl ketone solvent system not to be acidic at all. The problem with this type of fractionation is that many of the compounds in the MSF seem to have at least moderate solubility in both organic and non-organic solvents, so that very little fractionation is actually achieved by organic extraction of either acidic or basic solutions of MSF. Therefore, the gas chromatography described in the next section was performed on unfractionated ether soluble MSF.

6) Gas Chromatography to Establish the Presence of the Active Components in MSF

Using the conditions outlined under Methods for the F & M gas chromatograph, attempts were made to conclusively prove that the active inhibitors were actually present in MSF. In earlier attempts to use gas chromatography with the Aerograph 660, the lignin in the MSF was not removed and only one large peak was observed, which was originally erroneously taken as evidence of the homogeneity of the MSF sample. This time the lignin was removed as above before gas chromatography was performed to avoid fouling the column with non-volatile material. Figure 7 shows a typical gas chromatogram of ether soluble MSF, using the conditions described under Methods. The peaks which have been identified and their corresponding numbers on figure 7 are as follows:

Figure 7. A typical gas chromatographic pattern of ether soluble MSF, using an F and M model 400 gas chromatography apparatus as described under Methods. The active species are labelled. Identification was both by retention time and peak coincidence when both MSF and the compound were injected simultaneously.

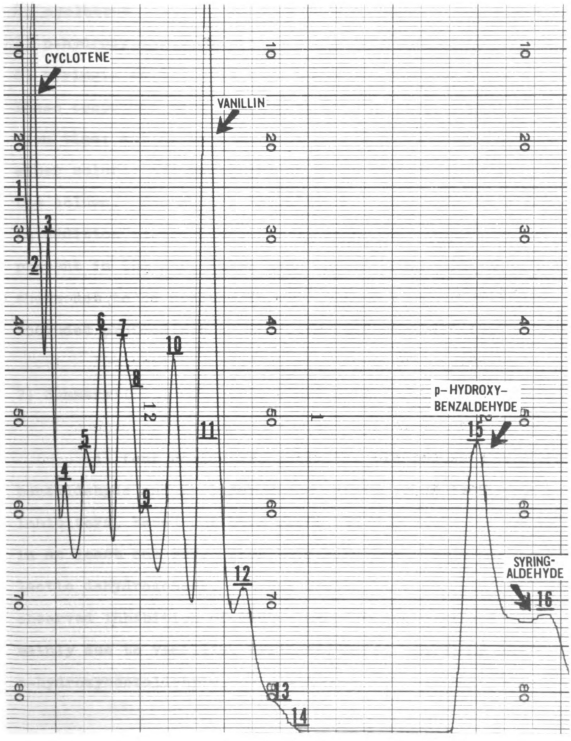

CYCLOTENE
VANILLIN
p-HYDROXY-
BENZALDEHYDE
SYRING-
ALDEHYDE
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 7

1) ether, the solvent peak
2) cyclotene
3) p-hydroxybenzoic acid
6) syringic acid
10) coumarin
11) vanillin
15) p-hydroxybenzaldehyde
16) syringaldehyde

These compounds were identified in two ways: 1) by retention time; and 2) by peak coincidence after applying both ether soluble MSF and compound to be tested together into the column. The active species are labelled on the figure. In addition to the compounds above which were proved to be present in the MSF, there were several compounds which were shown not to be present in significant amounts, and these included protocatachuic acid, caffeic acid and quinic acid.

7) Summary

All four compounds, vanillin, cyclotene, p-hydroxybenzaldehyde and syringaldehyde, found to be active as inhibitors, have thus been shown to be present in the MSF in at least one way. They have also been shown to inhibit lactic dehydrogenase. It was therefore concluded that the observed inhibition of lactic dehydrogenase by MSF was mainly due to vanillin, syringaldehyde, cyclotene and p-hydroxybenzaldehyde.

III. Elucidation of the Mechanism of Inhibition and Preparation and Testing of Analogues to Synthetically Develop an Improved Inhibitor

A) Introduction

At this stage of development, the next major questions were:

1) What are the critical structural features responsible for the inhibition?
2) Can a better inhibitor be prepared?

Since an answer to the first question is a prerequisite to a logical approach to the second, it was considered first. Inspection of the active inhibitors isolated from maple syrup revealed that they possessed the following common structural features (see Table 7):

1) A hydroxyl group, which could bind to the enzyme either through hydrogen bonding or an ionic bond, provided the hydroxyl is ionized. They are thus phenols and phenol analogs.
2) Generally, a substituent ortho to the phenolic hydroxyl such as methyl, methoxy or hydroxy, which may also bind through hydrogen bonding to the enzyme, or which may indirectly affect the hydroxyl, through electronic effects on the aromatic ring system.

3) Generally, an aldehyde group para to the hydroxyl, which may either bind directly to the enzyme, such as in a Schiff base, or decrease the pK of the hydroxyl group and aid binding to the enzyme in this manner.

These observations provided the starting point to determine the relation between structure and inhibition. By assaying other compounds having various combinations of these common structural features, it was expected that elimination of one or more of the required features could be accomplished.

B) Screening of Compounds Similar in Structure to the Naturally Occuring Inhibitors

All compounds to be tested were assayed in the original lactic dehydrogenase assay described under Methods, using a concentration of 4.5×10^{-4} M, the same concentration used for the experiments in table 6. Under these conditions pyrogallic acid was found to inhibit the enzyme 41%. All of the following compounds were found to be completely inactive as inhibitors.

1) vanillic acid (7)
2) syringic acid (4)
3) o-methoxyphenol
4) m-methoxyphenol
5) p-methoxyphenol
6) ferulic acid*
7) protocatachuic acid (4)
8) p-hydroxybenzoic acid (4)
9) gallic acid (7)
10) quinic acid (4)

*The structure of ferulic acid is o-methoxy-p-acrylyl phenol.

Note: The numbers following these compounds refer to the table in which their structures are given.

It can easily be seen from the above list that adjacent hydroxyl and methyl or methoxy groups are not sufficient to create an active inhibitor, since o-methoxyphenol, which contains this arrangement, does not inhibit, while pyrogallic acid and p-hydroxybenzaldehyde, which lack any methyl or methoxy groups, inhibit quite well. Therefore, structural similarity to lactate was not the common denominator of the active species.

Since the pK of phenolic hydroxyl groups varies widely depending upon the substituents on the ring (see Noller, 1957, chapter 24), the possibility that the pK might be a critical factor merited consideration. In view of the fact that only anionic substances have previously been found to inhibit lactic dehydrogenase (see literature review), this seemed an especially attractive possibility.

C) Determination of the pK of the Active and Inactive Compounds

Consistent with the above reasoning, all of the inactive compounds had pK's above 9.6, and so would be uncharged in the lactic dehydrogenase assay at pH 7.40. Furthermore, the active species had pK's of about 7.9 or below as summarized in Table 7, so that almost 50% of the molecules were ionized. In two cases, pyrogallic acid and cyclotene, the

Table 7. The structure, length of substituent group, pK and approximate K_i of the active naturally occuring inhibitors of lactic dehydrogenase. The pK values were determined experimentally as described under Methods.

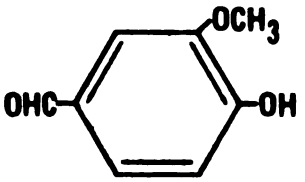
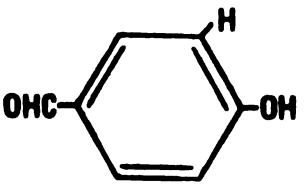
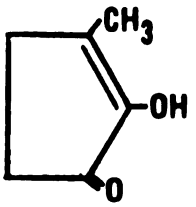
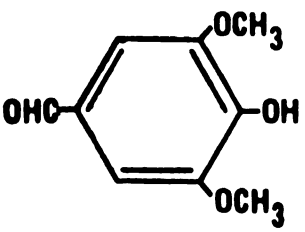
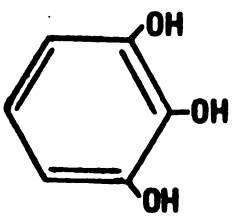
COMPOUND	STRUCTURE	SIDE CHAIN, LENGTH(Å)	pK	K_i (μM)
vanillin		3.959	7.60	96
p-hydroxy-benzaldehyde		1.084	7.90	414
cyclotene		2.639	6.90 10.00	~650
syringaldehyde		3.959	7.50	~500
pyrogallic acid		2.392	6.60 8.80	~600

Table 7

hydroxyl group appears to have two pK's. For pyrogallic acid the values may well represent titration of two different hydroxyl groups. The two pK's of cyclotene may well be due to a di-enol form of the molecule whose two hydroxyl groups titrate at different pH's.

Since a major common denominator of the active species was their low pK, synthesis of analogues having a low pK was undertaken.

D) Synthesis of "Methyl" Vanillin, "Ethyl" Vanillin and the Bisulfite Adduct of Vanillin

The two most active naturally occuring inhibitors, vanillin and p-hydroxybenzaldehyde, differ in structure only in the substituent ortho to the hydroxyl. To evaluate the effect of the substituent, the following series of six compounds was prepared with gradually increasing length of this substituent:

Substituent	Trival Name
H	p-hydroxybenzaldehyde
OH	protocatachualdehyde
CH_3	"methyl" vanillin
OCH_3	"methoxy" vanillin = vanillin
C_2H_5	"ethyl" vanillin
OC_2H_5	"ethoxy" vanillin

"Ethyl" and "methyl" vanillin were the only compounds in this list which could not be purchased commercially in pure form.

They were therefore synthesized by a slightly modified version of Adams revision (1923, 1924) of the Gatterman aldehyde synthesis. After the steam distillation, it was found necessary to pass the highly colored liquid through several consecutive charcoal adsorptions at 100°C to produce a clear, colorless supernatent from which the final product could be prepared. The "methyl" vanillin formed an amorphous precipitate which when left undisturbed for several days crystallized into small, flat white plates with a sharp melting point at 117.5°C, as compared to the value of 118°C of Adams (1924). Quantitative titration, thin layer chromatography in three solvent systems (methyl ethyl ketone:2N NH_4OH, 2:1; 20% KCl in H_2O; n-butanol:pyridine:dioxane:water, 70:20:5:5) designed to separate phenols, ultraviolet and infrared spectrophotometry all were consistent with a pure compound, and so it was used without further purification.

The "methyl" vanillin procedure was also used to prepare "ethyl" vanillin. Upon cooling the clear supernatent a white, amorphous precipitate formed much as above. Since conditions to form crystals from this amorphous precipitate could not easily be found, the suspension was lyophilized, yielding a fine white powder. Quantitative titration, infrared and ultraviolet spectrophotometry and thin layer chromatography in the above three solvent systems for phenols all were consistent with a pure preparation of "ethyl" vanillin and although it was not crystal-

line, it was used without further purification.

For reasons described in Section V E 1, it was desirable to prepare the sodium bisulfite addition product of vanillin, which was synthesized as follows. To 10 ml. of saturated aqueous sodium bisulfite was added crystalline vanillin with vigorous mixing. This order of addition was found to produce a purer product. A white precipitate was noted after some time, but addition of the vanillin was continued until a twofold molar excess of vanillin to bisulfite had been added, to insure complete reaction. The precipitate was collected by filtration and exhaustively washed with 100% ethanol, removing all unreacted vanillin. The adduct was then dried for 48 hours at 55°C, and was used without further purification. Yield was essentially quantitative. The pK's of the three new inhibitor analogues were as follows:

"methyl" vanillin ---------- 7.90

"ethyl" vanillin ---------- 7.90

bisulfite adduct of vanillin ---------- 7.65

They were therefore predicted to be good inhibitors of lactic dehydrogenase.

E) Synthesis of the p-nitroso and p-nitro Analogues of Syringaldehyde

In addition to the effect of the substituent ortho to the phenolic hydroxyl, the pK of aromatic phenols can be influenced by the substituents in the position para to the

hydroxyl. The aldehyde para to the hydroxyl groups in the naturally occuring inhibitors functions to lower the pK of the phenol. Therefore a more strongly electron-withdrawing group should theoretically produce a lower pK and as a result, a better inhibitor, unless the aldehyde group itself is somehow required for activity. It would be expected both to produce a lower pK and provide an excellent test for the essentiality of the aldehyde. (The bisulfite tests the requirement for carboxyl). Since the nitroso and nitro groups are good electron withdrawing groups, the synthesis of the p-nitroso and p-nitro derivatives of certain of the inhibitors was begun. The analogue of syringaldehyde was chosen due to the ready availability of the starting material, 2,6-dimethoxyphenol. Also, this analogue could be produced in greater yield and purified far more easily than the analogues of the other naturally occuring inhibitors.

The procedure of Cervinka and Kavka (1957) was considerably revised to synthesize 4-nitroso-2,6-dimethoxyphenol, since the limited solubility of the 2,6-dimethoxyphenol caused the reaction mixture to freeze at the low temperatures used by the above authors. The synthesis procedure was as follows. To 25 g. of 2,6-dimethoxyphenol was added a solution containing 48 g. NaOH in 450 ml. of deionized, distilled water. The suspension was mixed at $5^{\circ}C$ until dissolved. Then a solution containing 16 g. $NaNO_2$ in 450 ml. deionized, distilled water was added slowly, care being taken to maintain the temperature of $5^{\circ}C$. After 10

minutes, the dropwise addition of a solution containing 45 g. of H_2SO_4 in 100 ml. of water was begun, while passing a stream of CO_2 into the reaction mixture. Beginning five hours later 50 ml. of concentrated H_2SO_4 were slowly added over two hours, during which time a heavy precipitate formed. This precipitate was dissolved in concentrated NaOH, giving a very dark purple solution, and filtered. Acidification with H_2SO_4 to pH 3.0 was followed by extraction with n-butanol, which was subsequently air dried by placing in the hood overnight. This yielded the crude product, a dark brown in color.

This crude product was dissolved in 1M NaOH and the solution was adjusted to pH 4. The solution was then decolorized with charcoal, which, although drastically decreasing the yield, was the only way found to produce reasonably pure product. The clear filtrate from the charcoal step was further acidified to pH 2.0 and extracted with ether. Upon drying the ether solution, a small quantity of very light tan crystals was produced, which melted at 126-127°C. Quantitative titration of the crystals showed them to be at least 98% pure. They were used without further purification because of the limited quantity of material available.

Oxidation of a very small quantity of the 4-nitroso-2,6-dimethoxyphenol with H_2O_2 by the method of Travagli (1950) gave light tan crystals of 4-nitro-2,6-dimethoxyphenol. These titrated 99% pure and melted sharply at 116°C.

This compound was also assayed for inhibitor activity without further purification as described below.

As was predicted above, the pK's of these two analogues was considerably lower than either the naturally occuring inhibitors or the analogues described in section III D. The 4-nitroso-2,6-dimethoxyphenol had a pK of 6.55, while the 4-nitro-2,6-dimethoxyphenol titrated at 6.30. Since the lactic dehydrogenase assay is at pH 7.40, the effective anionic concentration of these analogues at a given molarity should be approximately twice that of a compound which titrates at pH 7.6 or above, as do the natural inhibitors. Therefore, all other factors being equal, these two compounds should be almost twice as effective inhibitors as those isolated. The results of the inhibition assays on these and the other inhibitor analogues are discussed below.

F) Activity of the Nitrogen Containing Analogues

The p-nitro and p-nitroso analogues of syringaldehyde were prepared in an effort to determine the importance and specificity of the aldehyde group for the observed inhibition. In addition to these, 4-nitroso-2-methylphenol and p-nitrophenol were commercially available (see Materials). In all cases, for the reasons discussed in Section III E, if the aldehyde group only functions to lower the pK of the phenolic hydroxyl, all of these compounds

should inhibit lactic dehydrogenase to a greater degree than their corresponding aldehydes.

At a concentration of 4.5×10^{-4} M, at which syringaldehyde inhibits lactic dehydrogenase 62%, the p-nitroso and p-nitro analogues inhibit 27% and 32% respectively. Unfortunately, not enough of these inhibitors could be prepared for a more detailed examination of their inhibition. However, it is apparent that rather than being better inhibitors than syringaldehyde, they fall short of being even as good, despite their lower pK.

The 4-nitroso-2-methylphenol purchased from Aldrich was technical grade, and despite attempts to purify it, the preparation remained somewhat impure. It was of interest to note, however, that 2.0×10^{-4} M "methyl" vanillin will inhibit lactic dehydrogenase approximately 80%, while this nitroso analogue inhibited only 26%. Although admittedly impure, the results with this compound support those cited above for the syringaldehyde analogues.

Since p-nitrophenol and p-hydroxybenzaldehyde contain only the phenolic hydroxyl and the two functional groups being compared, aldehyde and nitro, these two compounds are ideal to test the hypothesis concerning the importance of the aldehyde group in the inhibitors. At twice the concentration of p-hydroxybenzaldehyde necessary for 100% inhibition of lactic dehydrogenase, p-nitrophenol inhibited the enzyme only 40%. It was therefore concluded that the aldehyde portion of the inhibitor molecule is specifically

important structurally for activity, although the exact mechanism for this is an yet unknown.

As will be shown in Figure 25, the potency of the inhibitors increases with increasing electronegativity of the substituent in the 3 position of the ring. It was therefore concluded that the ideal compounds to test would be the 3-fluoro or 3-chloro p-hydroxybenzaldehydes. Unfortunately, these are very difficult to synthesize and are not available commercially. However, 2-chloro-4-nitrophenol was commercially available, and its activity is compared to that of vanillin in Figure 8. It can be seen that this compound is also considerably less potent than the aldehydehyde containing inhibitor, vanillin. This confirms the conclusions that the aldehyde group is required for maximum inhibitor potency.

It is clear, then, that the aldehyde portion of the naturally occurring inhibitors and the non-nitrogen containing analogues functions in some as yet unknown way to increase the activity of the inhibitors. This effect is in addition to merely functioning to withdraw electrons from the ring and thereby lower the pK of the phenolic hydroxyl group.

However, it is not irreplaceable, since cyclotene has no aldehyde, and the most potent inhibitor found, the bisulfite addition product of vanillin, has its aldehyde group altered, although it does contain a hydroxyl group in the same position which may serve the same function as the aldehyde in binding to the enzyme surface.

Figure 8. Comparison of vanillin and 2-chloro-4-nitrophenol as lactic dehydrogenase inhibitors. Revised lactic dehydrogenase assay was used as described under Methods and in Section IV A.

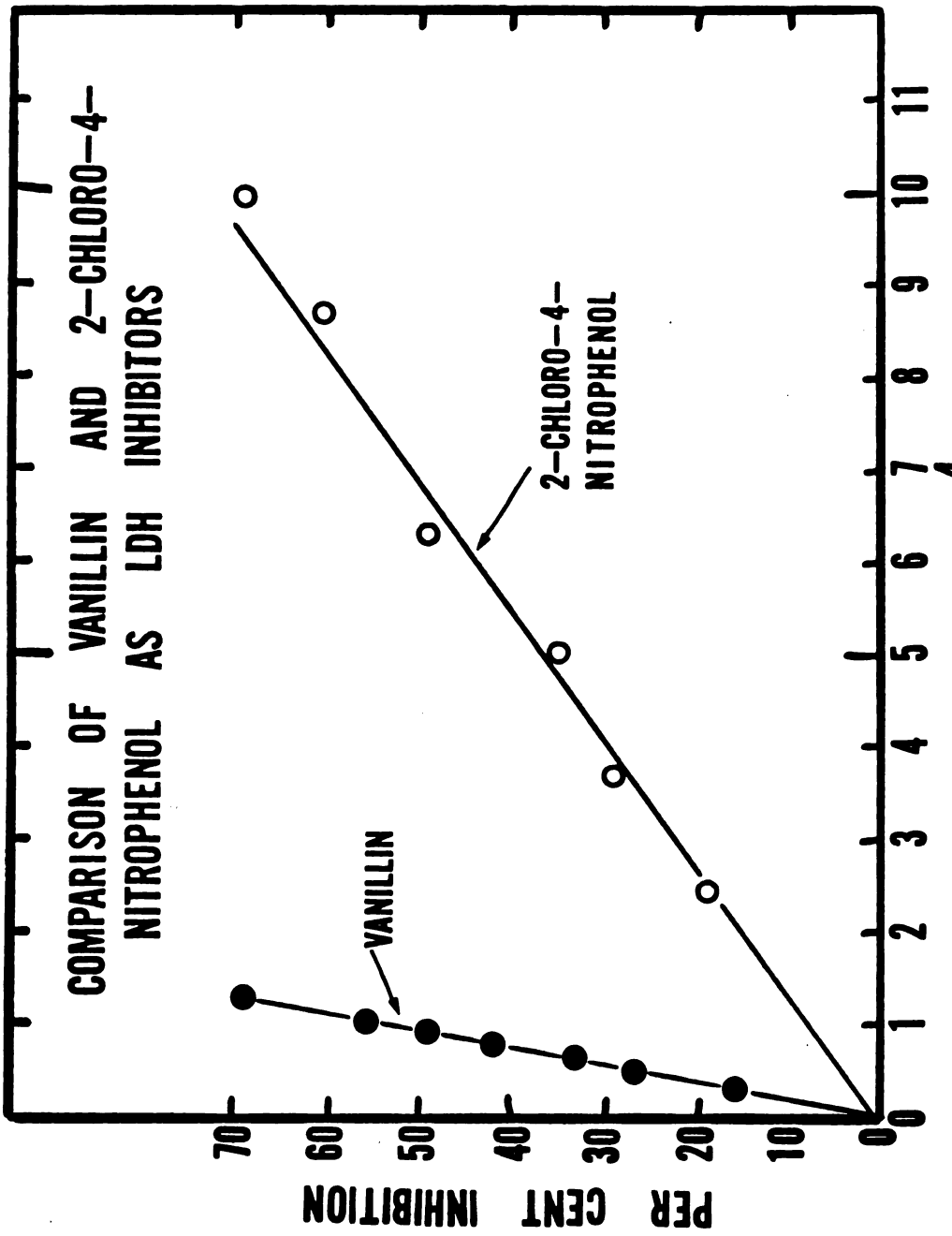

COMPARISON OF VANILLIN AND 2-CHLORO-4-NITROPHENOL AS LDH INHIBITORS
VANILLIN
2-CHLORO-4-NITROPHENOL
PER CENT INHIBITION
MOLARITY x 10^4
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
8
9
10
11

Figure 8

IV. Kinetic Evaluation of the Natural Inhibitors and the Synthetic Analogues and Proof for the Structural Basis of Inhibition

A) Requirement for a Modified Lactic Dehydrogenase Assay

Upon measuring enzyme control rates as a function of time in connection with the testing of inhibition by the MSF, it soon became apparent that the measured specific activity of the lactic dehydrogenase was decreasing with time. This loss in specific activity was found to be correlated with the length of time the enzyme was kept at the low concentration used for the pre-assay stock solution (about 5.6 μg/ml). Thus a new assay had to be developed to insure a stable native enzyme control for the study of the kinetics of the lactic dehydrogenase inhibitors.

As indicated in the literature review, there is evidence that at low concentrations lactic dehydrogenase may be inactivated due to dissociation (Millar, 1962) or due to surface denaturation depending on the type of vessel in which it is stored (Epstein, et al., 1964). A study of this inactivation is described below. The enzyme was brought to the pre-assay concentration of 5.6 μg/ml in either glass or plastic test tubes at 0°C and assayed at various times. As shown in figure 9, it is apparent that although glass may adversely affect dehydrogenase renatura-

Figure 9. Inactivation of lactic dehydrogenase upon standing at low concentrations. The enzyme was diluted to 5.6 μg/ml in 0.01M NaCl at time zero, in both glass and plastic tubes, and assayed at various times thereafter. See text for details.

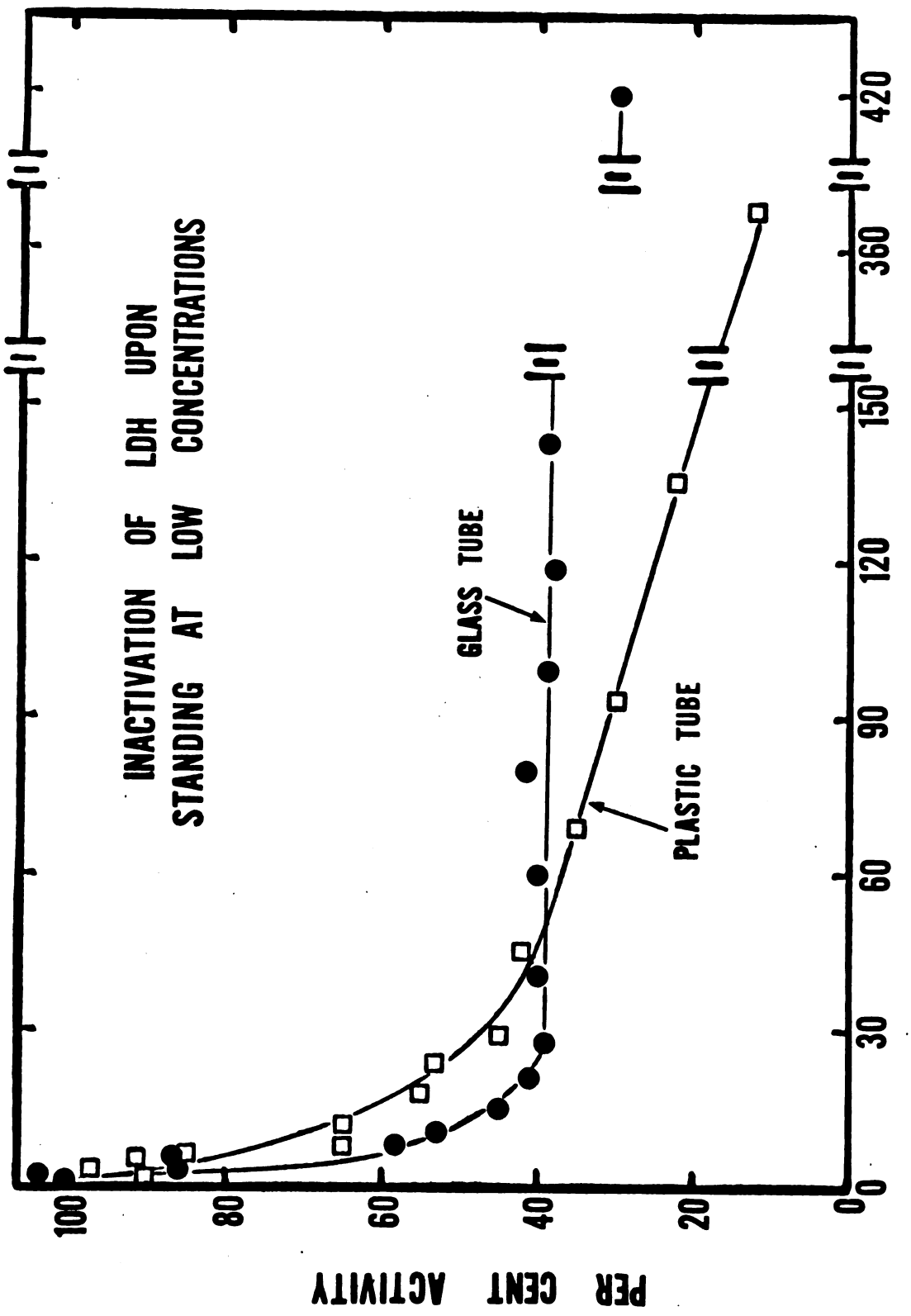

INACTIVATION OF LDH UPON
STANDING AT LOW CONCENTRATIONS
GLASS TUBE
PLASTIC TUBE
PER CENT ACTIVITY
TIME AFTER DILUTION, MINUTES
0
20
40
60
80
100
0
30
60
90
120
150
360
420

Figure 9

tion, for dilute solutions the enzyme is more stable in glass. After a very rapid loss of enzymatic activity over the first twenty minutes, the remaining activity seemed to remain stable in glass for at least two hours, whereas the enzyme stored in the plastic tubes continued to deteriorate. These results demonstrating a decrease in specific activity of lactic dehydrogenase at low concentration may support those of Hathaway and Criddle (1966) and Millar (1962) demonstrating a molecular change for the enzyme from tetramers to presumably inactive dimers at low concentration. A preliminary sucrose density gradient experiment raises some doubts about this, though, since no enzyme activity was found at the dimer position. (This assumes the dissociation is a "cold requiring process" and is reversible upon warming, as has been shown for several other enzymes in this laboratory (S. Constantinides and S. Blatti, private communication). Since the primary objective was to stablize the enzyme and not to explain its instability this interesting aspect of the study was not pursued further.

This decrease in specific activity could be overcome by increasing the concentration of the pre-assay enzyme solution tenfold, to 56 μg/ml. At this concentration, the specific activity of lactic dehydrogenase was found to remain constant for at least 48 hours. The increased amount of enzyme present in the cuvette under these conditions required a new assay protocol. This new assay which was used for all of the kinetic results described below is

described under Assays.

B) Stability of the Inhibitors

Prior to conducting detailed kinetic studies, it seemed advisable to test the inhibitors for stability, as the native enzyme had been tested. Solutions of the naturally occurring inhibitors which had been stored for several weeks were found to decrease in activity, as shown in Table 8.

	% Inhibition		
Inhibitor	Initially	After 1 week	After 2 weeks
vanillin	54	42	0
syringaldehyde	62	59	52
cyclotene	39	30	21

Table 8. Decrease in activity of three naturally occurring inhibitors stored at pH 7.50 at room temperature, while exposed to light. Vanillin was tested at an assay concentration of 1.0×10^{-4} M, while the other two compounds were at 4.5×10^{-4} M.

Also, both the naturally occuring and the synthetic inhibitors were very unstable in basic solution, even at pH's between 7 and 8. This observation explains the earlier loss of activity when the active region of the MSF thin layer chromatographs was subjected to rechromatography in the same methyl ethyl ketone solvent system, which had a pH of 11.0. Vanillin,

exposed to basic solutions, gave a spot which rechromatographed with an R_f of almost 1.0 in this solvent system, which correlated exactly with the R_f of the decomposition product of rechromatographed MSF. The mechanism of this base catalyzed decomposition is most likely an attack of the ionized hydroxyl group upon the reactive aldehyde portion of the inhibitors, forming an inactive polymerization product. In molecules with a sterically hindered hydroxyl or with the aldehyde absent, such as in syringaldehyde or cyclotene, the observed decomposition is slowed, as seen in Table 8.

It was also of interest to test the effect of air and light on the decomposition of the inhibitors. Solutions of the compounds in Table 8 were prepared at pH 5.5 in distilled water to minimize the base-catalyzed decomposition. It was found that over a period of two weeks, the samples kept in air exposed to light lost some activity, but that by storing the samples either under nitrogen or in the dark, full activity could be maintained for months. In addition, it was later found that solutions of the inhibitors kept at a pH between 7.0 and 7.5 were completely stable, provided that oxygen was excluded and the solutions were kept in the dark. These conditions were used to routinely store the stock solutions of the inhibitors in the animal testing described in Section V.

C) Reversibility of the Observed Inhibition

Although the previous results suggested that the inhibition by MSF was reversible, more direct proof of this reversibility was sought. Therefore, solutions of lactic dehydrogenase containing sufficient inhibitor to completely abolish all enzymatic activity were prepared. The medium contained 0.01M NaCl and potassium phosphate buffer at pH 7.40. Vanillin, p-hydroxybenzaldehyde and the bisulfite addition product of vanillin were chosen as representative inhibitors to be tested. The inhibitors were added to the enzyme solutions to yield a final inhibitor concentration of 1.0×10^{-3}M. The resulting solutions were divided into two portions. One was dialyzed against the phosphate buffer medium for 48 hours at 4°C, while the other remained in the test tube for 48 hours at 4°C. Assays showed that the nondialyzed controls were still completely inactive. However, the dialyzed samples had regained full activity. Subsequent addition of inhibitor to the reactivated enzyme once again inhibited its activity, demonstrating that no irreversible changes had occured from the exposure to high concentrations of the inhibitors. A final check of this conclusion was made. A sample of lactic dehydrogenase was stored for 48 hours at 4°C with sufficient vanillin to inhibit 75% of its activity at zero time. After 48 hours at 4°C it was inhibited to the same degree, which indicated that there were no further reactions, such as irreversible

inactivation, occuring with time. It was therefore concluded that the action of these inhibitors was reversible, and that they were apparently forming a non-covalent bond at the enzyme surface.

D) Lineweaver-Burke Reciprocal Plots for the Inhibitors at Various DPNH Concentrations

The next experiments were designed to kinetically characterize the mechanism of the inhibition. Figures 10-16 depict the Lineweaver-Burke reciprocal plots at various DPNH concentrations for the inhibitors shown in Table 9. It should be noted that the zero concentration inhibitor line on figures 11-16 has been copied from that of figure 10, which was determined very carefully and precisely. Therefore, the actual experimental points for this line are shown only on Figure 10. As can be seen from the shape of these plots, the inhibitors are non-competitive with respect to DPNH. It was shown in the literature review that in the case of substrate, there is a compulsory order of addition to the enzyme, pyruvate following DPNH. If this were the case for the inhibitors, one would expect uncompetitive inhibition. The observation of non-competitive inhibition indicates that the inhibitors may bind either to the enzyme-DPNH complex or to the enzyme alone, and may serve to explain in part why the inhibitors have such low K_i's.

The K_m for DPNH for lactic dehydrogenase calculated

Table 9. The structure, length of substituent group in ring position 3, pK and K_i of the inhibitors used in the kinetic studies described in detail in the text. The pK values were determined experimentally as described under Methods.

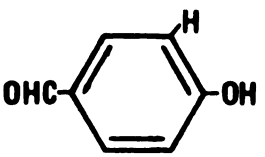
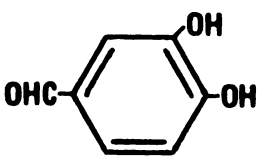
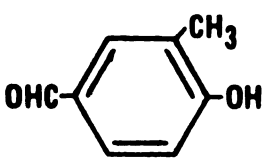
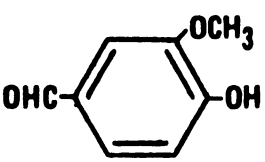
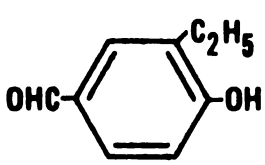
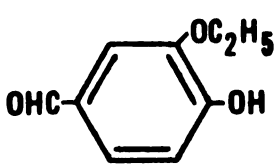
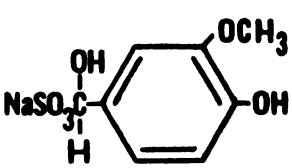
COMPOUND	STRUCTURE	SIDE CHAIN LENGTH(Å)	pK	K_i (μM)
p-hydroxy-benzaldehyde		1.084	7.90	414
protocatachuic aldehyde		2.392	7.75	174
"methyl" vanillin		2.639	7.90	270
vanillin		3.959	7.60	96
"ethyl" vanillin		4.175	7.90	241
"ethoxy" vanillin		5.495	7.65	108
bisulfite adduct of vanillin		3.959	7.65	14

Table 9

Figure 10. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by vanillin. Revised assay described in Section IV, A.

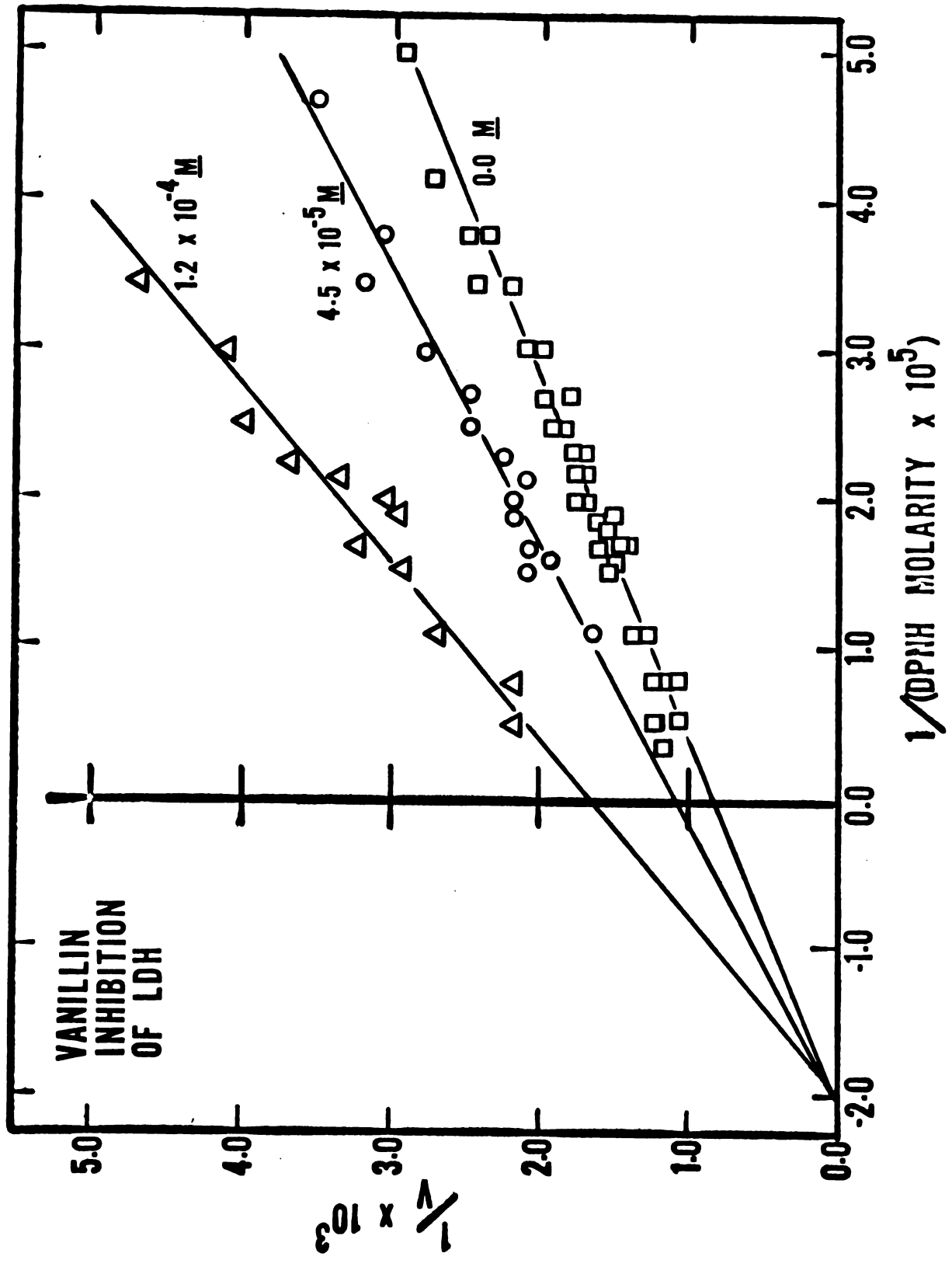

VANILLIN INHIBITION OF LDH
1.2 x 10⁻⁴ M
4.5 x 10⁻⁵ M
0.0 M
1/V x 10³
1/(DPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 10

Figure 11. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by "ethoxy" vanillin. Revised assay described in Section IV, A.

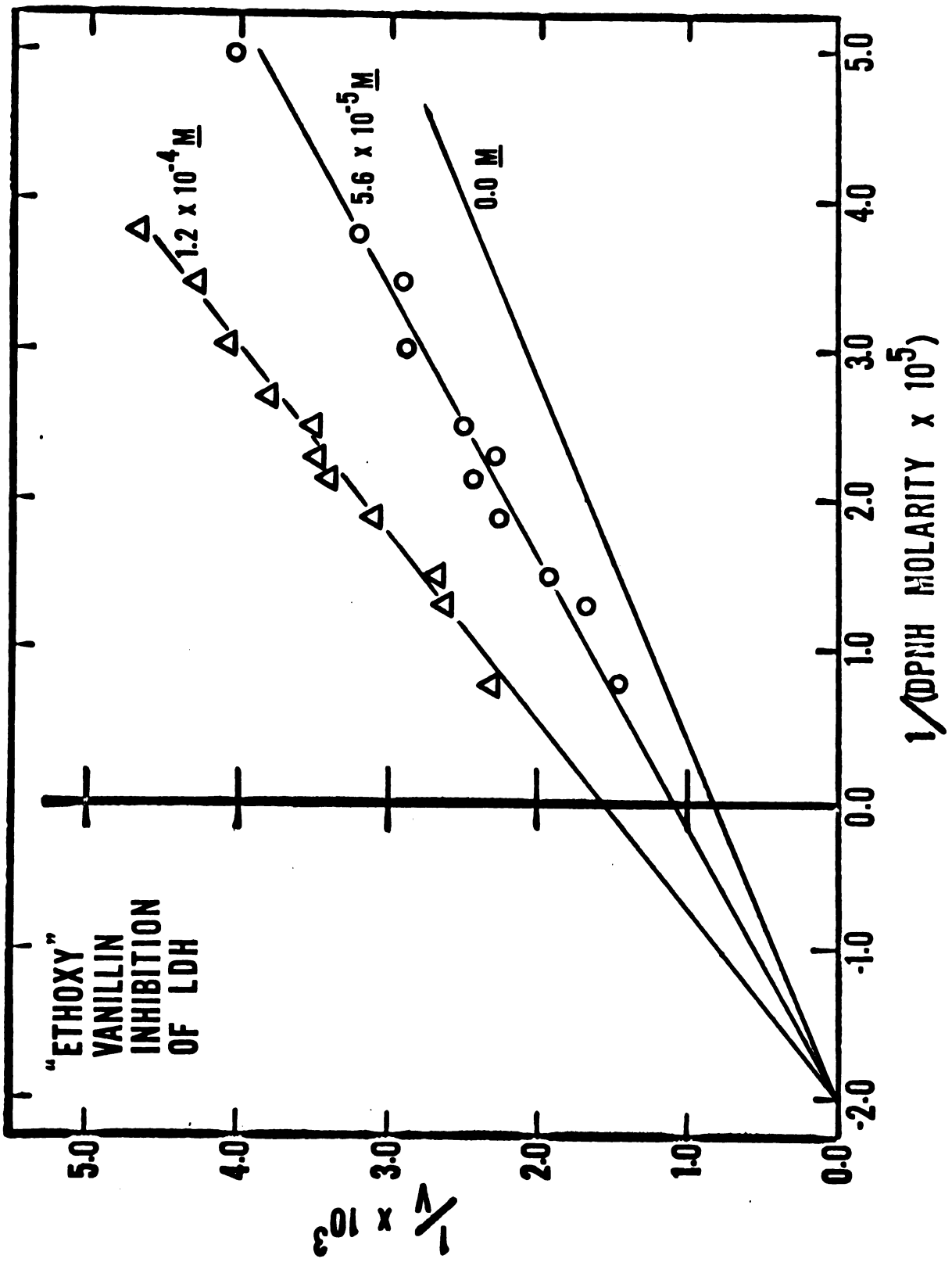

"ETHOXY" VANILLIN INHIBITION OF LDH
1.2 x 10⁻⁴ M
5.6 x 10⁻⁵ M
0.0 M
1/V x 10³
1/DPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 11

Figure 12. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by protocatachuic aldehyde. Revised assay described in Section IV, A.

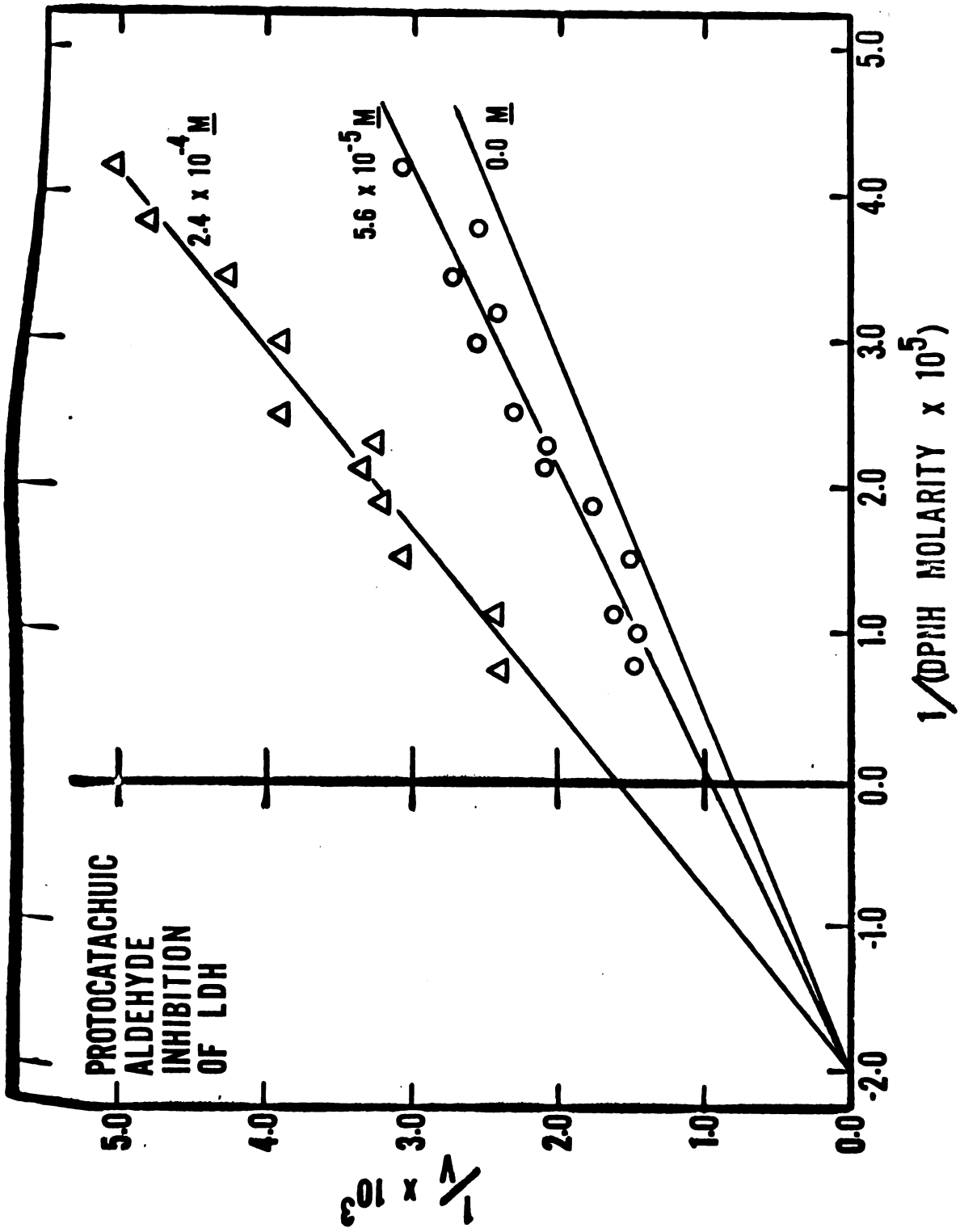

PROTOCATACHUIC
ALDEHYDE
INHIBITION
OF LDH
2.4 x 10⁻⁴ M
5.6 x 10⁻⁵ M
0.0 M
1/V x 10³
1/(DPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 12

Figure 13. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by p-hydroxybenzaldehyde. Revised assay described in Section IV, A.

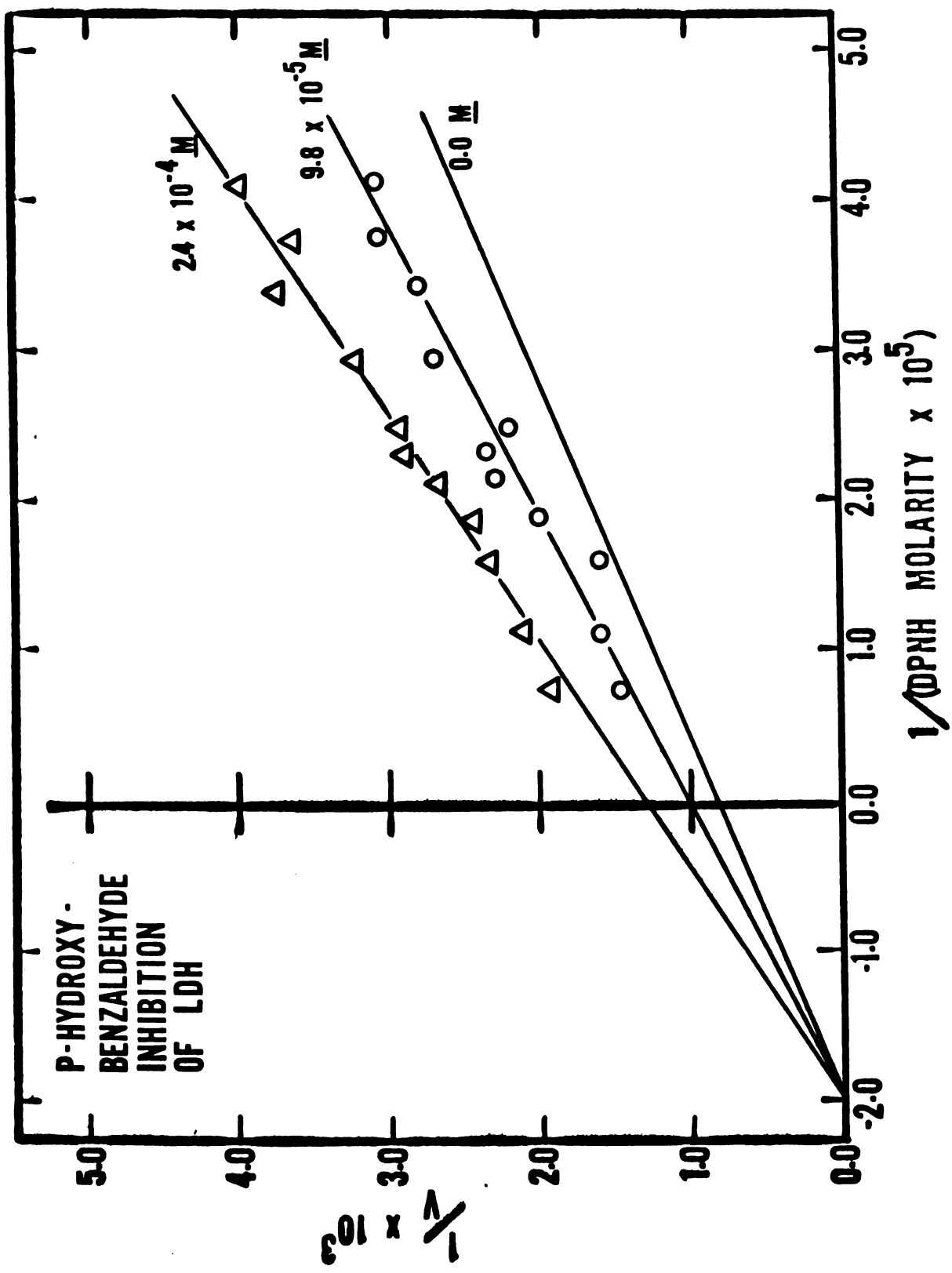

P-HYDROXY-
BENZALDEHYDE
INHIBITION
OF LDH
24 x 10⁻⁴ M
9.8 x 10⁻⁵ M
0.0 M
1/V x 10³
1/ΦPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 13

Figure 14. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by "methyl" vanillin. Revised assay described in Section IV, A.

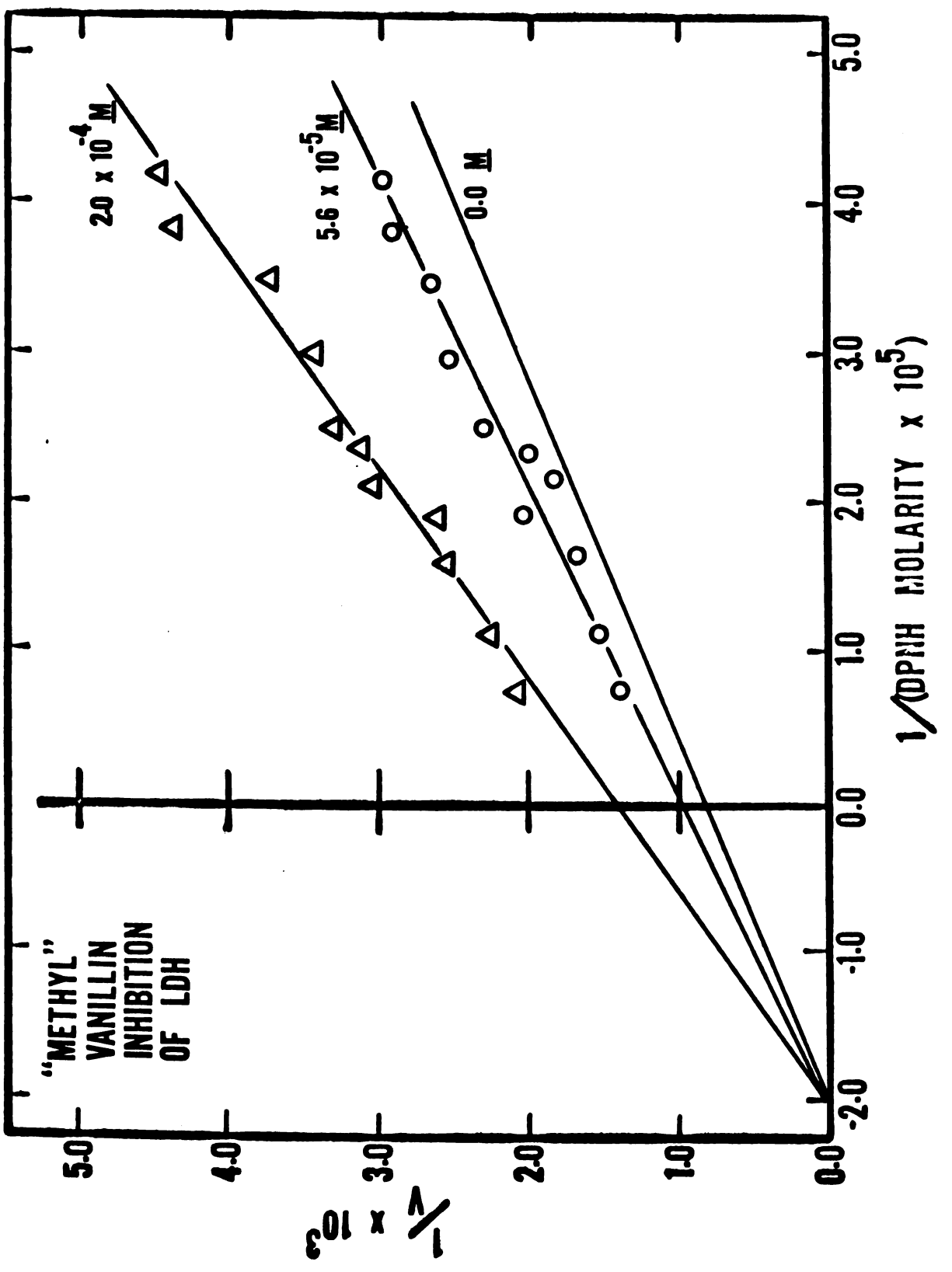

"METHYL" VANILLIN INHIBITION OF LDH
2.0 x 10⁻⁴ M
5.6 x 10⁻⁵ M
0.0 M
1/V x 10³
1/(DPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 14

Figure 15. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by "ethyl" vanillin. Revised assay described in Section IV, A.

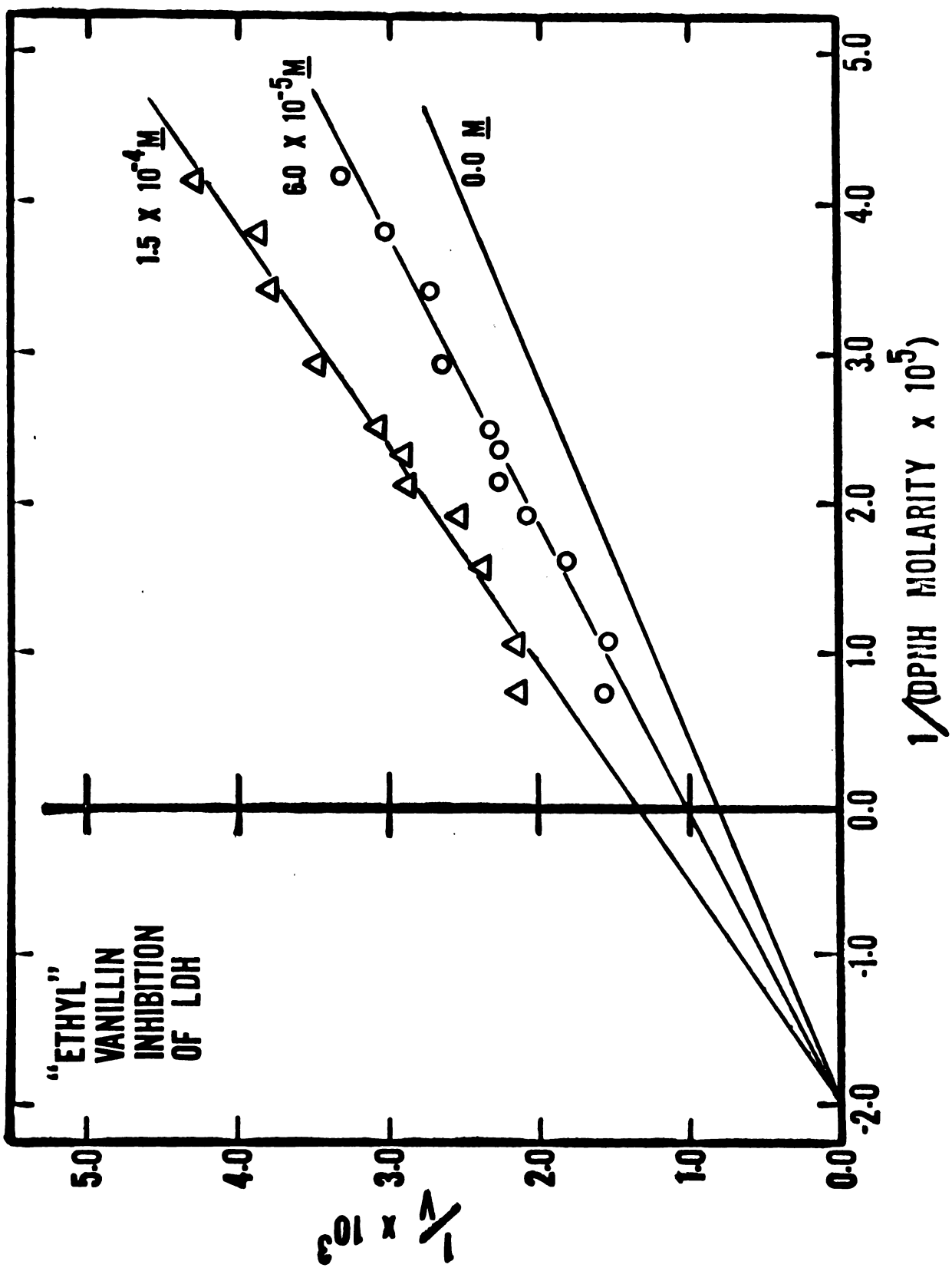

"ETHYL" VANILLIN INHIBITION OF LDH
1.5 X 10⁻⁴ M
6.0 X 10⁻⁵ M
0.0 M
1/V x 10³
1/(DPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 16. Lineweaver-Burke reciprocal plot demonstrating noncompetitive inhibition with respect to DPNH of lactic dehydrogenase by the bisulfite addition product of vanillin. Revised assay described in Section IV, A.

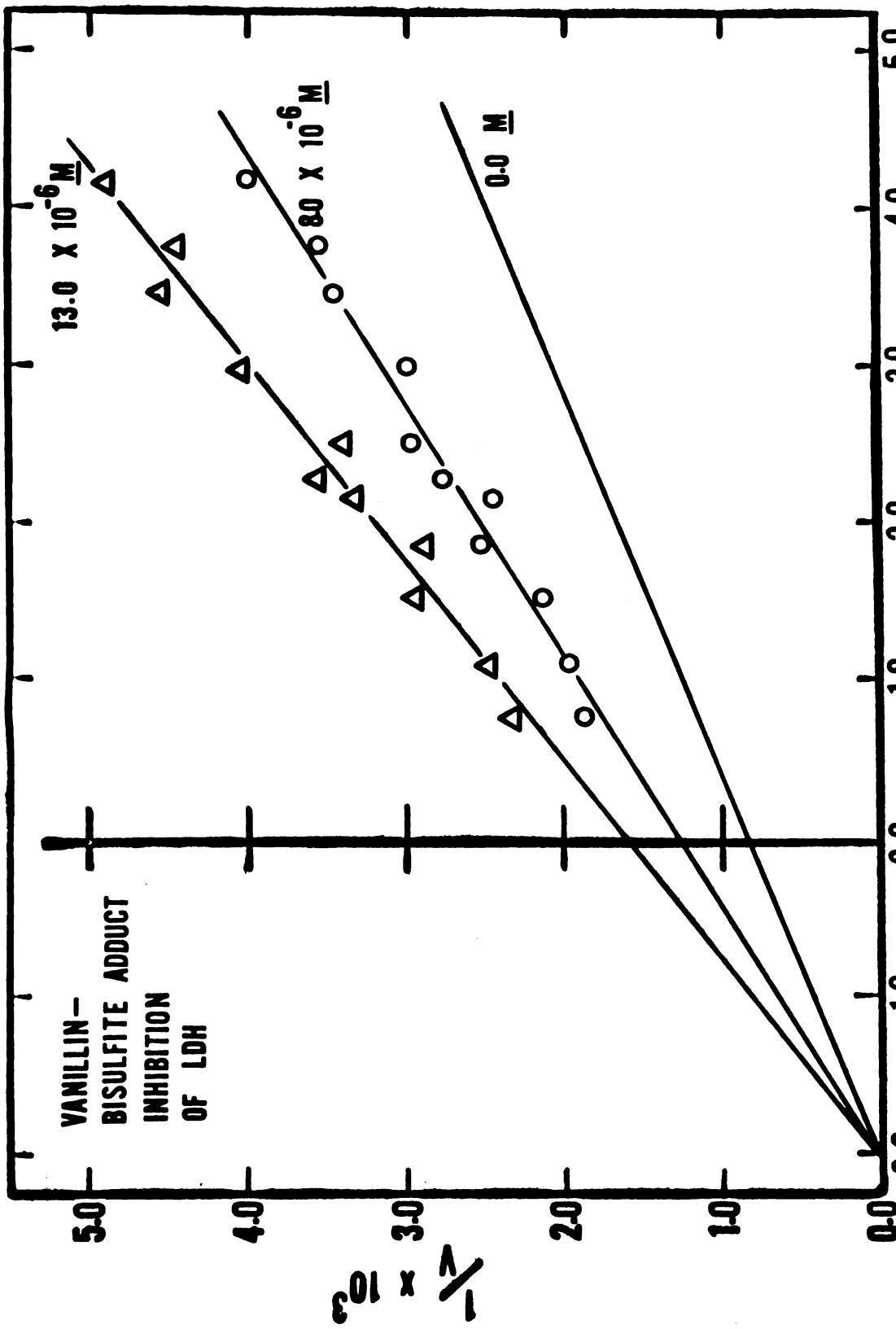

VANILLIN–
BISULFITE ADDUCT
INHIBITION
OF LDH
13.0 X 10⁻⁶ M
8.0 X 10⁻⁶ M
0.0 M
1/V x 10³
1/ΦPNH MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 16

from the intercept of the zero concentration inhibitor line is 5.0×10^{-6}M, agreeing well with the value reported by Hakala, et al. (1953) of 2.6×10^{-6}M. The K_i's for the inhibitors are essentially the same as those calculated from both sets of reciprocal plots for each inhibitor.

E) Lineweaver-Burke Reciprocal Plots for the Inhibitors at Various Pyruvate Concentrations

Figures 17-23 depict the reciprocal plots at various pyruvate concentrations for the series of natural and synthetic inhibitors whose structures are given in Table 9. As in the DPNH plots above, the zero inhibitor concentration line was determined for only the first figure, and was copied for the others. The shape of the plots shows that all of the inhibitors are competitive with respect to pyruvate, which agrees quite well with the known inhibition of lactic dehydrogenase as discussed in the literature review. An Eadie-Hofstee plot of lactic dehydrogenase with varying concentrations of pyruvate as shown in Figure 24 yields a K_m of 1.10×10^{-5}M for pyruvate. The intercept of the zero concentration inhibitor line on Figure 17 yields a K_m for pyruvate of 1.05×10^{-5}M. Both of these values are consistent with those in the literature; 5.2×10^{-5}M (Meister, 1950) and 1.7×10^{-5}M (Hakala, et al., 1953).

The length of the substituent side chain in position 3 of the aldehyde ring is also given in Table 9. It is

Figure 17. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by vanillin. Revised assay described in Section IV, A.

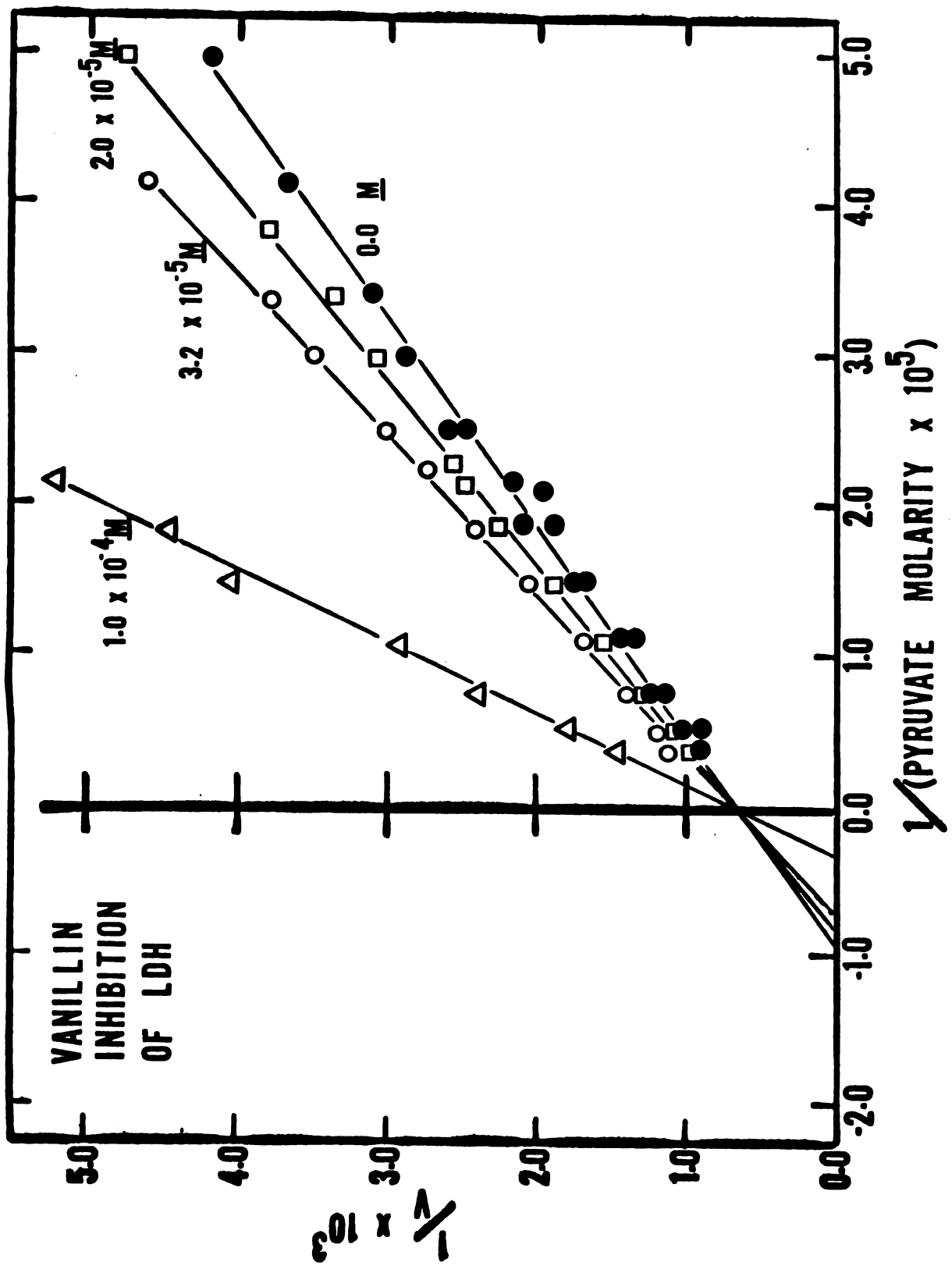

VANILLIN
INHIBITION
OF LDH
1.0 x 10⁻⁴M
3.2 x 10⁻⁵M
2.0 x 10⁻⁵M
0.0 M
1/V x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 17

Figure 18. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by "ethoxy" vanillin. Revised assay described in Section IV, A.

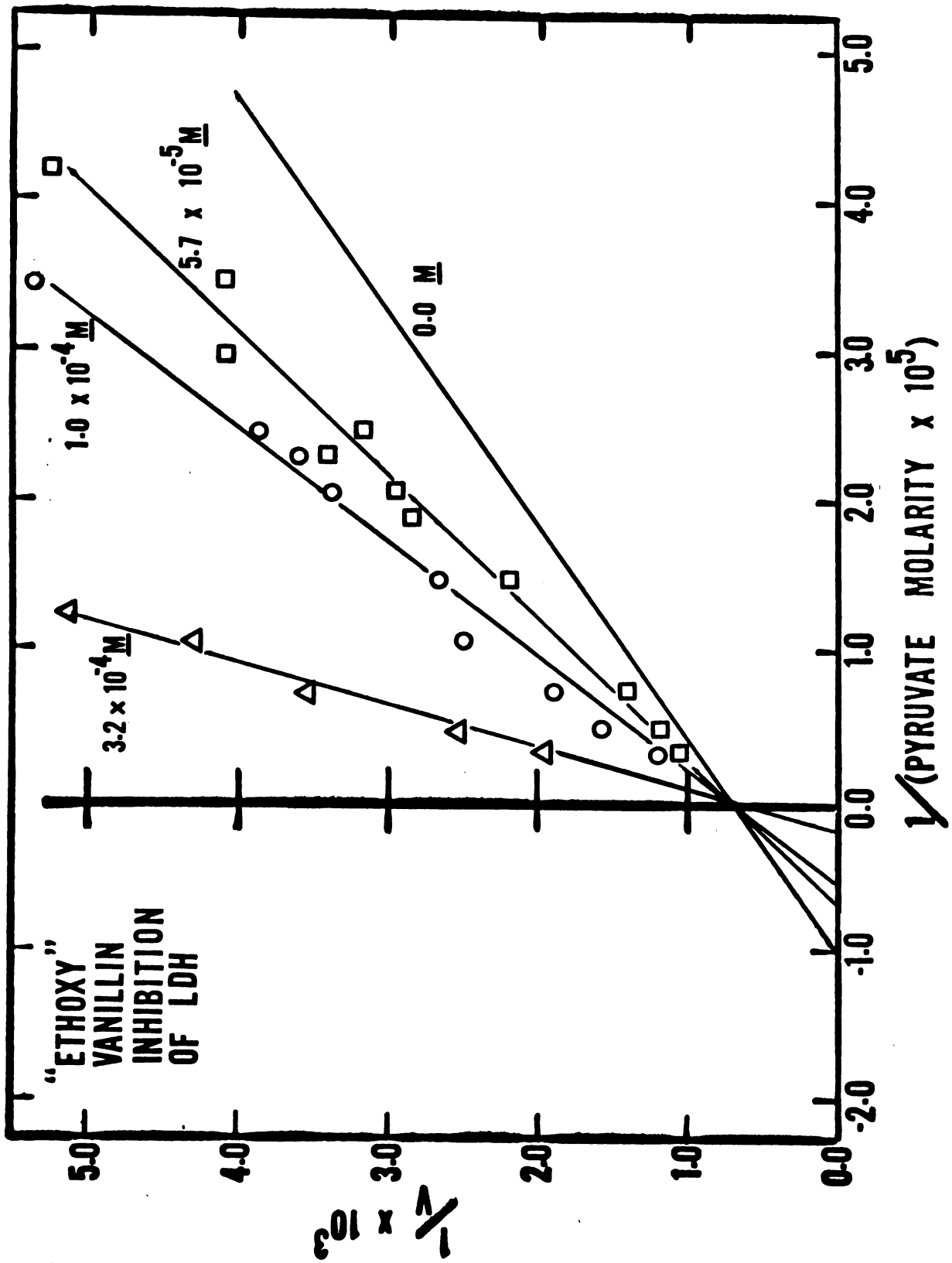

"ETHOXY" VANILLIN INHIBITION OF LDH
1.0 x 10⁻⁴ M
5.7 x 10⁻⁵ M
0.0 M
3.2 x 10⁻⁴ M
1/V x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 13

Figure 19. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by protocatachuic aldehyde. Revised assay described in Section IV, A.

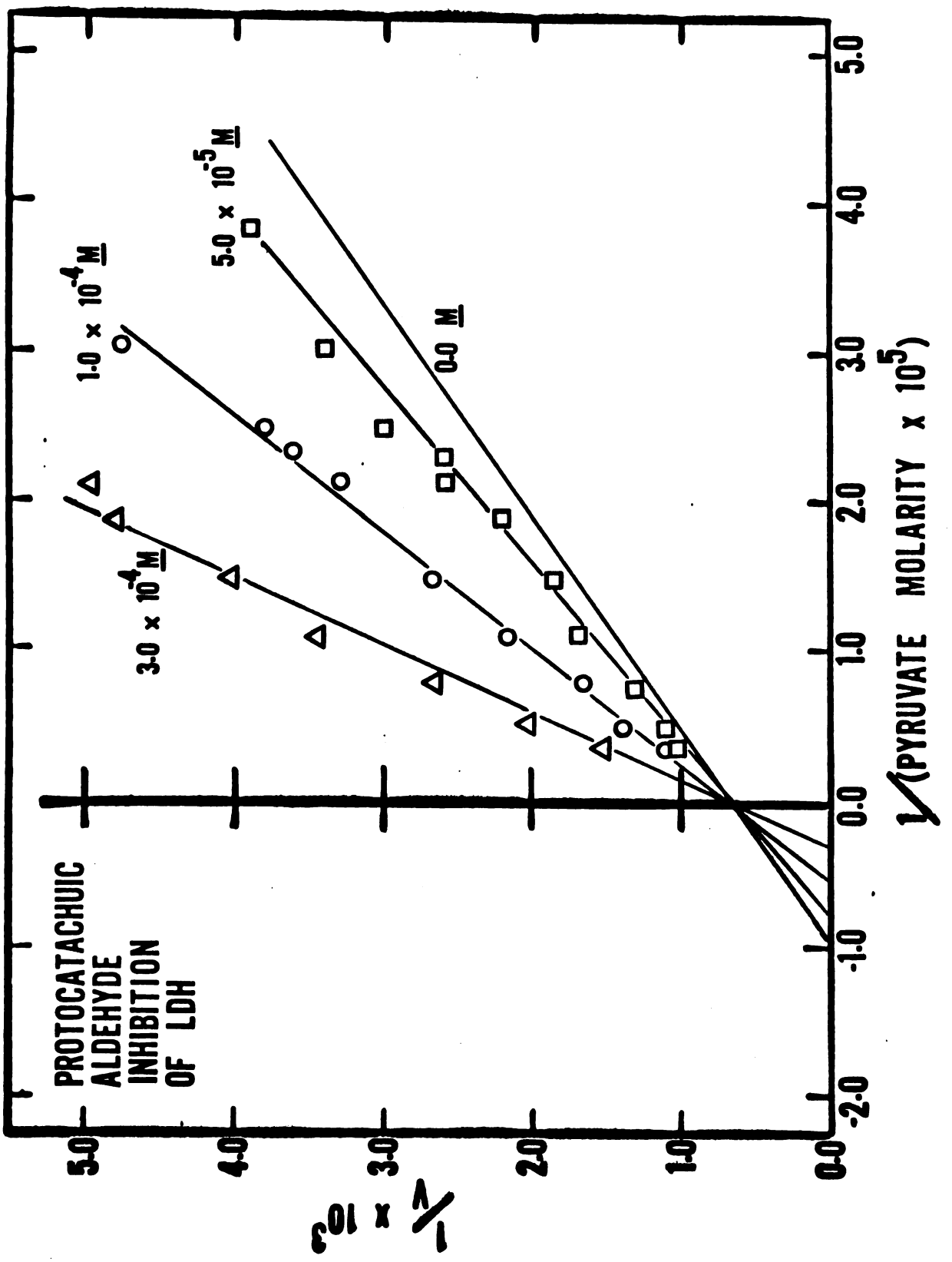

PROTOCATACHUIC
ALDEHYDE
INHIBITION
OF LDH
1.0 x 10⁻⁴ M
5.0 x 10⁻⁵ M
0.0 M
3.0 x 10⁻⁴ M
1/(PYRUVATE MOLARITY x 10⁵)
1/v x 10³
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 19

Figure 20. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by p-hydroxybenzaldehyde. Revised assay described in Section IV, A.

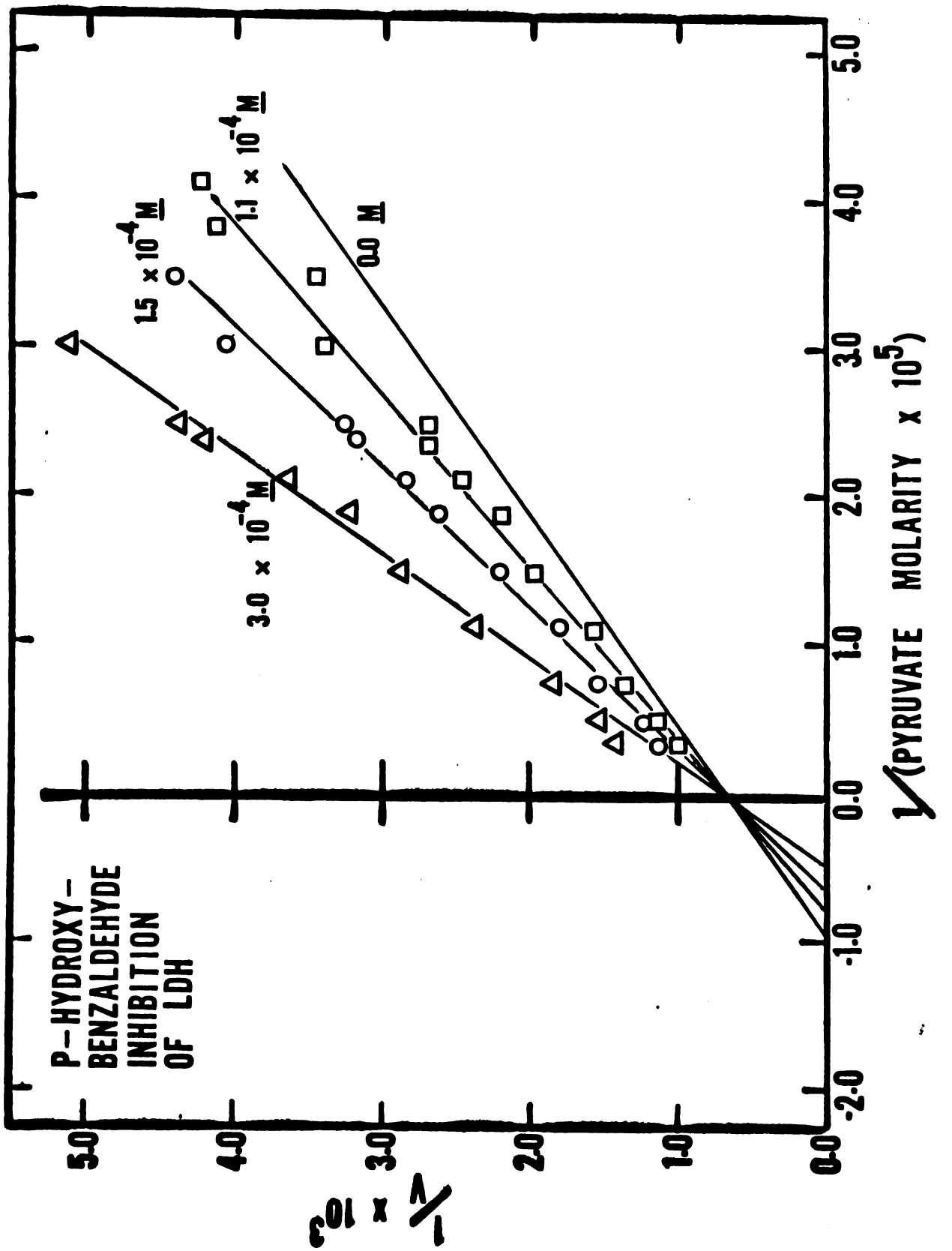

P-HYDROXY-
BENZALDEHYDE
INHIBITION
OF LDH
3.0 x 10⁻⁴ M
1.5 x 10⁻⁴ M
1.1 x 10⁻⁴ M
0.0 M
1/v x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 20

Figure 21. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by "methyl" vanillin. Revised assay described in Section IV, A.

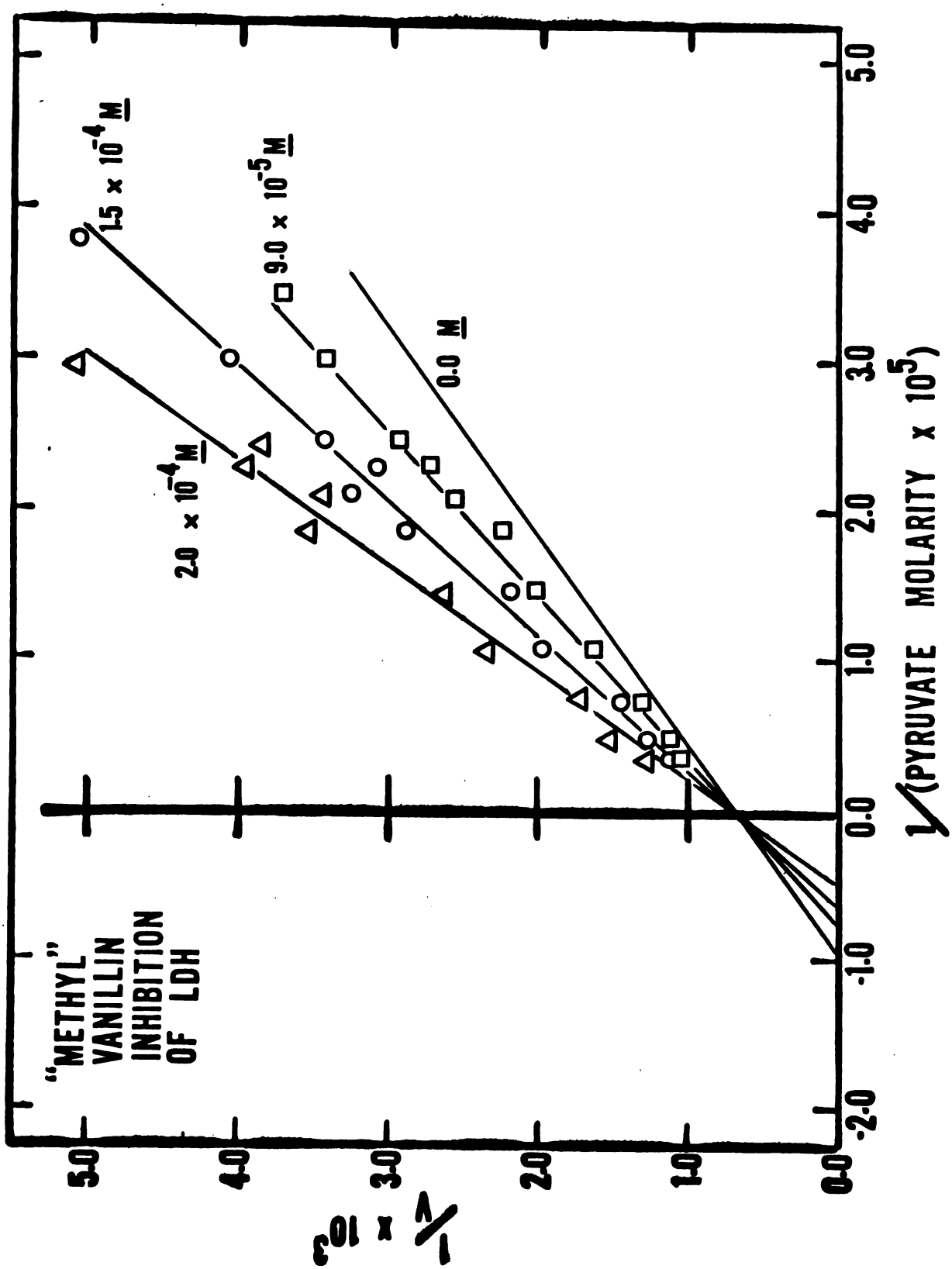

"METHYL" VANILLIN INHIBITION OF LDH
2.0 x 10⁻⁴ M
1.5 x 10⁻⁴ M
9.0 x 10⁻⁵ M
0.0 M
1/V x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-2.0
-1.0
0.0
1.0
2.0
3.0
4.0
5.0

Figure 21

Figure 22. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by "ethyl" vanillin. Revised assay described in Section IV, A.

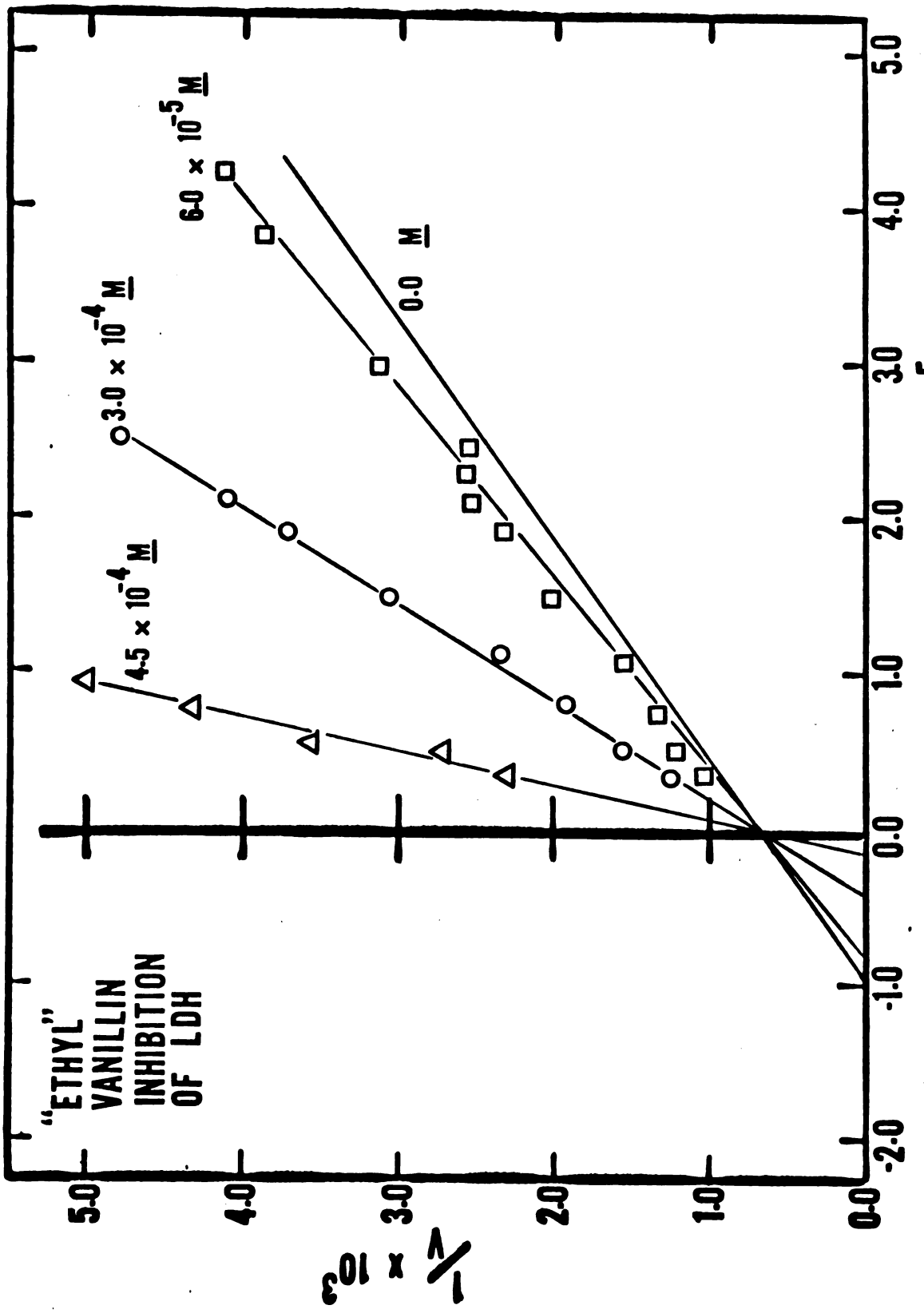

"ETHYL" VANILLIN INHIBITION OF LDH
6.0 x 10⁻⁵ M
3.0 x 10⁻⁴ M
4.5 x 10⁻⁴ M
0.0 M
1/V x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 22

Figure 23. Lineweaver-Burke reciprocal plot demonstrating competitive inhibition with respect to pyruvate of lactic dehydrogenase by the bisulfite addition product of vanillin. Revised assay described in Section IV, A.

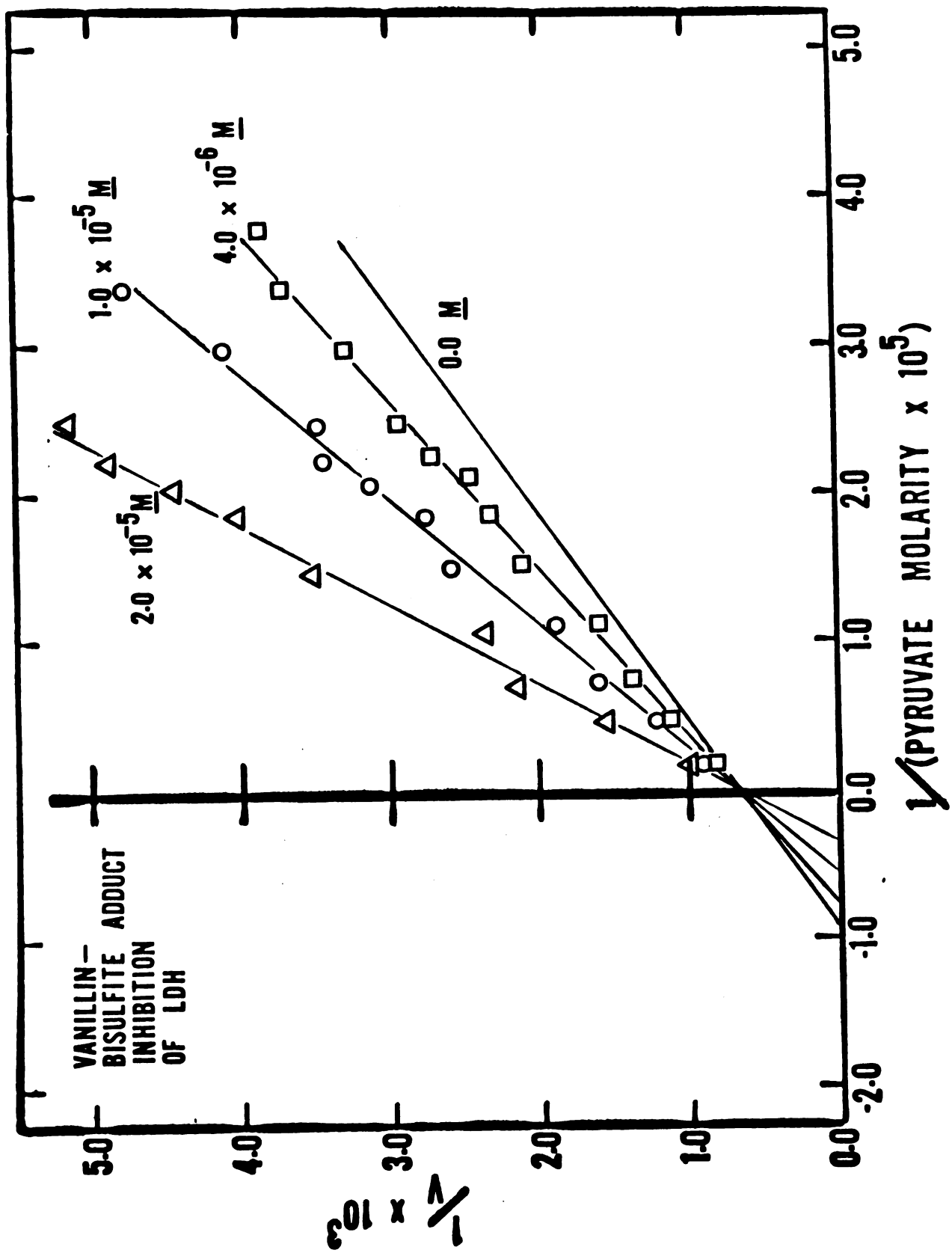

VANILLIN–BISULFITE ADDUCT INHIBITION OF LDH
1.0 x 10⁻⁵ M
4.0 x 10⁻⁶ M
0.0 M
2.0 x 10⁻⁵ M
1/V x 10³
1/(PYRUVATE MOLARITY x 10⁵)
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0

Figure 23

Figure 24. Eadie-Hofstee plot of lactic dehydrogenase as an alternate determination of the K_m for pyruvate. See text for details.

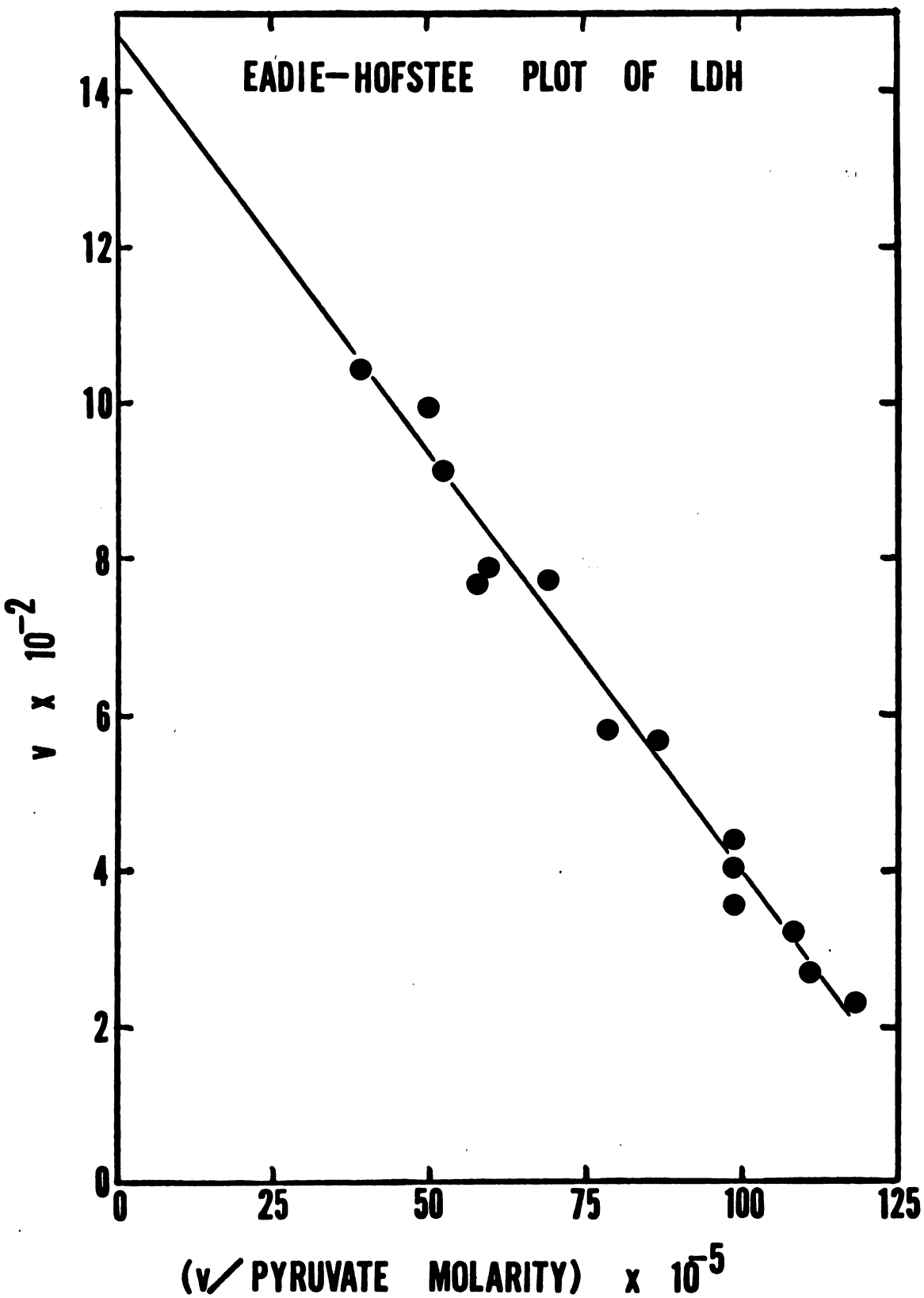

EADIE–HOFSTEE PLOT OF LDH
v x 10⁻²
0
2
4
6
8
10
12
14
0
25
50
75
100
125
(v/ PYRUVATE MOLARITY) x 10⁻⁵

Figure 24

readily apparent that the K_i's are not a direct function of the size of the substituent, and it was therefore suspected that the differences in the K_i's must be due to the electron withdrawing or donating effects of the various substituents.

F) The Molecular Basis of the Inhibition

1) The K_i's of the Inhibitors as a Function of the Relative Negative Charge on the 3 Position of the Aldehyde Ring

Such effects as electron withdrawing or donating are very difficult to quantitate, since aliphatic systems such as these substituents are not generally considered in the lengthy tables of such effects on aromatic systems. An additional problem is posed by the presence of a charged group in the ring, whose effect on the 3 position to which the substituents are attached cannot be accurately calculated without molecular orbital calculations, which have not been sufficiently sophisticated to even approach as complicated a problem as an aromatic ring system. These difficulties not withstanding, an approach to the quantitization of the electronic effects of the substituent groups can be made by the method of Taft (1956), who estimated the electronic effects of such groups in aliphatic systems. If we first assume that the contribution to the 3 position of the aldehyde ring from the ionized phenol to be some arbitrary value X, then we can base the effects of the substituent

groups on X. Furthermore, as was described in the literature review, it has previously been found that the presence of an electronegative group alpha to the anionic portion of the inhibitor produced a more potent inhibitor. One would therefore expect naively that the higher the relative charge on the atom directly attached to the 3 position of the ring, the better would be the inhibition. Calculating such a relative negative charge using the values of Taft (1956), where the greater negative charge is more negative in sign, and plotting them against the observed K_i's for the first six inhibitors in Table 9 yields the curve depicted in Figure 25. As can be seen, the K_i's are a function of the relative negative charge on the 3 position of the ring, although the interpretation of the exact nature of the curve is difficult, if not impossible.

2) Effect of pH on the Observed Inhibition by Vanillin

Since these phenolic inhibitors all have pK's between 7.60 and 7.90, they are not completely ionized at the pH routinely employed for the assay of lactic dehydrogenase, pH 7.40. The inhibition observed at a given concentration of the inhibitors should therefore increase with increasing pH of the assay. That is, at higher pH a larger fraction of the ionized anionic form, which is presumably the active inhibitor form, will be present. Vanillin, with a pK of 7.60, was chosen as a representative

Figure 25. The effect of ring substituents on the inhibition of lactic dehydrogenase. The K_i values are those given in Table 9. See text for a complete description of the calculation of the σ^* value on the alpha atom of ring position 3.

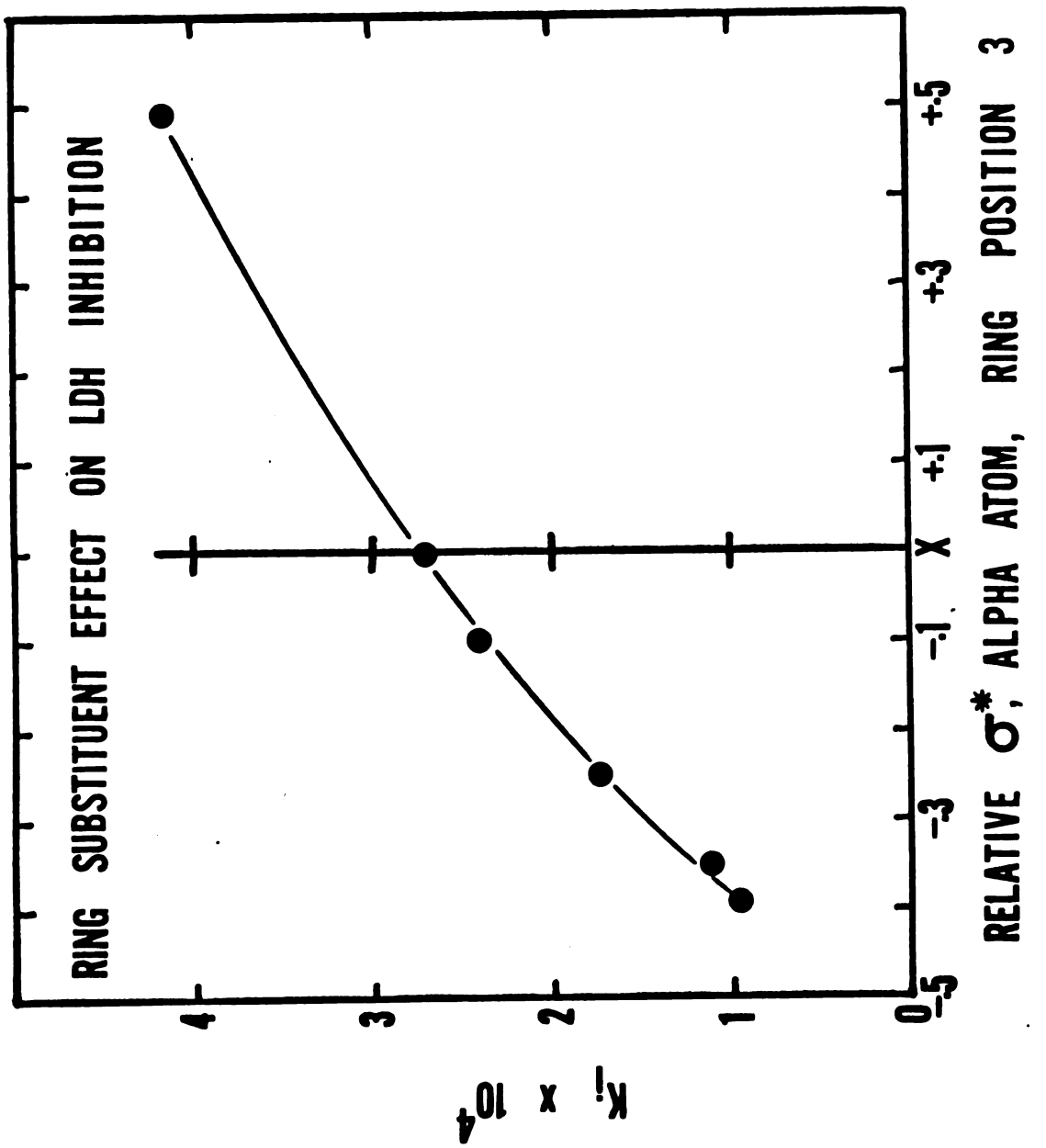

RING SUBSTITUENT EFFECT ON LDH INHIBITION
$K_i \times 10^4$
4
3
2
1
0
-5
-3
-1
X
+1
+3
+5
RELATIVE σ^*, ALPHA ATOM, RING POSITION 3

Figure 25

inhibitor, since it was readily available and could be assayed at low concentration, due to its high activity. Assays were performed at pH 7.00, 7.40, and 7.80, which corresponds to 20, 40 and 60% ionized vanillin (Figure 26). As can be seen, the increase from pH 7.00 to 7.40 resulted in a doubling of the inhibition as would be quantitatively predicted from the ionization of vanillin. In apparent contradiction to this working hypothesis, however, the further increase from pH 7.40 to 7.80 had no effect on the inhibition. It was therefore concluded that in this pH range, titration of groups on the enzyme surface was occuring. Presumably, these could be those groups responsible for the binding of the inhibitors. As discussed in the literature review, lysine and histidine are the residues presumed to be responsible for this binding. Of the two residues, it seems more likely that histidine would be titrated between pH 7.40 and 7.80, although the environmental electrostatic interactions could lower the pK of lysine sufficiently for it to titrate in this range.

It should be noted that maximum inhibition was achieved at pH 7.40, which is very close to physiological pH and is the pH at which all assays of the inhibitors have been performed, so the inhibitors would be expected to be effective <u>in vivo</u> as well as <u>in vitro</u>.

Figure 26. Effect of the assay pH on the vanillin inhibition of lactic dehydrogenase. Assays were as described under Methods, with the substitution of 0.10M potassium phosphate buffers at the indicated pH's for the standard assay buffer.

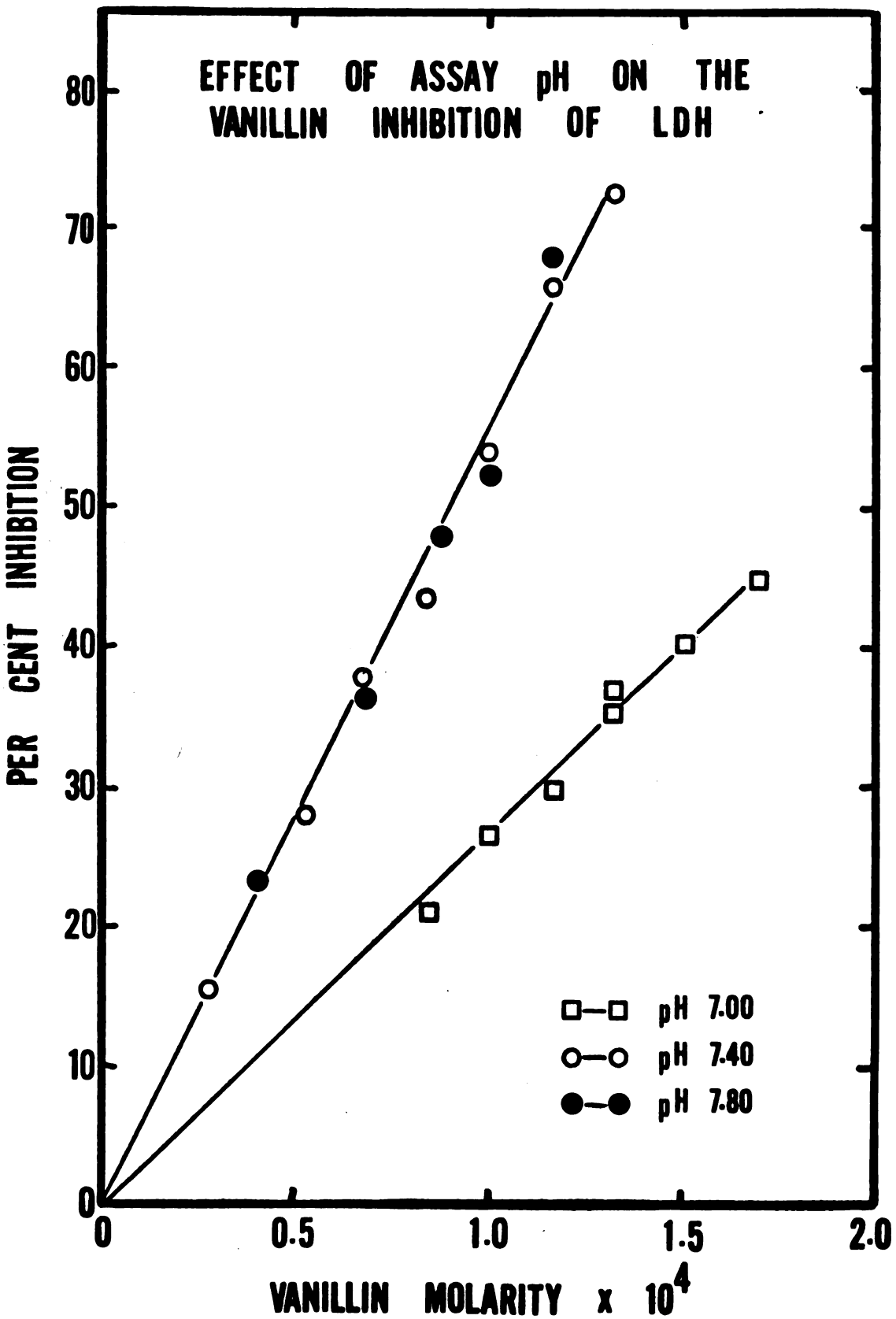

EFFECT OF ASSAY pH ON THE
VANILLIN INHIBITION OF LDH
PER CENT INHIBITION
VANILLIN MOLARITY x 10^4
0
10
20
30
40
50
60
70
80
0
0.5
1.0
1.5
2.0
pH 7.00
pH 7.40
pH 7.80

Figure 26

G) Comparison of the Vanillin Inhibition of the M_4 and H_4 Isozymes of Lactic Dehydrogenase

1) Introduction

Many of the previously discovered inhibitors of lactic dehydrogenase have different effects upon the two different lactic dehydrogenase isozymes, M_4 and H_4 (see literature review). It was therefore of interest to determine if these phenolic inhibitors exhibited such a selective inhibition. Again vanillin was chosen as a typical representative because it was the most active inhibitor.

2) Determination of the Purity of the Isozymes

Before such a study could be performed, it was necessary to demonstrate the purity of the isozyme samples to be used. Polyacrylamide gel electrophoresis of beef heart lactic dehydrogenase revealed the presence of two protein components. When these gels were stained for activity as described under Methods, both bands were shown to be active. These two components of beef heart lactic dehydrogenase are generally found to be active, (see literature review) and these results support this conclusion.

The two components were shown to be the H_4 and H_3M isozymes by comparing their electrophoretic mobility with hybrids produced by freezing rabbit muscle and beef heart

lactic dehydrogenase together in 1M NaCl at pH 7.00, after Salthe, et al. (1965). A densitometer tracing and integration of the beef heart enzyme electrophoresis showed the H_4 and H_3M isozymes to be present in a ratio of approximately 3:1. Previous work (Kaplan and Cahn, 1962) has shown that the individual isozymes in a mixture exhibit properties identical to those found for the pure parent type isozyme, and such activity is directly proportional to the amount of that isozyme present in the mixture. On this basis, this sample of beef heart lactic dehydrogenase contained one M subunit for each 15 subunits of the H type, and therefore was 94% pure heart isozyme. This enzyme was used to determine the effect of vanillin on the heart isozyme without further purification.

The conditions for polyacrylamide gel electrophoresis described under Methods could not be used to determine the purity of the rabbit muscle enzyme since at pH 8.60, this isozyme migrates toward the cathode and does not enter the gel. Therefore, polyacetate electrophoresis at this pH was employed. A densitometer tracing of the resulting pattern as above showed it to be essentially homogeneous M_4 lactic dehydrogenase, so it was used without further purification.

3) Vanillin Exhibits Greater Inhibition of the H_4 Isozyme

Figure 27 depicts the inhibition of both isozymes of

Figure 27. Comparison of the vanillin inhibition of the H_4 and M_4 lactic dehydrogenase isozymes. Assays were described under Methods.

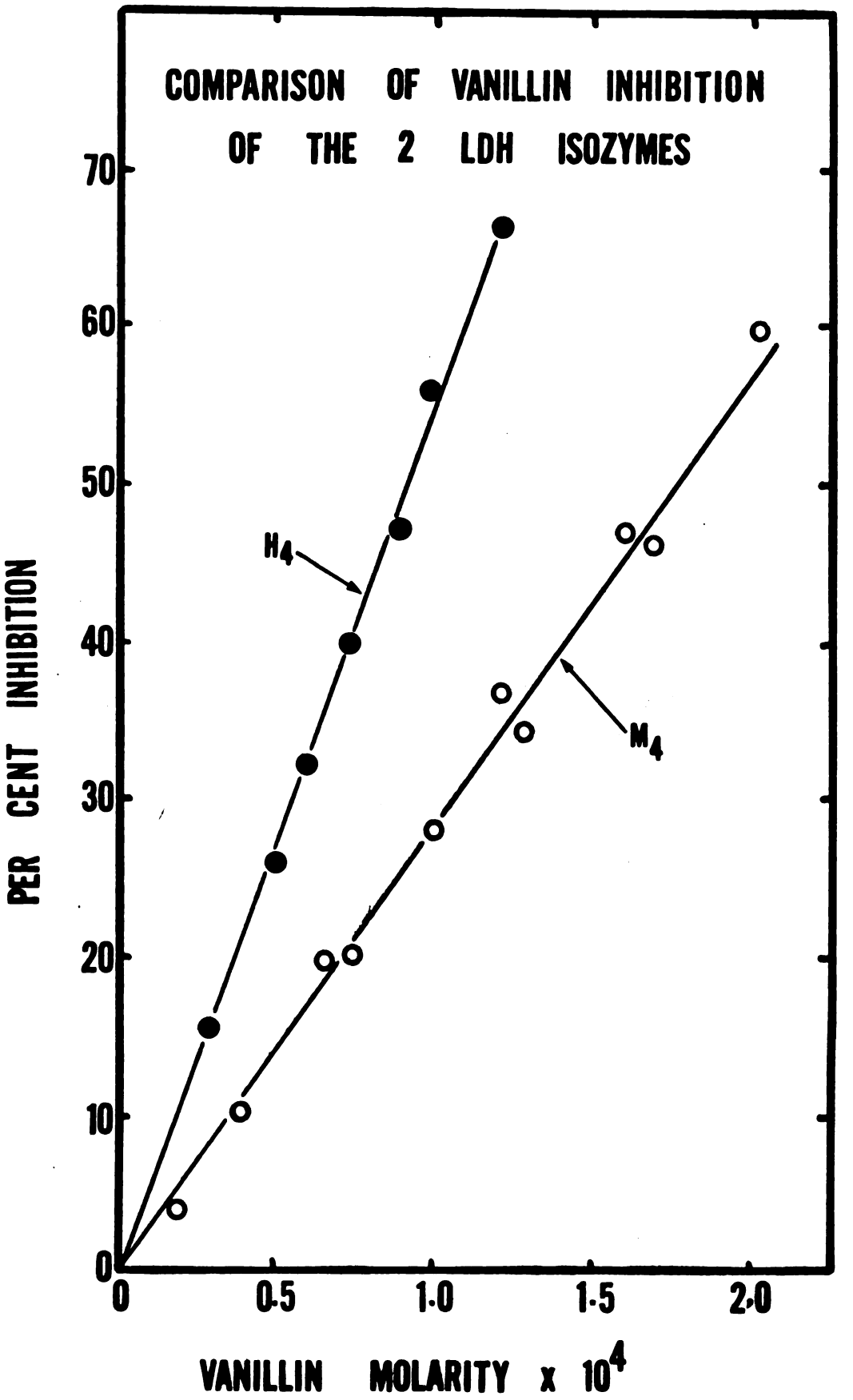

COMPARISON OF VANILLIN INHIBITION
OF THE 2 LDH ISOZYMES
PER CENT INHIBITION
VANILLIN MOLARITY x 10^4
H4
M4
0
10
20
30
40
50
60
70
0
0.5
1.0
1.5
2.0

Figure 27

lactic dehydrogenase by vanillin. As can be seen, the H_4 isozyme is inhibited to a somewhat greater degree than the M_4 isozyme. These results are in good agreement with those discussed in the literature review, since differences in either inhibition or activity of the two isozymes with various compounds are generally found to be small (Fondy and Kaplan, 1965; Kaplan, et al., 1960).

V. Direct Testing of the Theory of Inhibition of Cancer by Inhibition of Lactic Dehydrogenase

A) Testing Procedures

All testing performed in this laboratory followed the protocols described by the Cancer Chemotherapeutic National Service Center (CCNSC). The sarcoma 180 was from a line of tumor approved by CCNSC for use in a screening program of antitumor agents, and was kindly donated by Dr. Everett S. Beneke of the Michigan State University botany department. For the experiments described below, white, Swiss-Webster mice 15-25 days old and weighing approximately 20 grams were used. They were kept in metabolism cages with free access to common laboratory chow, with tap water for drinking, except where 13% glucose was specified.

The mice were first accustomed to the metabolism cages for at least two days prior to the beginning of the test. Tumors from carrier mice were excised and immediately placed in a solution of saline containing 1.0 mg/ml chloromycetin to prevent bacterial growth, where they were cut into small pieces approximately 1-2 mm square. These pieces were transplanted subcutaneously in the axilla region of a pre-weighed mice by trocar. These test animals were given a single intraperitoneal dose of compound to be tested daily on days 1-7. They were weighed again on day 8; weight change was

taken to be a measure of the toxicity of the compound under test. The mice were then sacrificed by cervical dislocation and the tumors excised and weighed on the Mettler balance as soon as possible.

The LD_{50} (the lethal dose for 50% of the animals) was used to provide a measure of toxicity. Determination of this quantity was as follows. The LD_{50} for each of the compounds described below was determined by using groups of six mice each and plotting the per cent mortality against dose to find the single dose level at which 50% of the animals died.

The metabolic rate of the compounds was determined as suggested by Koppanyi and Avery (1966). The procedure is to accurately determine the LD_{50} for a single dose and then to divide this dose evenly into several doses administered over several hours, and to calculate the extra amount necessary then for 50% mortality. The additional amount divided by the number of hours over which the administration proceded yields the metabolic rate of the compound in mg of compound metabolized/kg body weight/hour. It should be noted that this is only an approximate value, since compartmentalization and other factors can influence this value. However, the determination of a more exact metabolic rate requires an extensive study with radioactive tracers, and the expense for labeling the compounds described below was prohibitive in view of the limited value of the information to be gained.

B) Early Results Demonstrating MSF inhibition of Sarcoma 180 in Mice

1) Requirement for High Levels of Dietary Glucose for Inhibition

It had been reported that in order to effect a cancer cell in vivo by inhibition of lactic dehydrogenase, it was necessary for the organism to be maintained on a diet high in glucose, to force the use of glucose as the primary source of energy via glycolysis (Goldberg, Nitowsky and Colowick, 1965). To test this requirement in the present system, two groups of mice were inoculated with sarcoma 180. One group was given 13% glucose and the other tap water, while both were daily dosed with 50 mg/kg of MSF. After excision and weighing of the tumors, it was found that the group drinking plain tap water had tumors four times larger than the group on glucose (see Figure 28), supporting those results cited above. It should be noted that glucose had no apparent effect on a group of mice with no tumors, so the results described below all were obtained from mice maintained on 13% glucose rather than plain tap water.

2) LD_{50} for the MSF in Mice

Using MSF doses of 5, 20, 50, 200, 500 and 1000 mg/kg

Figure 28. Comparison of the sarcoma 180 tumors taken from mice on high and low glucose diets, as described in the text. Both groups were treated daily with 50 mg MSF/kg body weight.

1. Drinking 13% glucose.
2. Drinking plain tap water.

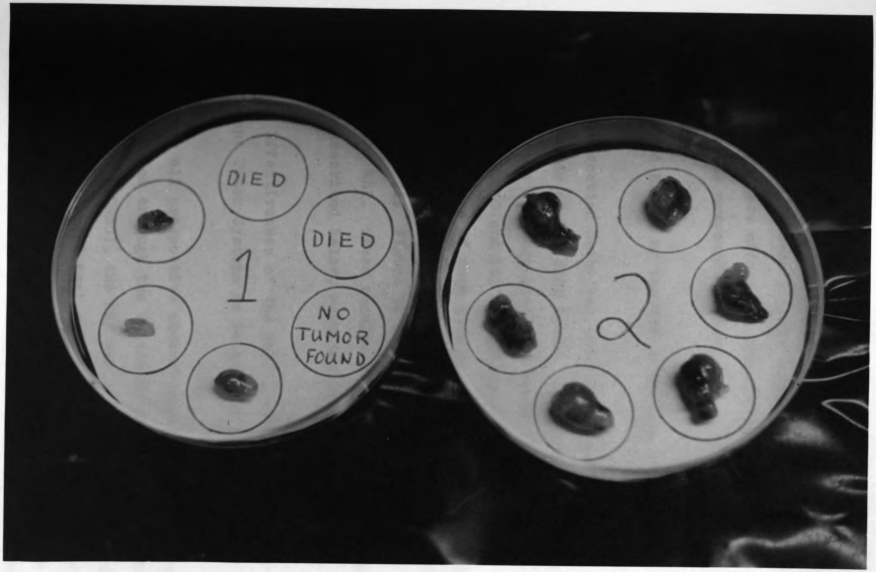


Figure 28

body weight, the LD_{50} was determined to be 750 mg/kg. The mice alive after ten days were sacrificed by cervical dislocation and the viscera grossly examined for evidence of toxicity. No gross effects on the viscera were observed with any of the compounds tested.

3) Pronounced Effect of the MSF on Sarcoma 180 in Mice

This section describes the direct testing of the MSF as an inhibitor of the S180 tumor. Seven groups of mice bearing sarcoma 180 were given the doses of MSF; namely 10, 20, 25, 35, 50, 50 and 100 mg/kg body weight/day. The external appearance of such mice is depicted in Figure 29. The excised tumors from these dosed animals are shown in Figures 30 and 31. Figure 32 depicts the dose response curve for the MSF. These results were quite encouraging, but as is described below, were not reproducible.

C) Slight Effectiveness of the Inhibitors Against Three Different Tumor Systems as Determined by CCNSC

Since all prospective cancer chemotherpeutic agents must be screened through the Cancer Chemotherapeutic National Service Center (CCNSC), the MSF was submitted for testing against three different test systems. In contrast to our observed inhibition of approximately 80%, CCNSC observed an inhibition of sarcoma 180 of only 6-20%. Furthermore, the

Figure 29. External appearance of sarcoma 180 bearing mice. Upper mouse was treated daily with 150 mg MSF/kg body weight. Alcohol was swabbed on the abdomen to facilitate visualization of the tumor. The lower mouse is a water treated control. The photograph was taken five days after transplantation of the tumors.

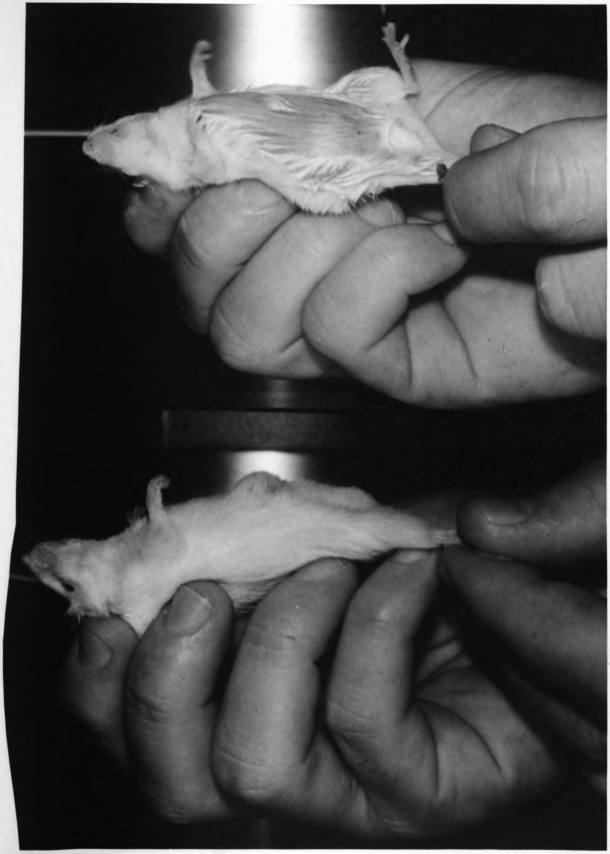


Figure 29

Figure 30. Sarcoma 180 tumors excised from mice treated with various doses of MSF, as follows:

1. 150 mg MSF/kg body weight/day
2. 65 mg MSF/kg body weight/day
3. 25 mg MSF/kg body weight/day
4. control, injected with water only

Experimental details are described in the text.

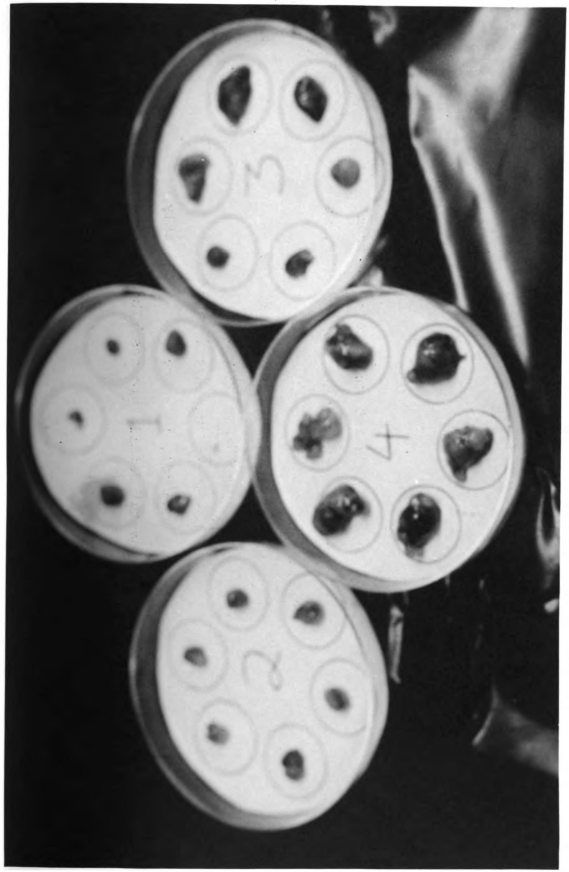


Figure 30

Figure 31. Sarcoma 180 tumors excised from mice treated with various doses of MSF, as follows:

1. 50 mg MSF/kg body weight/day
2. 35 mg MSF/kg body weight/day
3. 25 mg MSF/kg body weight/day
4. 10 mg MSG/kg body weight/day
5. control, injected with water only

Experimental details are described in the text.

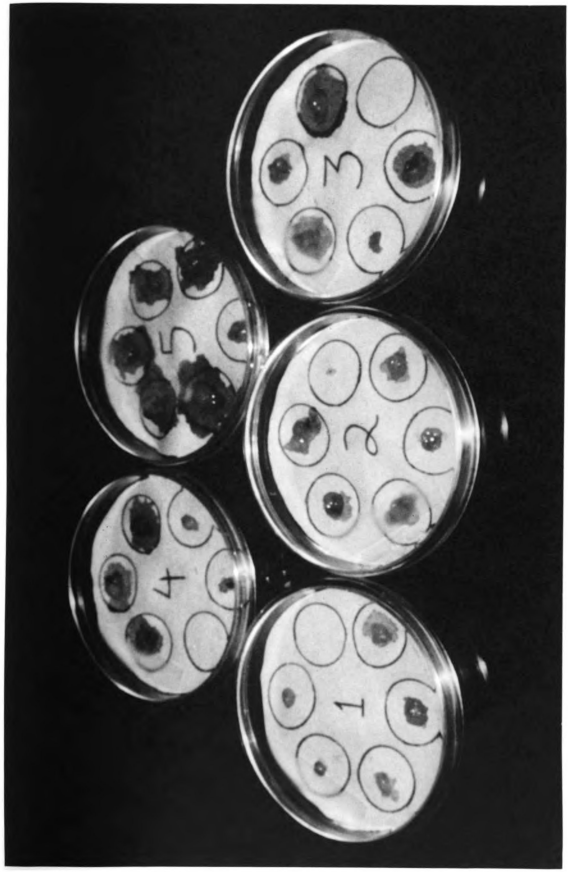


Figure 31

Figure 32. Dose response curve for sarcoma 180 in mice treated with MSF as described in the text.

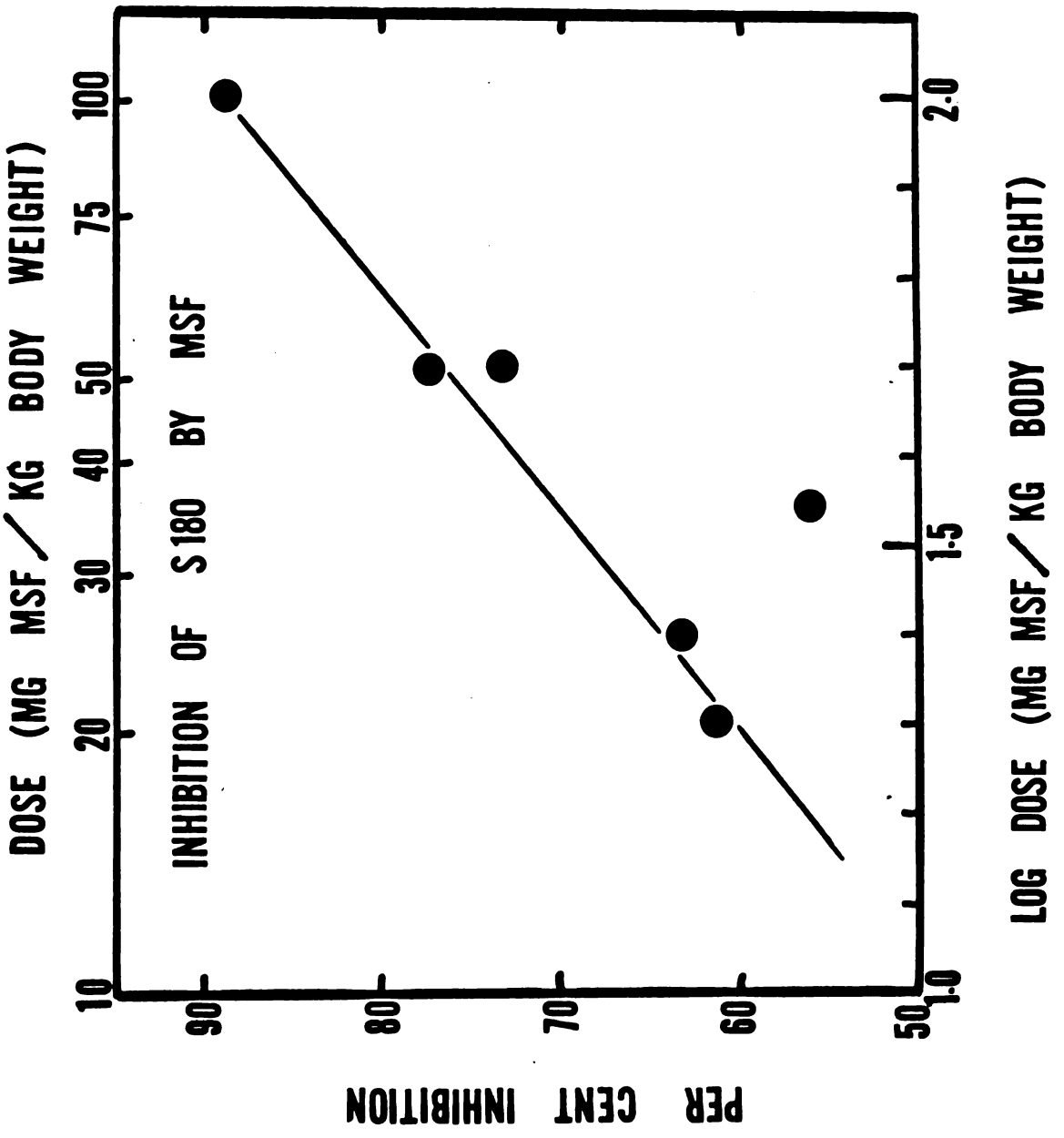

INHIBITION OF S180 BY MSF
PER CENT INHIBITION
DOSE (MG MSF / KG BODY WEIGHT)
LOG DOSE (MG MSF / KG BODY WEIGHT)
10
20
30
40
50
75
100
90
80
70
60
50
1.0
1.5
2.0

Figure 32

inhibition was not proportional to dose, and so this was felt not to be significant inhibition. No toxicity was noted. It should be noted that the metabolic studies described in Section V C2 were performed after the cancer inhibition studies.

The leukemia 1210 system was inhibited only 3-8%, and once again the inhibition was not proportional to the dose. Therefore, the MSF seemed to be ineffective in this test system as well. Although the Walker 256 intramuscular carcinoma was inhibited 10% at 100 mg/kg, the rats lost weight excessively and so the slight therapeutic value was outweighed by its toxicity. At this point, efforts were intensified to purify the inhibitors and assume their stability in the cancer test section (see Section IV B).

After the active inhibitors of lactic dehydrogenase had been isolated from the MSF, it was felt that they merited a test in the above systems, and they were tested against both the sarcoma 180 and leukemia 1210 systems. Vanillin, the most potent natural inhibitor, and cyclotene, another of the original naturally occurring inhibitors, were tested. Cyclotene was completely without effect against sarcoma 180 and gave between 5-15% inhibition of the leukemia 1210. However, the leukemia bearing mice treated with cyclotene lost considerable weight, indicating toxicity, and so the cyclotene was not tested further. Vanillin inhibited the sarcoma 180 and leukemia 1210 systems 1-16% and 10-11% respectively, but neither of these

are considered significant inhibition in these test systems.

D) Why is Vanillin Essentially Ineffective Against Cancer?

1) The LD_{50} and the Rapid Rate of Metabolism of Vanillin in Mice

In an effort to answer this question, it was felt that a knowledge of the rate of metabolism of vanillin in mice would be helpful. Using the techniques outlined in Section V A above, the LD_{50} for vanillin was determined to be 760 mg/kg, and the metabolic rate was estimated at 525 mg/kg/hour. It can easily be seen that since the mice can metabolize almost four times the test dosages each hour, and are dosed only once every 24 hours, vanillin could not be expected to inhibit cancer in such a test. Increasing the dose could not be effective either, since the metabolic rate approaches the LD_{50}, and the toxicity would be expected to outweigh the theraputic value achieved by such a procedure.

2) Metabolism of Vanillin in Mice

a) Introduction

Since it was now known that vanillin is rapidly

metabolized by mice, it was of interest to determine its metabolic products. Bray and coworkers (1952) determined the metabolic products of ortho, meta, and para hydroxybenzaldehyde. For p-hydroxybenzaldehyde, they found 67% excreted as the ether soluble acid, 4% as the ester glucuronide, 16% as the ether glucuronide and 9% as the ethereal sulfate. Since vanillin is quite similar in structure to p-hydroxybenzaldehyde, it was felt these results would probably be quite similar to those found for vanillin.

b) Spectral Analysis of Urinary Vanillin Metabolites

Urine was collected from 12 mice, after which they were each given a 600 mg/kg dose of vanillin. Urine samples were then taken every hour until the color of the urine again appeared the same as the control (four hours for vanillin). Figure 33 shows the pattern of excretion of the metabolites. This pattern remained the same if the urine optical density is measured at 260, 290 or 480 mμ, although the major metabolic product, vanillic acid (see below), absorbs only at 260 and 290 mμ. The metabolism of the mice must be affected in such a way as to cause an excretion of some colored product together with the metabolic products of vanillin. It should be noted that at the third hour, the peak of absorption in the urine had shifted from near 270 mμ to 250 mμ, as if the

Figure 33. Pattern of urinary excretion of the metabolic products of vanillin in the mouse. Spectral analysis of the urine is described in the text.

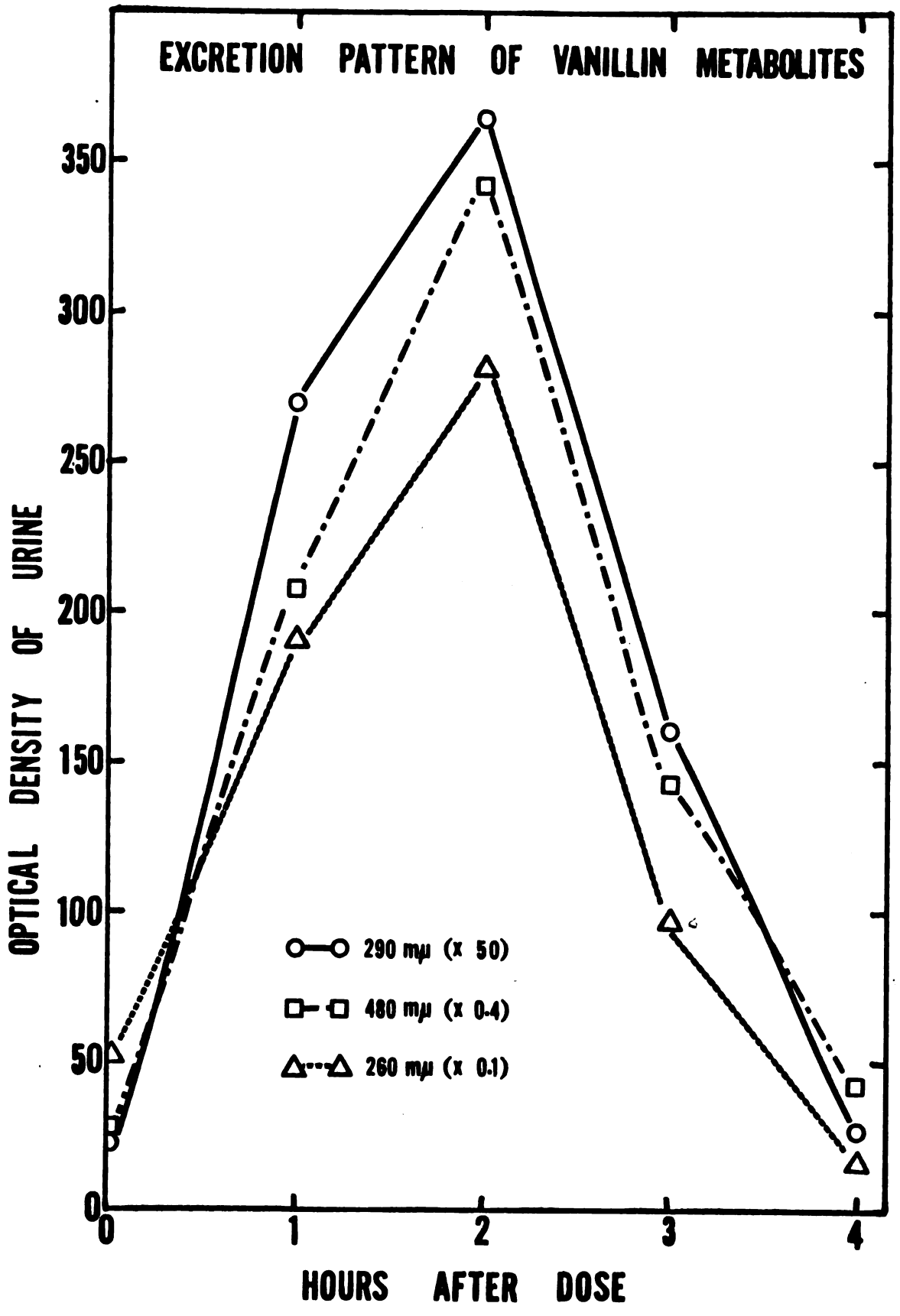

EXCRETION PATTERN OF VANILLIN METABOLITES
OPTICAL DENSITY OF URINE
350
300
250
200
150
100
50
0
0
1
2
3
4
HOURS AFTER DOSE
290 mμ (x 50)
480 mμ (x 0.4)
260 mμ (x 0.1)

Figure 33

product had been altered in some way. The largest increase in the amount of conjugates was noted at this time. The curve observed in Figure 33 should decrease logarithmically, but apparently the products are excreted so quickly that this is not seen. By 4 hours, the optical density has returned to the pre-dose level, and excretion of metabolites is apparently complete.

c) Chromatography of Ether Extracts of Both Hydrolyzed and Nonhydrolyzed Urine from Vanillin Treated Mice

To further characterize the metabolism of vanillin in mice, chromatography was employed. The following scheme was used to determine the products of the metabolism of vanillin in mice. 0.2 ml. of each urine sample were mixed with 0.04 ml. of concentrated HCl and extracted with six consecutive 0.5 ml. portions of ether. This ether extract was concentrated to 100 μL, and 25 μL portions of each urinary extract were subjected to thin layer chromatography on silicic acid in three different solvent systems:

1) n-butanol:acetic acid:water, 8:2:2 (v/v/v), pH 2.80
2) n-butanol:pyridine:dioxane:water, 70:20:5:5 (v/v/v/v), pH 7.50
3) methyl ethyl ketone:2N ammonium hydroxide, 2:1 (v/v, organic phase), pH 11.00

The phenolic compounds were revealed by spraying the air

dried chromatography plates with 5% $FeCl_3$. The extracted, acidified urine was hydrolyzed anaerobically for 12 hours at 110°C, and then re-extracted with another six consecutive 0.5 ml. portions of ether, which were then concentrated and chromatographed as above. Standards of vanillin and vanillic acid were run with each chromatogram.

By far the major product of vanillin metabolism was vanillic acid. Three spots present in the nonhydrolyzed extracts disappeared after hydrolysis, and it was presumed that these were the conjugates as found by Bray and his coworkers (1952) for p-hydroxybenzaldehyde. These conjugates greatly increased in amount at the third hour,,which agrees with the shift seen in the spectrum of the third hour urine. It is interesting to note that although no free vanillin was ever excreted, hydrolysis revealed the release of vanillin as well as vanillic acid from conjugates, indicating that many different conjugates are formed, although only three were detected by chromatography before hydrolysis.

E) Decreasing the Rate of Metabolism of Vanillin by Forming Its Bisulfite Addition Product

1) Introduction

Although vanillin is a potent inhibitor of lactic dehydrogenase, it seems unlikely that it can inhibit cancer

because it is metabolized so rapidly. Since it is apparent that the major site of the metabolism of vanillin is at the aldehyde group, attempts were made to alter this part of the molecule to decrease the metabolic rate. The nitro and nitroso analogues were not further tested because of their decreased inhibitory activity for lactic dehydrogenase. On the other hand, the bisulfite addition product of vanillin both altered the aldehyde functional group and increased the activity. It was therefore of interest to determine the metabolism of this compound.

2) The LD_{50} and Rate of Metabolism of the Bisulfite Adduct in Mice

The LD_{50} for the bisulfite addition product of vanillin was determined to be 1850 mg/kg, and the metabolic rate to be 310 mg/kg/hour. In addition to an increased LD_{50} as compared to vanillin, it has a considerably slower metabolic rate, as would be predicted from the knowledge that it is at the aldehyde group where metabolism occurs, as shown above. Since the bisulfite adduct has no aldehyde, its metabolism would be necessarily slower.

3) Metabolism of the Adduct by Mice

Urinary metabolites of the adduct were analyzed in the same way as described for vanillin. Doses of 400 and

1400 mg/kg were used. Figure 34 depicts the pattern of excretion following these doses. The delayed 400 mg/kg dose shows the effect of 400 mg/kg for five days prior to the collection of the urine. A drastic change in the pattern of metabolism is seen. Whereas the initial doses both yield the predicted exponential decrease in urinary output of metabolites, the delayed dose shows a slight rise at one hour, followed by a later slow rice (see Section V E 4). It should be noted that the initial 400 mg/kg dose peaks one hour earlier than the initial 1400 mg/kg dose. This can be most easily explained by noting that at 1400 mg/kg, the mice are unconscious for about one hour, and appear to be completely concerned with survival, exhibiting greatly increased respiration and pulse, with complete loss of coordination and control of bodily functions.

The metabolites are the same as vanillin, even to the shift noted from the acid to the conjugates after three hours, although the shift occured at four hours at the 1400 mg/kg dose, probably because of the reasons outlined above.

Since the curves in Figure 34 both return to the base line by 10 hours, it was assumed that the entire dose had been eliminated by then. However, a second 1400 mg/kg dose 24 hours later gave almost 60% mortality. Apparently, some small fraction of the dose is stored, and a second high dose raises the stored amount to a lethal level. However, 400 mg/kg can be given for up to two weeks with no

Figure 34. Pattern of urinary excretion of the metabolic products of the bisulfite addition product of vanillin in the mouse. Spectral analysis of the urine is described in the text. The delayed 400 mg/kg dose curve represents the pattern of excretion following the sixth daily dose of 400 mg/kg, demonstrating a drastic change from the first dose of 400 mg/kg.

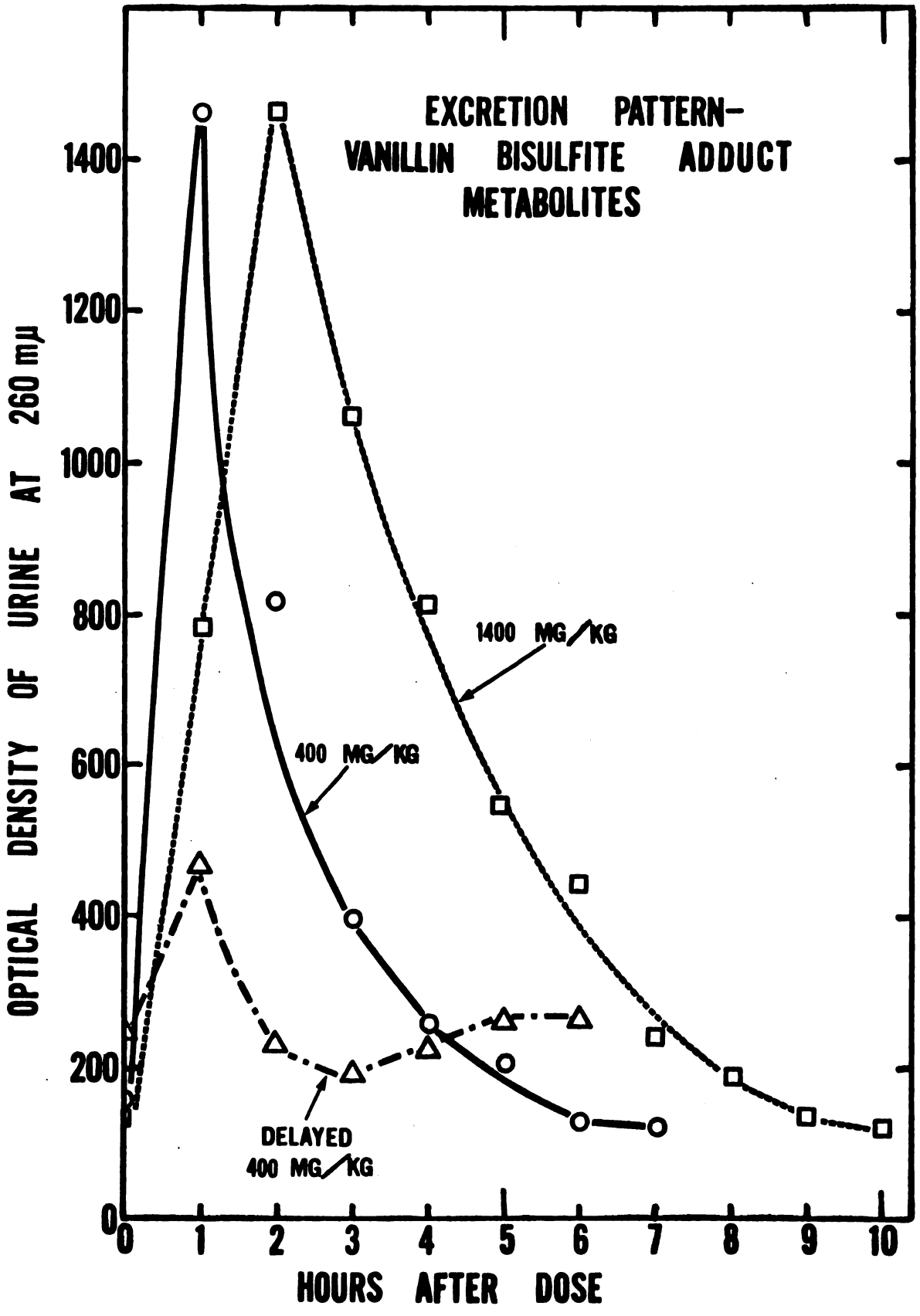

EXCRETION PATTERN-
VANILLIN BISULFITE ADDUCT
METABOLITES
OPTICAL DENSITY OF URINE AT 260 mμ
HOURS AFTER DOSE
1400 MG/KG
400 MG/KG
DELAYED
400 MG/KG
0
200
400
600
800
1000
1200
1400
0
1
2
3
4
5
6
7
8
9
10

Figure 34

mortality, so even the small stored amount must be slowly metabolized.

4) Induction of the Drug Metabolizing Enzymes

Since the pattern of metabolism seen for an initial dose of 400 mg/kg and that seen after five days of this dose is so different, it seemed likely that the change was due to induction of some enzymes responsible for the metabolism of the adduct. To test this conclusion, a group of mice were given 400 mg/kg once a day for 12 days, and then were given no dose for two days to be sure all remaining adduct had been excreted. The metabolic rate of the adduct was then re-determined for this group, and it was found to be 660 mg/kg/hour, more than double the value obtained with mice which have never been exposed to such compounds. It was therefore concluded that these compounds induce enzymes responsible for their own metabolism. This is obviously an undesirable characteristic for a compound which is to be used for long term treatment of cancer.

5) Lack of Effectiveness of the Vanillin Bisulfite Adduct in the Treatment of Cancer

Tested in sarcoma 180, leukemia 1210, and Walker 256 intramuscular carcinomas, the vanillin bisulfite adduct exhibited the same disappointing lack of inhibition described

above for the other tested compounds. The rate of metabolism may still be too high to allow response at the tested dosages (100, 200 and 300 mg/kg/day).

Discussion

Testing Inhibition of Cancer by Lactic Dehydrogenase Inhibition

In the course of studies with the Krebs cycle metabolites, a potent inhibitor of lactic dehydrogenase was observed. This inhibitor was isolated and purified not only to characterize its inhibition of the enzyme, but also to test the hypothesis that compounds which inhibit lactate production should inhibit cancer. The three elements of this theory are as follows:

1) Warburg (1924) found that cancer cells have an abnormally high production of lactic acid, even under aerobic conditions.
2) Warburg (1956) showed that of all tissues tested, only cancer cells demonstrated a high production of lactate under aerobic conditions (so-called aerobic glycolysis).
3) Boxer and Devlin (1961) showed that the high rate of aerobic glycolysis in tumor cells is caused by the absence in such cells of α-glycerolphosphate dehydrogenase.

If conditions could be found under which the glycolysis of tumors was specifically inhibited, the growth of tumors might be specifically inhibited without interfering with the

growth of cells which depend solely on oxidative metabolism (Papaconstantinou and Colowick, 1961a). Such specific inhibition of glycolysis can only be accomplished by inhibition of lactic dehydrogenase, as discussed by the above authors. Any inhibitors to be tested for carcinostatic activity should have the following characteristics maximized:

1) Potency as a lactic dehydrogenase inhibitor.
2) Lack of toxicity *in vivo*.
3) A slow rate of metabolism, to allow sufficient time for the compound to affect the tumor before metabolism removes it.

Previous workers have tested many compounds for carcinostatic activity (Novoa, *et al.*, 1959; Papaconstantinou and Colowick, 1961a; Goldberg and Colowick, 1965; Busch and Nair, 1957; Davis and Busch, 1958; Ottolenghi and Denstedt, 1958; Fiume, 1960). These compounds have all failed to exhibit significant carcinostatic activity, since they were generally rather inactive lactic dehydrogenase inhibitors (Busch and Nair, 1957), and exhibited high toxicity in animals. Metabolic rates were, in general, not measured.

The substituted phenolic inhibitors of lactic dehydrogenase described in this thesis are quite potent as enzymatic inhibitors, having K_i's as low as 14 μM. Two were also shown to have quite low toxicities (LD_{50}'s of 760 and 1850 mg/kg) compared to those previously tested. However, the metabolic rates for these two compounds, vanillin and its bisulfite

adduct, were 525 and 310 mg/kg/hour. It should be noted that the adduct has excellent characteristics in two of the three categories:

1) It is the most potent lactic dehydrogenase inhibitor reported to date (except for the peptide inhibitors of Wacker and Schoenberger, 1966a, 1966b).
2) It has the lowest toxicity reported to date of any lactic dehydrogenase inhibitor.

It fails to meet the third criterion, however, since its rate of metabolism is so high. As might be expected from this high metabolic rate, the compounds are without reproducible effect on cancer, thus making impossible direct confirmation of the above theory using these substituted phenolic inhibitors of lactic dehydrogenase.

Mechanism of the Inhibition of Lactic Dehydrogenase

During the course of this investigation, four inhibitors of lactic dehydrogenase were found to be present in a methylene chloride extract of maple syrup, namely vanillin, syringaldehyde, cyclotene and p-hydroxybenzaldehyde. It was shown that structural similarity to either lactate or pyruvate was not the common denominator of these active species, since many other structural analogues did not function as inhibitors. Each of the active inhibitors was found to have a pK of 7.9 or below, while the inactive

compounds all titrated above pH 9.6. It was therefore concluded that in order to function as lactic dehydrogenase inhibitors, these compounds were required to function as anions. This conclusion agrees well with that of Winer and Schwert (1959), who concluded that "The information which is available on inhibitors for the lactic dehydrogenase system indicates that only anionic substances function as inhibitors."

The two most active naturally occuring inhibitors were vanillin and p-hydroxybenzaldehyde, which differ in structure only in the substituent on the 3 position of the aldehyde ring. In an effort to obtain a greater inhibition, as well as to define the mode of action of these inhibitors, analogues of these two compounds were tested, differing in structure only in the substituent on position 3, as follows (the K_i's are in μM in parenthesis): p-hydroxybenzaldehyde (414), protocatachualdehyde (174), "methyl" vanillin (270), "ethyl" vanillin (241), "methoxy" vanillin (= vanillin) (96), "ethoxy" vanillin (108), and the bisulfite adduct of vanillin (14). These compounds were found to be completely reversible inhibitors of lactic dehydrogenase.

The kinetic properties of this series of inhibitors were examined. They were found to be competitive with respect to pyruvate and non-competitive with respect to DPNH. From purely steric considerations, the K_i's would be expected to be proportional to the size of the substitu-

ent groups, but this was found not to be the case. This suggested that the differences in the K_i's must be due to the electron withdrawing or donating effects of the various substituents. Ottolenghi and Denstedt (1958) found that most substrate analogue inhibitors of lactic dehydrogenase contain both an anionic moiety and an alpha electronegative group, which could correspond to the 3 position of the aldehyde ring in the series of inhibitors under investigation here. It was found that the K_i's of this series was proportional to the relative negative charge on the atom directly attached to the 3 position of the ring, which supports the results of Ottolenghi and Denstedt (1958). From pH 7.00 to pH 7.40, the amount of vanillin in the ionized form increases from 20 to 40%, and the observed inhibition of lactic dehydrogenase similarly increases two-fold, suggesting that the anionic form of the molecule is the active inhibitory species. Since further raising the pH from 7.40 to 7.80 does not increase the inhibition, it was concluded that the functional groups on the enzyme surface responsible for the binding of the inhibitors, are titrated in this pH range. Nygaard (1956) and Winer and Schwert (1959) postulated these groups to be imidazole and lysine, of which it is more likely the imidazole which is titrated between pH 7.40 and 7.80, although local electrostatic interactions could lower the pK of lysine sufficiently for it to titrate in this pH range.

Since the aldehyde moiety of the inhibitors may

function merely to lower the pK of the phenolic hydroxyl group, the nitro and nitroso derivatives of syringaldehyde, "methyl" vanillin and p-hydroxybenzaldehyde were tested, since they should have a lower pK than their aldehyde-containing analogues. Since they were not as active, it was concluded that the aldehyde group functions in some way to increase the potency of the inhibitors, in addition to withdrawing electrons from the ring to lower the pK of the phenolic hydroxyl group.

Vanillin, chosen as a representative inhibitor of the series, exhibited greater inhibition of the H_4 isozyme, although there was less than a two-fold difference between the inhibition of the two lactic dehydrogenase isozymes.

During the course of this investigation, it was found that samples of lactic dehydrogenase stored at low concentrations (about 5.6 μg/ml.), showed a rapid loss of activity to approximately 40% of the activity of a control sample stored at higher concentration. This observation may support those of Hathaway and Criddle (1966) and Millar (1962) demonstrating a molecular change for the enzyme from tetramers to presumably inactive dimers at low concentrations.

Summary

1) The bisulfite adduct of vanillin was both the most potent (K_i = 14 μM) low molecular weight lactic dehydrogenase inhibitor yet reported (the specific polypeptide inhibitors are more potent) and the least toxic (LD_{50} = 1850 mg/kg).

2) The rate of metabolism in mice of vanillin and of the bisulfite adduct of vanillin was very great (525 and 310 mg/kg/hour). As result, at the 300 mg/kg level used for testing inhibition of cancer, the drug is reduced to base line levels about 3 hours after administration, based on metabolic products in the urine as determined both spectrophotometrically and using thin layer chromatography.

3) For this reason, they could not be expected to and did not show any significant reproducible carcinostatic activity against Walker 256 intramuscular, leukemia 1210 or sarcoma 180 carcinomas in tests performed by CCNSC.

4) However, 3 series of tests on the MSF in our laboratory gave 75-80% inhibition of sarcoma 180 growth, although tests on MSF by CCNSC did not show significant inhibition. By any standards of scientific objectivity, the testing performed in this laboratory was reproducible. Previous workers have also had considerable difficulty in

achieving reproducible results in this field. In this case, the explanation may lie in the use of different sarcoma 180 tumor lines in this laboratory and at CCNSC. This conclusion is impossible to test, since the tumor line used for testing in this laboratory is no longer available.

5) The inhibition of lactic dehydrogenase by a commercial malic acid preparation was traced to the presence of an impurity.

6) Four compounds, vanillin, p-hydroxybenzaldehyde, cyclotene and syringaldehyde, were isolated from maple syrup, the commercial source of malic acid, and found to be active as lactic dehydrogenase inhibitors.

7) In an effort to obtain a greater inhibition than that achieved with the naturally occuring inhibitors, the following series of seven analogues was tested (the K_i's, in μM, are given in parenthesis): p-hydroxybenzaldehyde (414), protocatachualdehyde (174), "methyl" vanillin (270), "ethyl" vanillin (241), "methoxy" vanillin (= vanillin) (96), "ethoxy" vanillin (108) and the bisulfite adduct of vanillin (14).

8) The kinetic properties of this series of inhibitors was examined:

a) They were reversible inhibitors of lactic dehydrogenase.

b) They were competitive with pyruvate and non-competitive with DPNH.

c) The K_i <u>was not</u> proportional to the length of

the substituents on the ring, as would be expected from purely steric considerations.

d) The K_i was found to be a function of the relative negative charge on the 3 position of the aldehyde ring.

e) The inhibition increased when the assay pH was raised from 7.00 to 7.40, due to an increased concentration of the anionic form of the inhibitors, presumed to be the active form.

f) Vanillin, chosen as a representative inhibitor, inhibited the H_4 isozyme of lactic dehydrogenase approximately twice as much as the M_4 isozyme.

9) Nitro or nitroso groups could not fully substitute for the aldehyde moiety of the inhibitors. It was therefore concluded that the aldehyde group, although not indispensible (since cyclotene and the most potent inhibitor, the bisulfite addition product of vanillin, lack any aldehyde group), apparently functions to increase the activity of the inhibitors, in addition to withdrawing electrons from the ring to lower the pK of the phenolic hydroxyl group.

10) It was found that lactic dehydrogenase shows a marked loss of activity to about 40% of the control when left standing at low concentrations (about 5.6 μg/ml.), but is quite stable for up to 48 hours at higher concentrations (about 56 μg/ml.).

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APPENDIX

The research described in the body of this thesis was performed in the laboratory of Dr. W. C. Deal, whose major interests lie in the field of physical biochemistry, specifically the structure of the glycolytic enzymes. Therefore, much work in this laboratory utilizes the Spinco analytical ultracentrifuge, both for determining molecular weights by equilibrium centrifugation techniques and for determining sedimentation coefficients, both for native molecules and for dissociated subunits. Both molecular weight and sedimentation coefficient are generally found to be dependent upon concentration, so that to achieve accuracy, the values must be determined at several protein concentrations and then extrapolated to zero concentration. It is immediately apparent that the accuracy of the extrapolation is of vital importance in determining the correct value at zero concentration. It was for this reason that the following computer program was developed.

The program is written in Fortran 63, an advanced form of the basic Fortran, and is presented as Figure 35. The first six comment cards describe the required form of the imput data. The intercept must have the highest Y value and the Nth, or last, point must have the greatest X and least Y value. In other words, the line must have a negative slope. The program was designed this way to handle

Figure 35. The computer program as it is described in the body of the appendix, written in Fortran 63. The following pages are a continuation of the program and therefore have no legends.

```
      PROGRAM LESTCHI
C     TERCEPT MUST HAVE HIGHEST Y + NTH POINT MUST HAVE GREATEST X, LEAST Y
C     THE RUN NUMBER MUST ONLY CONTAIN THE DIGITS 0-9, NO LETTERS AT ALL
C     1ST DATA CARD HAS THE TOTAL N IN COLUMNS 1+2, RUN NUMBER IN COLUMNS 3-8
C     ALSO 9-15 HAS CENTRI. RUN NO., 16-23 DATE OF RUN, 24-31 DATE OF
C     3600 RUN, 32 HAS 1 OR 2, 1 FOR SED. COEFF., 2 FOR M.W.
C     THE OTHER DATA CARDS HAVE X IN 1-4, Y IN 5-10(NO DECIMAL POINTS)
C     Y AXIS BEGINS AT 7000 BELOW Y OF FINAL POINT, ENDS AT 12000 ABOVE Y(1)
C     X AXIS BEGINS AT ZERO, ENDS AT X OF FINAL POINT
C     EXCLUSION LIMITS 3,7,11,15 ARE LINES AT 12,3,6,9 O CLOCK RESPECTIVELY
      DIMENSION X(30),Y(30),NUM(10),YC(30),XC(30),ZUM(10),V(30),NNUM(5),
     1 YD(30), CRITVAL(30),YINCHS(30),SUBSCRPT(30)
      COMMON X,Y
      TYPE REAL MOVE
      TYPE INTEGER SUBSCRPT
      CALL FAULT(1)
      CALL PLOT(200,0,3)
  101 READ 1,N,(NUM(KK),KK=1,6),NULTRA,NDU,NDC,IC,(X(J),Y(J),J=1,N)
      IF(EOF,60) 999,998
    1 FORMAT(I2,6I1,A7,A8,A8,I1/(F4.2,F6.0))
  998 BLIP = -.05
      IN = 0
      NNN = X(N)
      DIV = 9.0/X(N)
      POSITION = 1.0
      CALL PLOT(0,0,0,100.,10C.)
      CALL PLOT(0,-11.0,2)
      CALL PLOT(0,0,0)
      CALL PLOT(0,POSITION,2)
      MOVE = POSITION
      DO 370 J1 = 1,NNN
      MOVE = MOVE + DIV
      CALL PLOT(0,MOVE,1)
      CALL PLOT(BLIP)
  370 CALL PLOT(0)
      CALL PLOT(0,POSITION,2)
      NNN = (19000.0 + Y(1) - Y(N))/1000.0
      DIV = 10.0/FLOATF(NNN)
      MOVE = 0.0
      BLIP = POSITION - 0.05
      DO 371 J2 = 1,NNN
      MOVE = MOVE + DIV
      CALL PLOT(MOVE,POSITION,1)
      CALL PLOT(MOVE,BLIP)
  371 CALL PLOT(MOVE,1.0)
      SLY = (1000.)/((Y(1)) - (Y(N)) + 19000.)
      SLX = (900.)/(X(N))
      P = 4./SLY
      QQ = 4./SLX
      CALL PLOT(0,POSITION,2)
      CALL PLOT(0,0,0,SLY,SLX)
      DO 500 J =1,N
      V(J) = Y(J) - Y(N) + 7000.
      YINCHS(J) = (V(J) - P)/(100./SLY)
```

```
      CALL PLOT((V(J) + P),X(J),2,SLY,SLX)
      CALL PLOT((V(J) + P),(X(J) + QQ),1,SLY,SLX)
      CALL PLOT((V(J) - P),(X(J) + QQ),1,SLY,SLX)
      CALL PLOT((V(J) - P),(X(J) - QQ),1,SLY,SLX)
      CALL PLOT((V(J) + P),(X(J) - QQ),1,SLY,SLX)
      CALL PLOT((V(J) + P),X(J),1,SLY,SLX)
  500 CONTINUE
      PRINT 400, (NUM(KK),KK=1,6),NULTRA,NDU,NDC,(NUM(KK),KK=1,6)
  400 FORMAT(1H1,* L. C. RUN *,6I1,*  ULTRACENTRIFUGE RUN *,A7,* ON *,A8
     1*     3600 RUN ON *,A8,5X,*PROGRAM DATED 2/24/67*,14X,*RUN *,6I1)
      GO TO(1007,1009),IC
 1007 PRINT 1008
 1008 FORMAT(1H-,* SEDIMENTATION COEFFICIENT VS. CONCENTRATION*,//)
      GO TO 1011
 1009 PRINT 1010
 1010 FORMAT(1H-,*  MOLECULAR WEIGHT VS. CONCENTRATION*,//)
 1011 PRINT 1000
 1000 FORMAT(1H-,15X,*RAW DATA*,/)
      PRINT 1001
 1001 FORMAT(1H ,9X,*POINT*,5X,*X*,10X,*Y*)
      DO 1002 J=1,N
 1002 PRINT 1003,J,X(J),Y(J)
 1003 FORMAT(1H ,10X,I2,5X,F5.2,5X,F6.0)
      PRINT 1004
 1004 FORMAT(1H-,11X,*CALCULATIONS*,//)
      IME = 0
      C = Y(N)
      B = X(N)
      DO 944 J = 1,N
  944 SUBSCRPT(J) = J
  305 SUMXY = 0.0
      DO 2 I=1,N
    2 SUMXY = SUMXY +X(I)*Y(I)
      SUMX = 0.0
      DO 3 K=1,N
    3 SUMX = SUMX + X(K)
      SUMY = 0.0
      DO 4 L=1,N
    4 SUMY = SUMY + Y(L)
      Q = N
      TOPSL = Q*SUMXY-SUMX*SUMY
      SUMX2 = 0.0
      DO 5 II=1,N
    5 SUMX2 = SUMX2 + X(II)**2.
      SUMX22 = (SUMX)**2.
      DENOM = Q*SUMX2 - SUMX22
      IF(ABSF(DENOM)-.0001) 9,9,100
  100 CONTINUE
      TOPCEPT = SUMY*SUMX2 - SUMX*SUMXY
      SLOPE = TOPSL/DENOM
      TERCEPT = TOPCEPT/DENOM
      IME = IME + 1
      PRINT 8, IME,SLOPE,TERCEPT
    8 FORMAT(1H ,5X,I2,2X,8HSLOPE = ,F12.2,2X,13H INTERCEPT = ,F11.2)
```

```
      JJJ = 15
  300 DO 301 J=1,N
      A = JJJ/100.
      YC(J) = (SLOPE)*(X(J)) + (TERCEPT)
      YD(J) = Y(J) - YC(J)
      CRITVAL(J) = ABSF(YD(J)) - A*(YC(J))
      IF(CRITVAL(J) - .00001) 302,302,303
  303 V(J) = Y(J) - C + 7000.
      GO TO(800,800,803,800,800,800,807,800,800,800,811,800,800,800,815)
     1, JJJ
  803 CALL PLOT(V(J),X(J),2,SLY,SLX)
      CALL PLOT((V(J) + 3.*P),X(J),1,SLY,SLX)
      GO TO 800
  807 CALL PLOT(V(J),X(J),2,SLY,SLX)
      CALL PLOT(V(J),(X(J) + 3.*QQ),1,SLY,SLX)
      GO TO 800
  811 CALL PLOT(V(J),X(J),2,SLY,SLX)
      CALL PLOT((V(J) - 3.*P),X(J),1,SLY,SLX)
      GO TO 800
  815 CALL PLOT(V(J),X(J),2,SLY,SLX)
      CALL PLOT(V(J),(X(J) - 3.*QQ),1,SLY,SLX)
  800 KLM = J
      IF(J-N) 801,802,802
  801 N = N - 1
      DO 850 LL =KLM,N
      X(LL) = X(LL+1)
      Y(LL) = Y(LL+1)
  850 CONTINUE
      GO TO 804
  802 N = N-1
  804 CONTINUE
      PRINT 304,SUBSCRPT(J),JJJ
  304 FORMAT(1H ,9X,*POINT *,I2,* EXCLUDED WITH LIMIT = *,I3,* PER CENT*
     1,/)
      IF(J-(N+1))805,305,305
  805 DO 969 LL = KLM,N
  969 SUBSCRPT(LL) = SUBSCRPT(LL+1)
      GO TO 305
  302 CONTINUE
  301 CONTINUE
      JJJ = JJJ - 4
      IF(JJJ-1) 306,306,300
  306 PRINT 307
  307 FORMAT(1H-,40HALL POINTS NOW GOOD TO WITHIN 3 PER CENT,5(/))
      PLTCEPT = TERCEPT - C + 7000.
      CALL PLOT(PLTCEPT,.0,2,SLY,SLX)
      YCAL = SLOPE*B + TERCEPT
      YPLTCAL = YCAL - C + 7000.
      CALL PLOT((YPLTCAL),B,1,SLY,SLX)
      CALL PLOT(.0,.0,2,SLY,SLX)
      CALL PLOT(0,-1.0,0,.02,45.)
      CALL PLOT(-2500.,1.,2,.02,45.)
      CALL PLOT(-3750.,1.,1,.02,45.)
      CALL PLOT(-3750.,.8,1,.02,45.)
```

```
      CALL PLOT(-3750.,1.5,1,.02,45.)
      CALL PLOT(-2500.,1.5,1,.02,45.)
      CALL PLOT(-2500.,.8,1,.02,45.)
      CALL PLOT(-2500.,2.,2,.02,45.)
      CALL PLOT(-2500.,2.5,1,.02,45.)
      CALL PLOT(-2500.,2.,2,.02,45.)
      CALL PLOT(-3750.,2.,1,.02,45.)
      CALL PLOT(-3750.,2.5,1,.02,45.)
      CALL PLOT(-3125.,2.,2,.02,45.)
      CALL PLOT(-3125.,2.3,1,.02,45.)
      CALL PLOT(-2500.,3.25,2,.02,45.)
      CALL PLOT(-3750.,3.,1,.02,45.)
      CALL PLOT(-2500.,3.25,2,.02,45.)
      CALL PLOT(-3750.,3.5,1,.02,45.)
      CALL PLOT(-3125.,3.375,2,.02,45.)
      CALL PLOT(-3125.,3.125,1,.02,45.)
      CALL PLOT(-2500.,4.,2,.02,45.)
      CALL PLOT(-3750.,4.,1,.02,45.)
      CALL PLOT(-3750.,4.5,1,.02,45.)
      CALL PLOT(0,-1.0,2)
      CALL PLOT(0,-3.0,0,.02,45.)
      CALL PLOT(-3750.,6.0,2)
      CALL PLOT(-2500.,6.0,1)
      CALL PLOT(-2500.,6.5)
      CALL PLOT(-3125.,6.5)
      CALL PLOT(-3125.,6.0)
      CALL PLOT(-3125.,6.2)
      CALL PLOT(-3750.,6.5)
      CALL PLOT(-2500.,7.0,2)
      CALL PLOT(-3750.,7.0,1)
      CALL PLOT(-3750.,7.5)
      CALL PLOT(-2500.)
      CALL PLOT(-3750.,8.0,2)
      CALL PLOT(-2500.,8.0,1)
      CALL PLOT(-3750.,8.5)
      CALL PLOT(-2500.)
      CALL PLOT(-2500.,9.2,2)
      CALL PLOT(-3750.,9.2,1)
      CALL PLOT(-2500.,9.5,2)
      CALL PLOT(-3750.,9.5,1)
      CALL PLOT(-2900.,9.0,2)
      CALL PLOT(-2900.,9.7,1)
      CALL PLOT(-3250.,9.0,2)
      CALL PLOT(-3250.,9.7,1)
      CALL PLOT(0,-3.0,2)
      CALL PLOT(0,-4.3,0,.02,45.)
  599 KK = 0
 1020 KK = KK + 1
      IF(NUM(KK)-1)1020,1111,1111
 1111 KK=KK-1
  501 KK = KK + 1
      ZZ = KK
      ZUM(KK) = NUM(KK)
      IF((ZUM(KK)) - 0.1) 502,502,503
```

```
503 LLL = NUM(KK)
    GO TO(601,602,603,604,605,606,607,608,609), LLL
502 CALL PLOT(-2500.,(10.+ZZ-1.),2,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
601 CALL PLOT(-2500.,(10.+ZZ-1.),2,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
602 CALL PLOT(-2500.,(10.+ZZ-1.),2,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.),2,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
603 CALL PLOT(-2500.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
604 CALL PLOT(-2500.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.2),2,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.2),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
605 CALL PLOT(-2500.,(10.+ZZ-1.),2,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
606 CALL PLOT(-2500.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3125.,(10.+ZZ-1.5),1,.02,45.)
    CALL PLOT(-2500.,(10. + ZZ -1.5),2,.02,45.)
    CALL PLOT(-2500.,(10. + ZZ -1.),1,.02,45.)
    CALL PLOT(-2750.,(10. + ZZ - 1.),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
607 CALL PLOT(-2500.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.),1,.02,45.)
    CALL PLOT(-3750.,(10.+ZZ-1.2),1,.02,45.)
    IF(ZZ- 5.8) 501,501,610
608 CALL PLOT(-2500.,(10.+ZZ-1.5),2,.02,45.)
    CALL PLOT(-2500.,(10.+ZZ-1.),1,.02,45.)
```

```
            CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
            CALL PLOT(-3750.,(10.+ZZ-1.5),1,.02,45.)
            CALL PLOT(-2500.,(10.+ZZ-1.5),1,.02,45.)
            CALL PLOT(-3125.,(10.+ZZ-1.5),2,.02,45.)
            CALL PLOT(-3125.,(10.+ZZ-1.),1,.02,45.)
            IF(ZZ- 5.8) 501,501,610
        609 CALL PLOT(-3125.,(10.+ZZ-1.),2,.02,45.)
            CALL PLOT(-3125.,(10.+ZZ-1.5),1,.02,45.)
            CALL PLOT(-2500.,(10.+ZZ-1.5),1,.02,45.)
            CALL PLOT(-2500.,(10.+ZZ-1.),1,.02,45.)
            CALL PLOT(-3750.,(10.+ZZ-1.),1,.02,45.)
            CALL PLOT(-3750.,(10. + ZZ - 1.),2,.02,45.)
            CALL PLOT(-3750.,(10. + ZZ - 1.5),1,.02,45.)
            CALL PLOT(-3500.,(10. + ZZ - 1.5),1,.02,45.)
            IF(ZZ- 5.8) 501,501,610
        610 IN = IN + 1
            IF(IN-1.5) 593,593,550
        593 CALL PLOT(0,-4.3,2,.02,45.)
            CALL PLOT(.0,.0,0,SLY,SLX)
            CALL PLOT(PLTCEPT,60./SLX,2,SLY,SLX)
            CALL PLOT(-3000.,9.0,0,.02,45.)
            ENCODE(8,898,XXX) TERCEPT
        898 FORMAT(F6.0)
            DECODE(8,899,XXX) (NUM(KK), KK=1,6)
        899 FORMAT(6I1)
            GO TO 599
        550 CALL PLOT(-3000.,9.0,2,.02,45.)
            CALL PLOT(0.0,9.0,0,.02,45.)
            CALL PLOT(0.0,8.9,2,.02,45.)
            CALL PLOT(0.0,8.0,1,.02,45.)
            CALL PLOT(200.0,8.15,1,.02,45.)
            CALL PLOT(0.0,8.0,2,.02,45.)
            CALL PLOT(-200.0,8.15,1,.02,45.)
            CALL PLOT(35000.0,0.0,2,.02,45.)
            GO TO 200
          9 PRINT 10
         10 FORMAT(1H ,10X,38HTHE DENOMINATOR EQUALS ZERO-CHECK DATA, 5(/))
        200 CONTINUE
            GO TO 101
        999 PRINT 1006
       1006 FORMAT(1H1)
            CALL PLOT(35000.0,0.0,-1,.02,45.)
            END
LOAD
RUN,2,5000,0,D
```

the vast majority of cases in which this is found. The first data card has the total number of points in columns 1 and 2, the run number in columns 3-8, containing only the digits 0-9 with no letters, the ultracentrifuge run number in columns 9-15, the date of the last ultracentrifuge run in columns 16-23, and the date of the computer run in columns 24-31. Column 32 has either a 1 or 2 punch, depending on which sort of data is involved, sedimentation coefficient or molecular weight, respectively. Each of the other data cards contain the X value (concentration) in columns 1-4, and the Y value (sedimentation coefficient or molecular weight) in columns 5-10, with the decimal points being excluded. Decimal points are automatically placed by the computer after columns 2 and 10, so data sould be entered accordingly.

A flow chart tracing the path of calculations and other operations described by the program is presented in Figure 36. The symbols are standard for computer flow charts, as follows:

1) A rectangular box designates any mathematical operation.
2) A rectangular box with convex vertical sides designates either an input or output operation, such as READ or PRINT.
3) A diamond shaped box designates an arithmetic operation involving a decision, such as an IF or computed GO TO statement. In this case, the

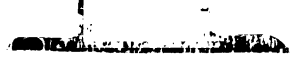
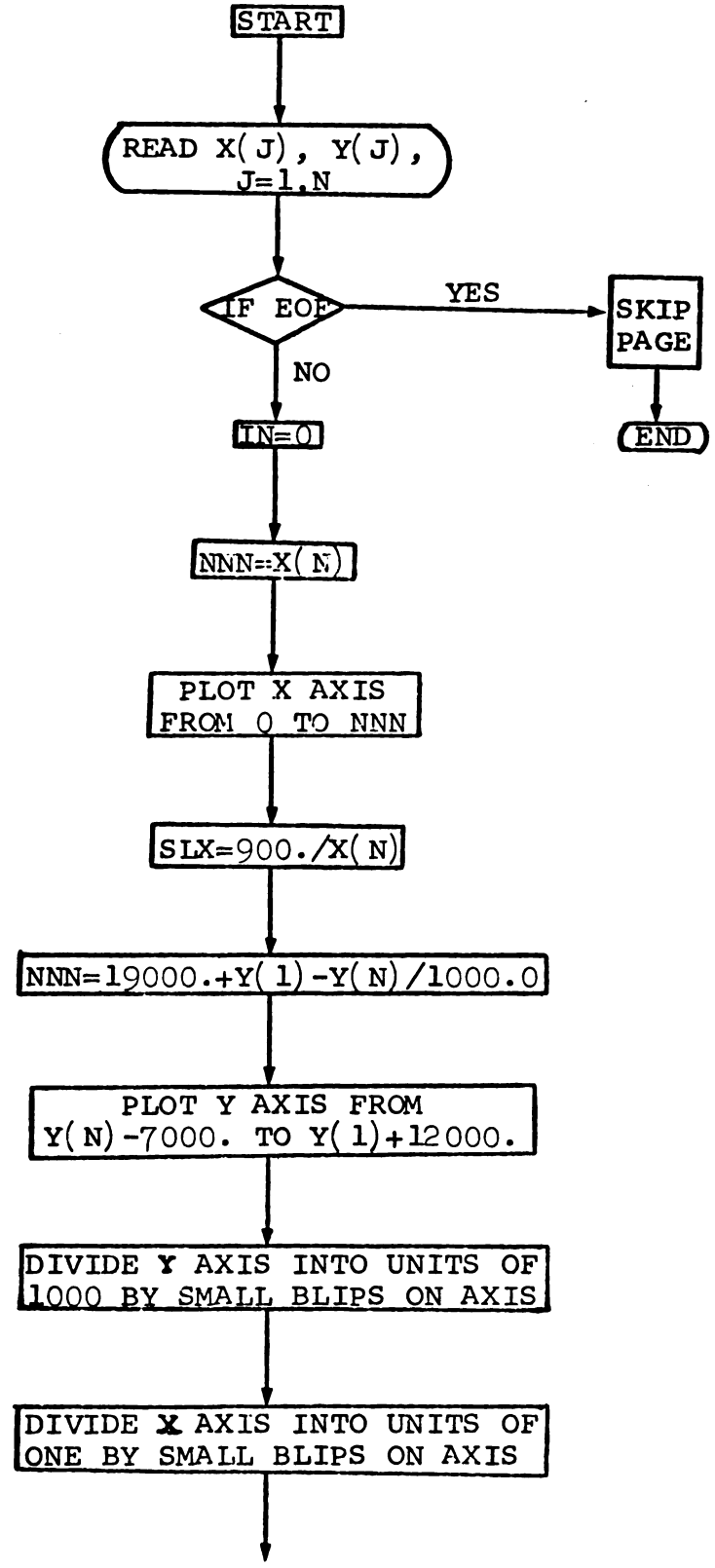
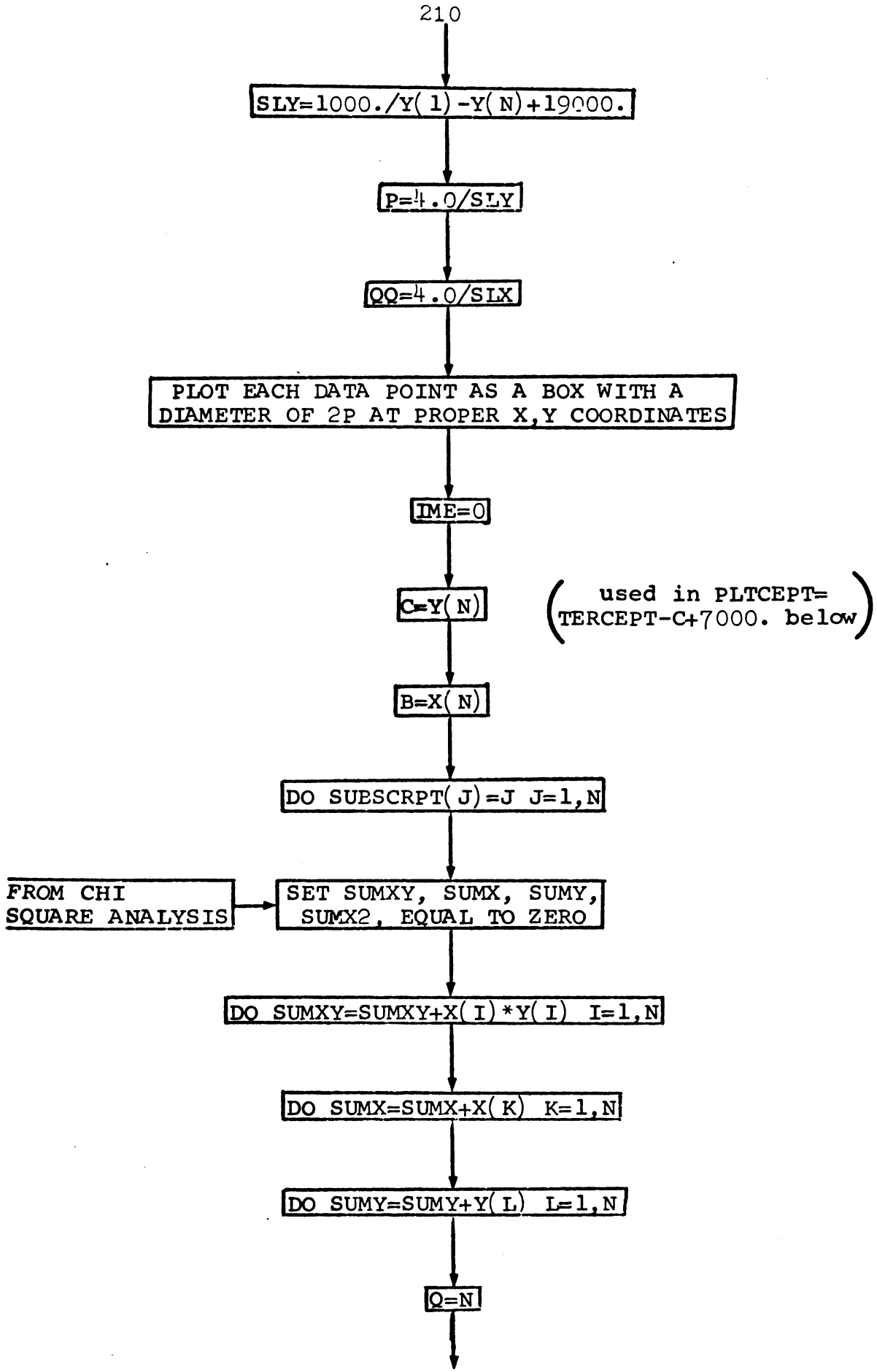
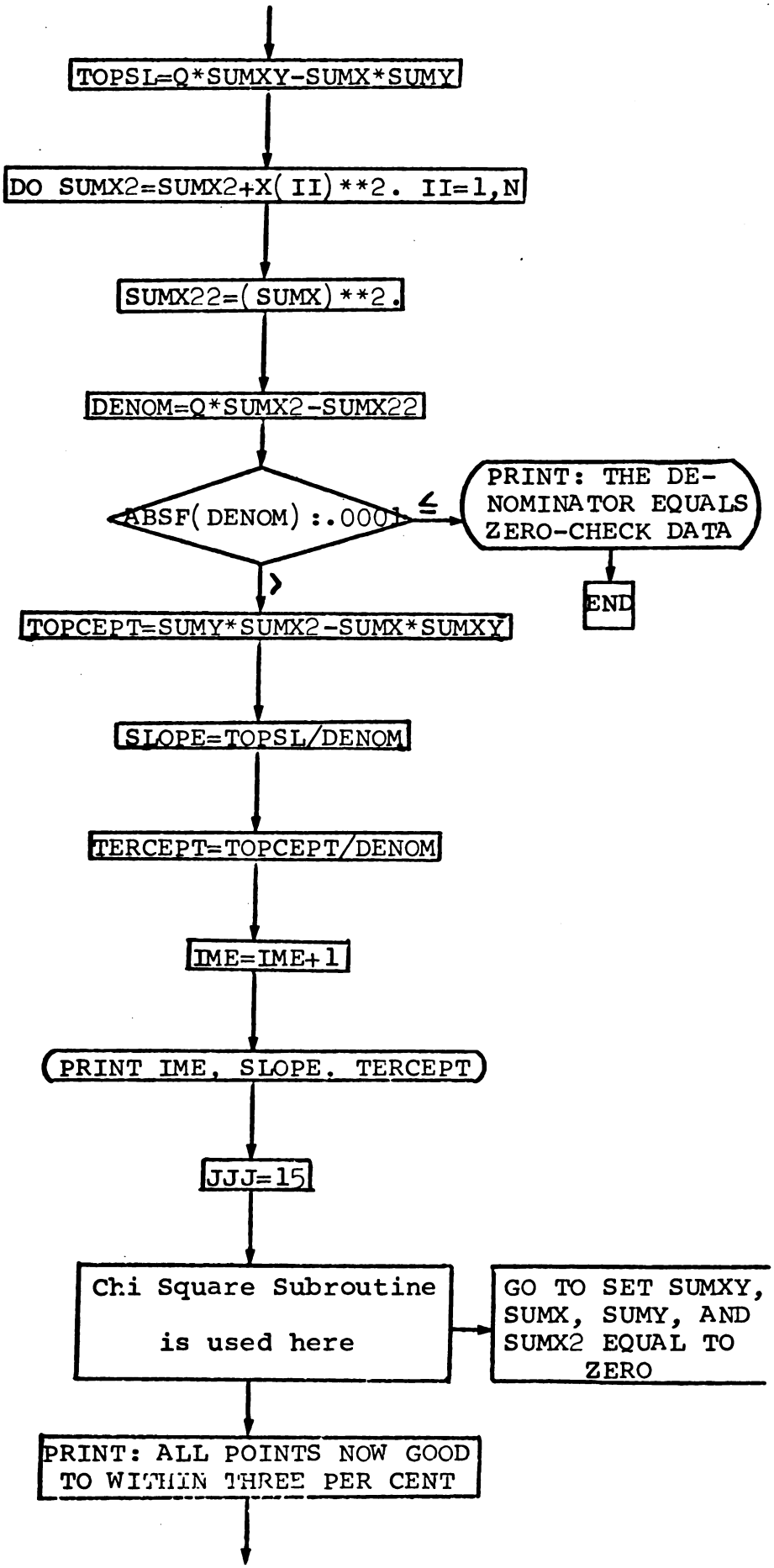


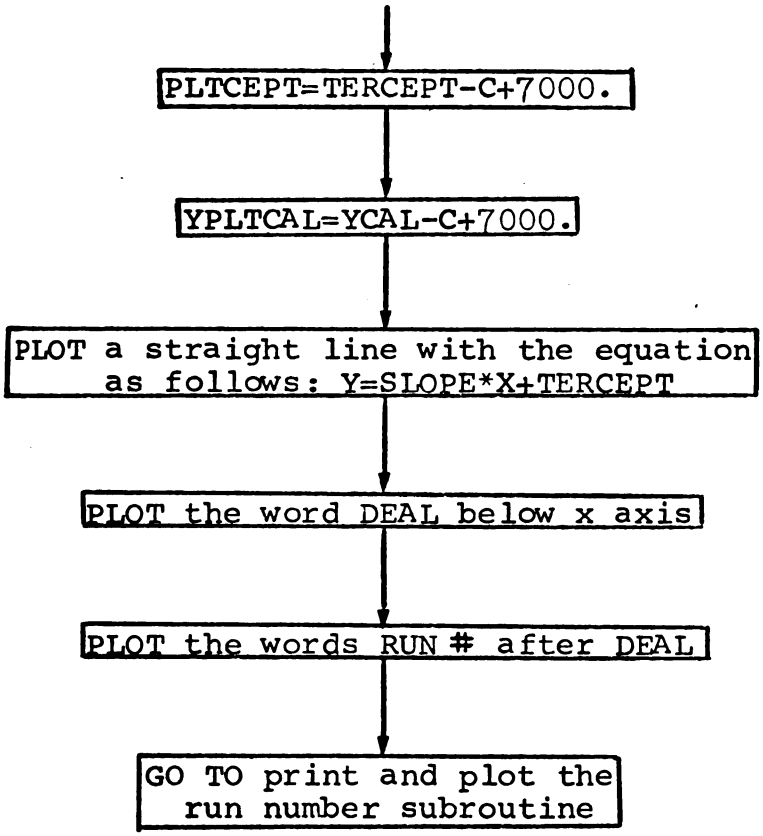
Figure 36. The flow sheet for the computer program, as it is described in the body of the appendix. The following pages are a continuation of the program and therefore have no legends.

Calculation Section



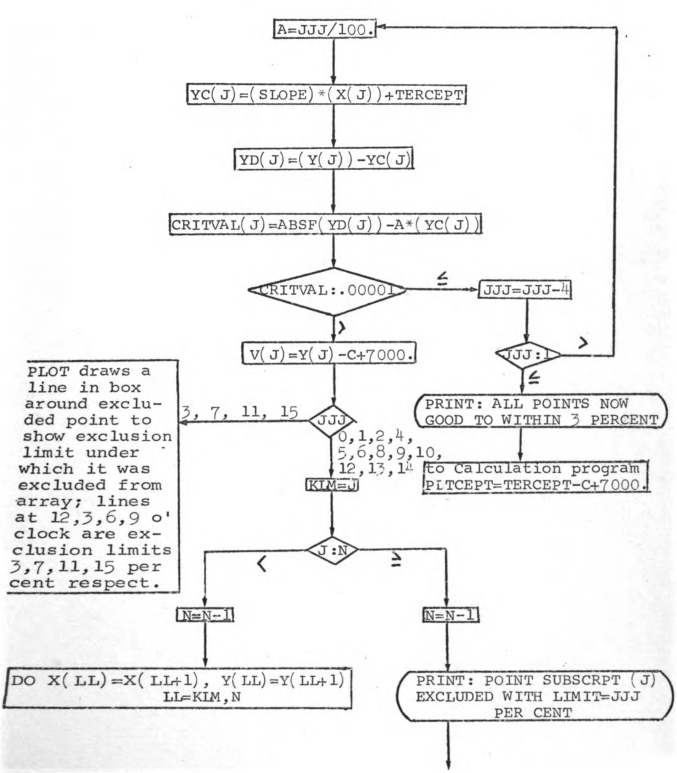

SLY=1000./Y(1)-Y(N)+19000.
P=4.0/SLY
QQ=4.0/SLX
PLOT EACH DATA POINT AS A BOX WITH A DIAMETER OF 2P AT PROPER X,Y COORDINATES
IME=0
C=Y(N)
(used in PLTCEPT= TERCEPT-C+7000. below)
B=X(N)
DO SUBSCRPT(J)=J J=1,N
FROM CHI SQUARE ANALYSIS
SET SUMXY, SUMX, SUMY, SUMX2, EQUAL TO ZERO
DO SUMXY=SUMXY+X(I)*Y(I) I=1,N
DO SUMX=SUMX+X(K) K=1,N
DO SUMY=SUMY+Y(L) L=1,N
Q=N

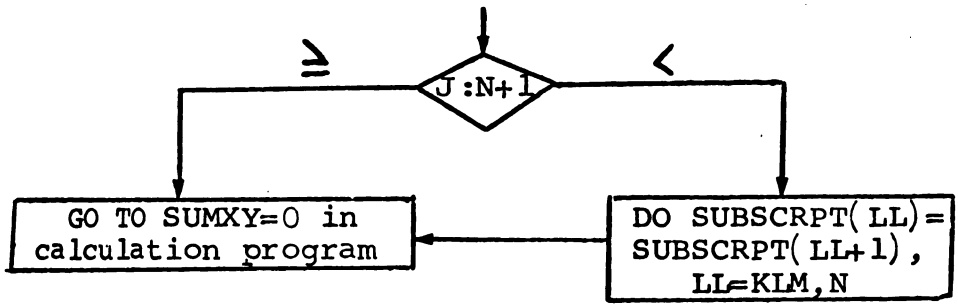

TOPSL=Q*SUMXY-SUMX*SUMY
DO SUMX2=SUMX2+X(II)**2. II=1,N
SUMX22=(SUMX)**2.
DENOM=Q*SUMX2-SUMX22
ABSF(DENOM) :.0001
≤
PRINT: THE DE-
NOMINATOR EQUALS
ZERO-CHECK DATA
END
>
TOPCEPT=SUMY*SUMX2-SUMX*SUMXY
SLOPE=TOPSL/DENOM
TERCEPT=TOPCEPT/DENOM
IME=IME+1
PRINT IME, SLOPE, TERCEPT
JJJ=15
Chi Square Subroutine
is used here
GO TO SET SUMXY,
SUMX, SUMY, AND
SUMX2 EQUAL TO
ZERO
PRINT: ALL POINTS NOW GOOD
TO WITHIN THREE PER CENT


PLTCEPT=TERCEPT-C+7000.
YPLTCAL=YCAL-C+7000.
PLOT a straight line with the equation as follows: Y=SLOPE*X+TERCEPT
PLOT the word DEAL below x axis
PLOT the words RUN # after DEAL
GO TO print and plot the run number subroutine

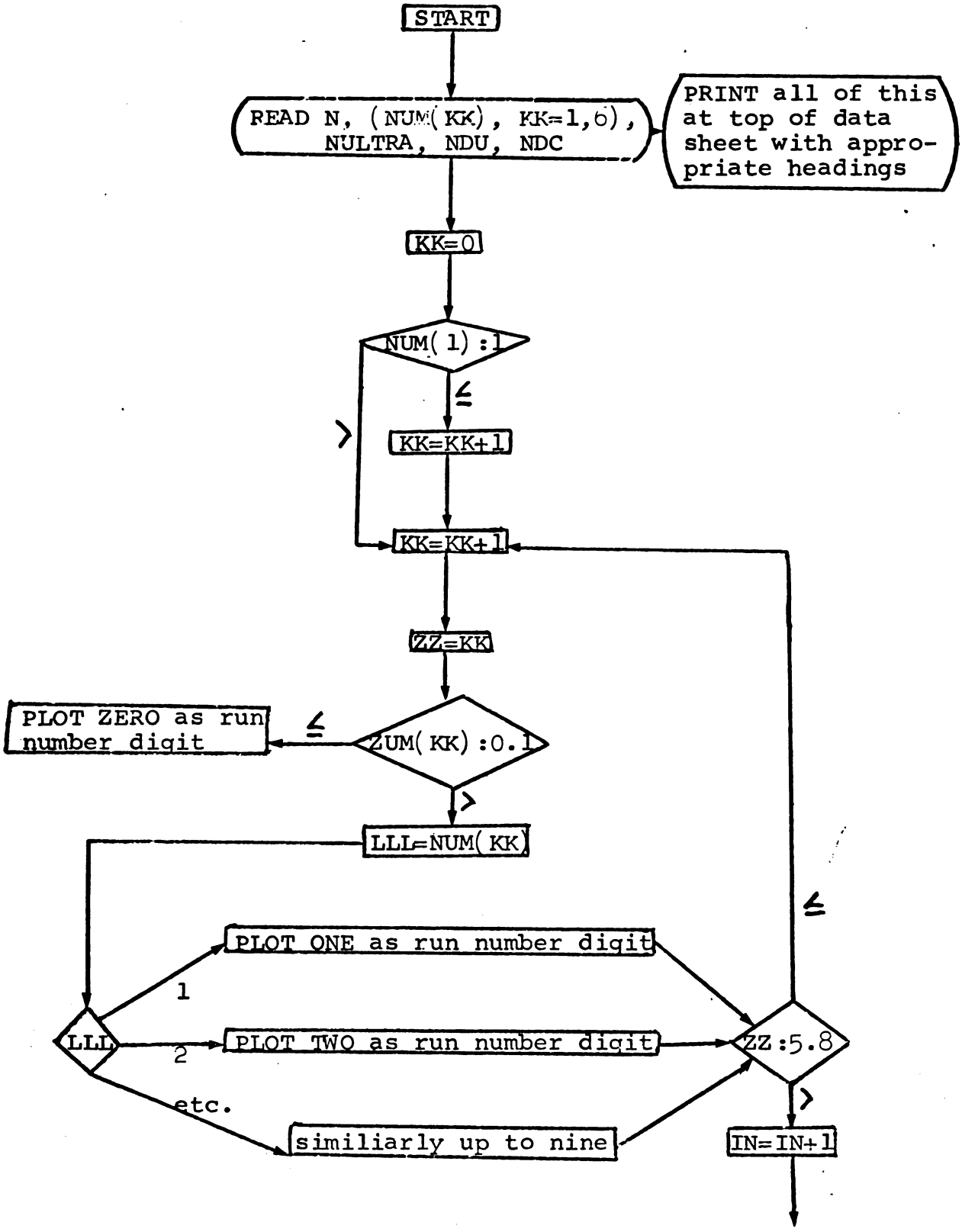
Modified Chi Square Analysis

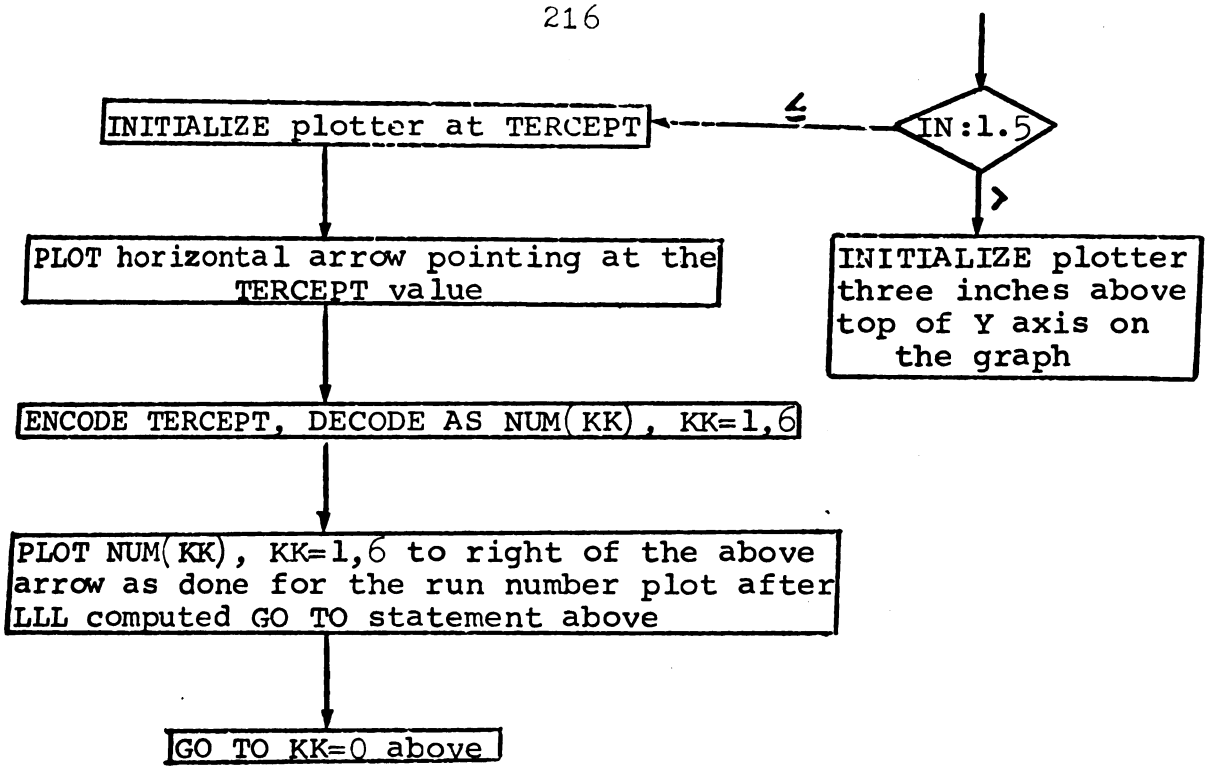
This entire section of the program is in one DO loop of the form DO CONTINUE (i.e., DO whole subroutine), J=1,N




≥
J :N+1
<
GO TO SUMXY=0 in calculation program
DO SUBSCRPT(LL)= SUBSCRPT(LL+1), LL=KLM,N

Print and Plot the Run Number Subroutine




IN:1.5
≤
>
INITIALIZE plotter at TERCEPT
INITIALIZE plotter three inches above top of Y axis on the graph
PLOT horizontal arrow pointing at the TERCEPT value
ENCODE TERCEPT, DECODE AS NUM(KK), KK=1,6
PLOT NUM(KK), KK=1,6 to right of the above arrow as done for the run number plot after LLL computed GO TO statement above
GO TO KK=0 above

type of alternative is expressed over the arrow leading from this to the subsequent choice operation. For instance, an IF statement has two exiting arrows, and over one may read "less than or equal to" and over the other "more than." In all cases, the path of calculations and other operations follows the path of the arrows from one operation to another. Re-enteries into the program are described where they occur in as much detail as space allows.

The operations performed on the data after it has been entered into the computer are described below:

1) A least square analysis is performed, and the best straight line through all of the input data points is calculated.
2) Each point is then tested by a modified chi-square analysis to determine if it is more than 15 per cent from the Y value calculated by the computer for the X value of that point on the line calculated in 1 above.
3) If all points are within 15 per cent of the line, the exclusion limit is progressively lowered to 11, 7 and 3 per cent. Once a point is excluded (see 4), all remaining points are retested at all exclusion limits again following recalculation of the new best straight line by least squares.
4) If at any time a point is further from the calculated line than the exclusion limit, this point is dropped from the array, and a new least squares

line is calculated for the remaining points, which are then retested as in 2 and 3 above. A new line is calculated each time a point is excluded, and the final line acceptable has all points remaining good to at least 3 per cent.

5) For each line that is calculated, both the slope and intercept on the Y axis are calculated and printed out. In addition, each point that is excluded is printed out along with the exclusion limit at that point in the execution.

6) The computer plots the final (best) straight line calculated, including all the points but indicating by means of lines in the boxes which points have been excluded. The Y axis is divided into units of 1000, and the X axis is divided into units of one by small blips. An arrow is drawn pointing to the Y intercept, and its numerical value is printed adjacent to this arrow. The words DEAL and the run number are printed just below the X axis.

Three typical sets of results are presented on the following pages. Each set of data consists of both printed and plotted results. The data presented was provided as follows:

1) L.C. 6 - pyruvate kinase subunit molecular weight extrapolated to zero concentration, by Dr. M. S. Kayne.

L. C. RUN 000006 ULTRACENTRIFUGE RUN 698L050 ON 3/12/65

3600 RUN ON 2/20/67 PROGRAM DATED 2/24/67

MOLECULAR WEIGHT VS. CONCENTRATION

RAW DATA

POINT	X	Y
1	1.00	68574
2	1.00	61177
3	1.25	59091
4	1.50	61280
5	1.50	53109
6	1.75	55097
7	1.75	57886
8	2.00	57982
9	2.25	55919
10	2.50	53552
11	2.75	56335
12	3.00	56825
13	3.25	65030
14	3.50	54227
15	3.75	53702
16	4.00	48120
17	4.25	56312
18	4.50	51715
19	4.75	58736
20	5.00	53336
21	5.00	44007

CALCULATIONS

1 SLOPE = -2287.08 INTERCEPT = 62848.04
POINT 13 EXCLUDED WITH LIMIT = 15 PER CENT

2 SLOPE = -2391.60 INTERCEPT = 62665.15
POINT 1 EXCLUDED WITH LIMIT = 11 PER CENT

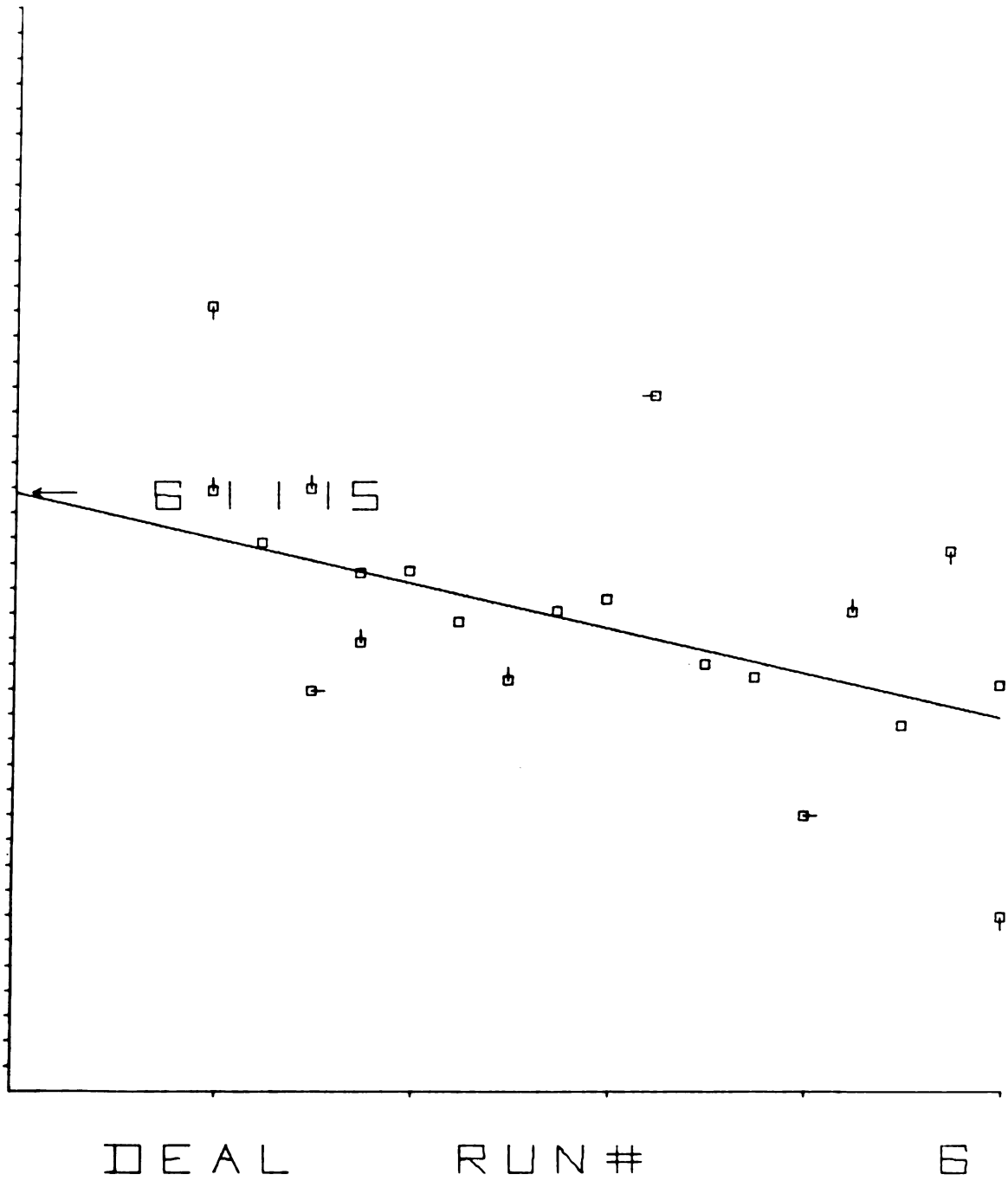
3 SLOPE = -1904.69 INTERCEPT = 60793.19
POINT 19 EXCLUDED WITH LIMIT = 11 PER CENT

4 SLOPE = -2351.51 INTERCEPT = 61677.04
POINT 21 EXCLUDED WITH LIMIT = 11 PER CENT

5 SLOPE = -1809.39 INTERCEPT = 60549.97
POINT 5 EXCLUDED WITH LIMIT = 7 PER CENT

6 SLOPE = -2072.75 INTERCEPT = 61581.97
POINT 16 EXCLUDED WITH LIMIT = 7 PER CENT

```
 7  SLOPE =     -1767.41   INTERCEPT =    61097.19
    POINT  2 EXCLUDED WITH LIMIT =   3 PER CENT

 8  SLOPE =     -1584.50   INTERCEPT =    60445.93
    POINT  4 EXCLUDED WITH LIMIT =   3 PER CENT

 9  SLOPE =     -1306.27   INTERCEPT =    59380.30
    POINT  6 EXCLUDED WITH LIMIT =   3 PER CENT

10  SLOPE =     -1477.08   INTERCEPT =    60066.27
    POINT 10 EXCLUDED WITH LIMIT =   3 PER CENT

11  SLOPE =     -1589.85   INTERCEPT =    60671.36
    POINT 17 EXCLUDED WITH LIMIT =   3 PER CENT

12  SLOPE =     -1819.58   INTERCEPT =    61115.05

ALL POINTS NOW GOOD TO WITHIN 3 PER CENT
```


6 1 1 5
DEAL RUN# 6

L. C. RUN 000007 ULTRACENTRIFUGE RUN 5018PHL ON 2/10/65

3600 RUN ON 2/20/67 PROGRAM DATED 2/24/67

MOLECULAR WEIGHT VS. CONCENTRATION

RAW DATA

POINT	X	Y
1	2.50	61945
2	3.00	55522
3	3.50	53973
4	4.00	51588
5	4.50	48797
6	5.00	51678
7	6.00	42926
8	7.00	40710
9	7.50	37180
10	8.00	36297
11	8.50	34862
12	9.00	34653
13	10.00	31237

CALCULATIONS

1 SLOPE = -3872.41 INTERCEPT = 68104.03
POINT 1 EXCLUDED WITH LIMIT = 3 PER CENT

2 SLOPE = -3646.14 INTERCEPT = 66377.47
POINT 6 EXCLUDED WITH LIMIT = 7 PER CENT

3 SLOPE = -3557.16 INTERCEPT = 65482.15
POINT 9 EXCLUDED WITH LIMIT = 3 PER CENT

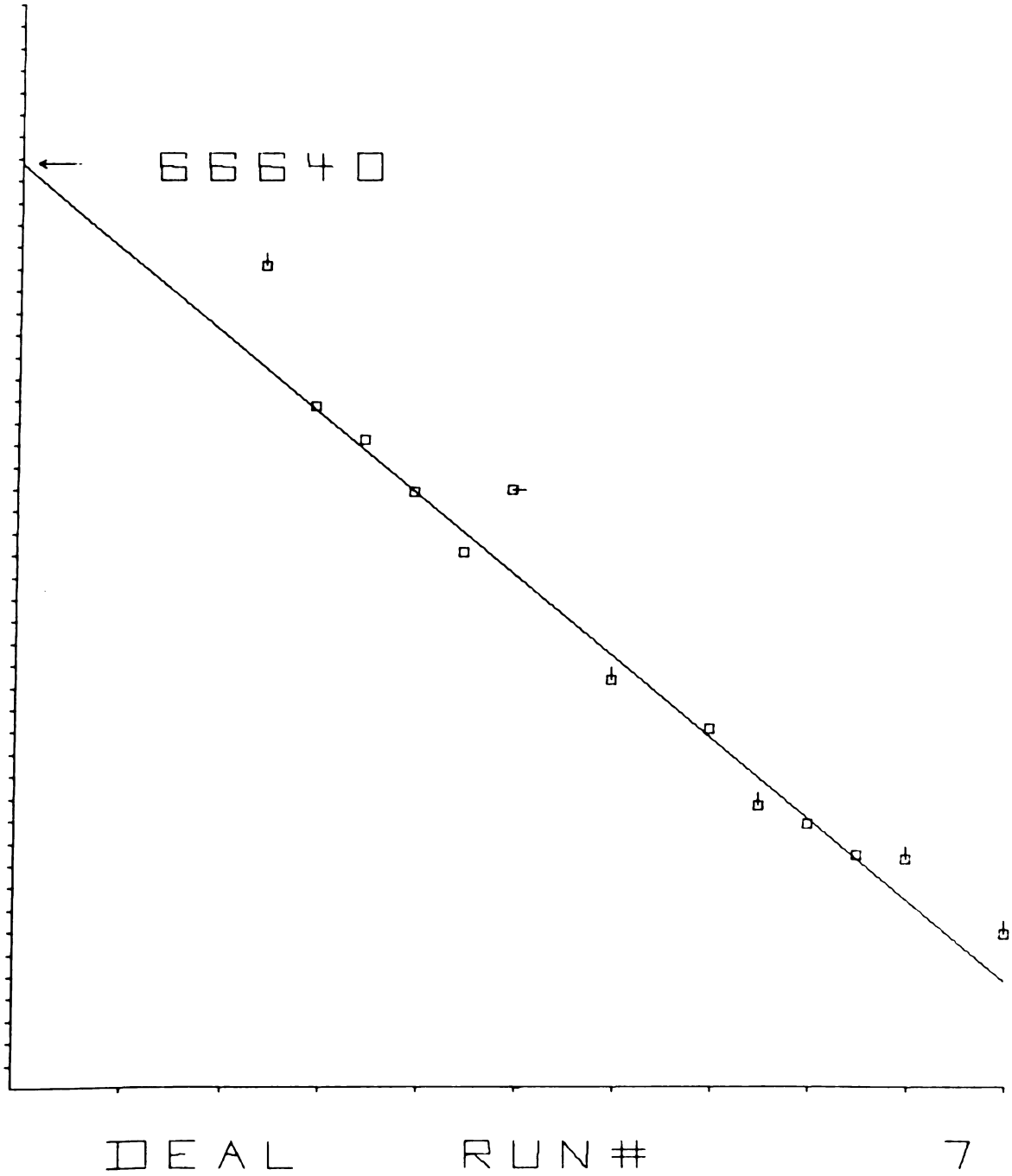
4 SLOPE = -3524.14 INTERCEPT = 65434.76
POINT 7 EXCLUDED WITH LIMIT = 3 PER CENT

5 SLOPE = -3533.54 INTERCEPT = 65646.41
POINT 13 EXCLUDED WITH LIMIT = 3 PER CENT

6 SLOPE = -3623.72 INTERCEPT = 66066.06
POINT 12 EXCLUDED WITH LIMIT = 3 PER CENT

7 SLOPE = -3759.24 INTERCEPT = 66639.97

ALL POINTS NOW GOOD TO WITHIN 3 PER CENT


66640
IDEAL
RUN#
7

L. C. RUN 000011 ULTRACENTRIFUGE RUN 722RCL0 ON 5/ 6/65

3600 RUN ON 2/20/67 PROGRAM DATED 2/24/67

MOLECULAR WEIGHT VS. CONCENTRATION

RAW DATA

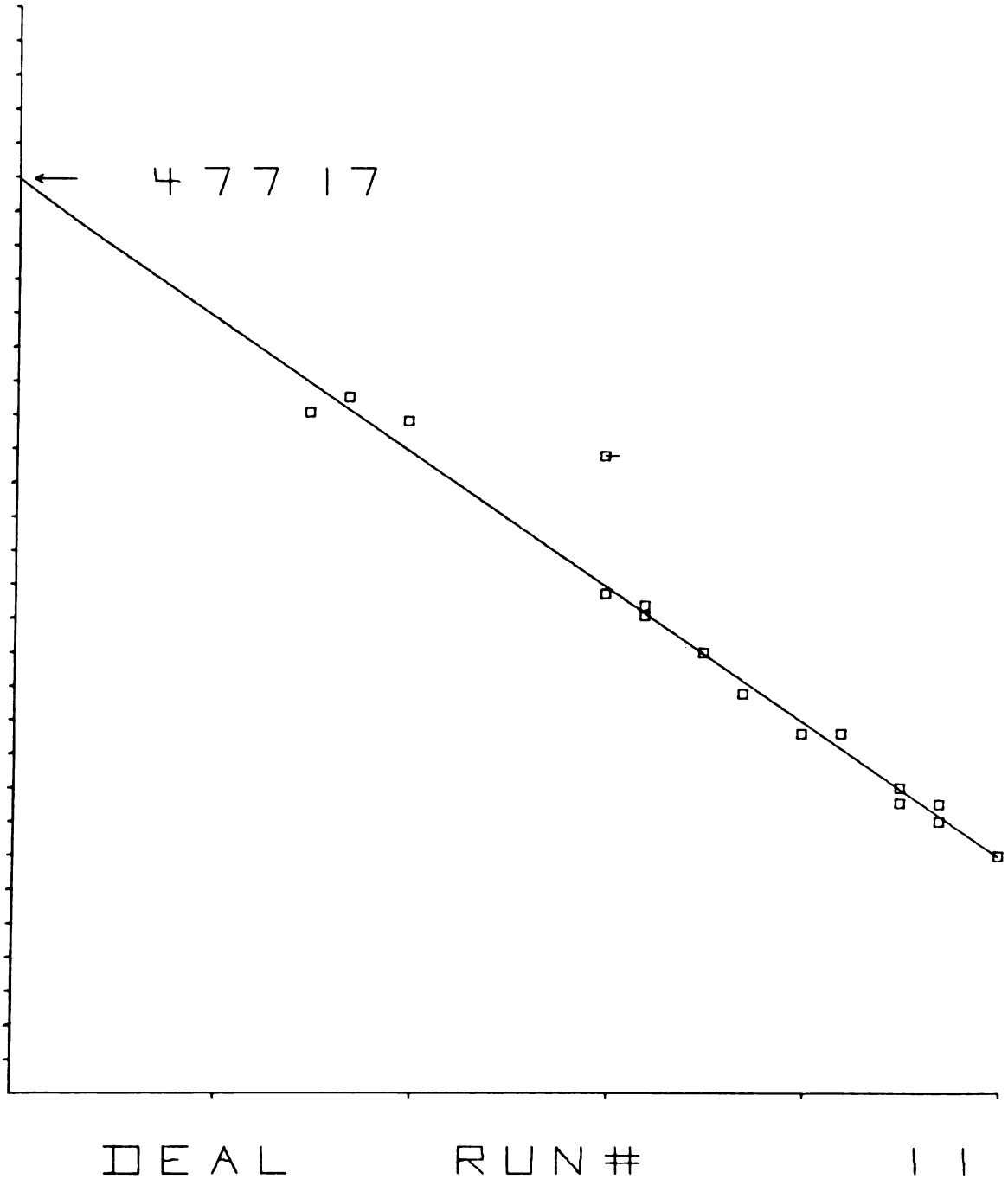
POINT	X	Y
1	1.50	40787
2	1.70	41238
3	2.00	40533
4	3.00	39517
5	3.00	35422
6	3.20	34788
7	3.20	35066
8	3.50	33686
9	3.70	32459
10	4.00	31278
11	4.20	31283
12	4.50	29678
13	4.50	29228
14	4.70	28665
15	4.70	29184
16	5.00	27639

CALCULATIONS

1 SLOPE = -4135.40 INTERCEPT = 48355.47
POINT 4 EXCLUDED WITH LIMIT = 7 PER CENT

2 SLOPE = -4023.01 INTERCEPT = 47717.50

ALL POINTS NOW GOOD TO WITHIN 3 PER CENT


← 4 7 7 1 7
DEAL
RUN#
1 1

2) L.C. 7 - phosphorylase a subunit molecular weight extrapolated to zero concentration, by Dr. W. C. Deal.

3) L.C. 11 - pyruvate kinase subunit (different conditions) molecular weight extrapolated to zero concentration, by Dr. M. S. Kayne.

To determine the value of the computer program, it is of interest to judge the difference in the results produced by drawing the best estimated line and having the computer draw the best calculated line. It can be seen that for L.C. 11, the results would be nearly equal. However, for L.C. 7, the best estimated line extrapolates to approximately 71,000, whereas the value determined to be correct to within three per cent was 66,640, and this difference amounts to an error of approximately 7 per cent. For L.C. 6, the data scatters so badly that extrapolation by eye could give values ranging from 57,000 to 68,000, but the computer calculated the best straight line through ten of the points to extrapolate to 61,115. For both of these last two sets of data, the computer program proved to be an invaluable aid in determining the best extrapolated molecular weight, even from badly scattered data.

It should be noted in closing that the computer is limited to straight line extrapolations, which may not always be the best data-fitting curve. The human element must determine this, but even in these cases, the accurate plot produced by the computer provides a representation of the data to simplify visual estimation of the best curve.