INHIBITION OF A MICROBIAL D-GLUCOSE-6-PHOSPHATE DEHYDROGENASE BY DIETHYLSTILBESTROL AND STRUCTURAL ANALOGUES

By

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CHAPTER I

INTRODUCTION

The concept that estrogens may regulate metabolism by influencing certain enzymes is not new (28, 31) and some of the precise mechanisms underlying these effects have recently been elucidated. Of particular interest are the potent synthetic estrogens such as diethylstilbestrol and structurally analogous stilbenoid compounds which have organic structures quite dissimilar to those of the natural estrogens.

One example of the extensive effects of hormones in mammals is the addition of trace amounts of synthetic estrogens to the diet of fattening steers which results in a greatly increased rate of weight gain and efficiency of feed utilization (6). Possible mechanisms of this effect include alteration by the hormone of the population and metabolism of the complex rumen microflora as well as a direct influence on the overall metabolism of the animal.

Knowledge of the influence of synthetic estrogens on microbial metabolism is somewhat limited. Low concentrations of several stilbenoid compounds are bactericidal to species of Staphylococcus and Streptococcus (5). Diethylstilbestrol has been reported to inhibit both respiration and fermentation of bakers yeast utilizing glucose as substrate (30). Cellulose digestion by ovine rumen microorganisms in an artificial rumen was stimulated by diethylstilbestrol (4). Durham and Perry (12) showed that although diethylstilbestrol inhibited the rate

of oxidation of several substrates by Aerobacter aerogenes, it did not inhibit the total oxygen consumption. This inhibition could not be overcome by addition of several organic metabolites, electron carriers, or metallic ions (11).

Although several "isolated" enzymes are inhibited by synthetic estrogens (7, 10, 23, 25, 27, 33, 36), the mechanisms of inhibition thus far delineated differ considerably. Hochster and Quastel (15) showed that, in the presence of MnO_2, diethylstilbestrol inhibited several dehydrogenase reactions. They postulated that diethylstilbestrol was oxidized to a quinone form by MnO_2 and that the quinone-quinol system functioned as a competitive electron carrier. Yielding and Tomkins (32, 34, 35, 36) found that, in the presence of low concentrations of reduced pyridine nucleotides, either synthetic or natural estrogens caused a disaggregation into subunits of crystalline glutamic dehydrogenase. Disaggregation of the protein was accompanied by inhibition of glutamic dehydrogenase activity and stimulation of alanine dehydrogenase activity. Diethylstilbestrol also inhibits pyruvate kinase (20) and aldehyde dehydrogenase (24) by causing extensive alteration of structure, but no disaggregation of the apoenzyme was detected. McKerns and Bell have reported that stilbenoid compounds inhibit bovine adrenal glucose-6-phosphate dehydrogenase by binding to the nicotinamide adenine dinucleotide phosphate-accepting sites on the apoenzyme (26).

The studies reported herein were undertaken to delineate the mode of action of diethylstilbestrol, hexestrol, dienestrol and benzestrol on a partially purified D-glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) (17) from A. aerogenes.

CHAPTER II

MATERIALS AND METHODS

Organism:

A laboratory stock culture of Aerobacter aerogenes incapable of growth on a medium containing diethylstilbestrol as the sole carbon and energy source was used throughout the study as the source of enzyme. This culture was identical to that used in previous studies by Durham and co-workers (10, 11, 12).

Substrates:

Nicotinamide adenine dinucleotide phosphate (NADP) and the crystalline barium salts of D-glucose-6-phosphate and D-gluconate-6-phosphate were purchased from the Sigma Chemical Company. The sodium salts of the two sugar-phosphates were obtained by precipitating the barium as $BaSO_4$ by equimolar addition of Na_2SO_4.

Synthetic estrogens:

Trans-Diethylstilbestrol (trans-3,4-bis(p-hydroxyphenyl)-3-hexene), meso-hexestrol (meso-3,4-bis(p-hydroxyphenyl)-n-hexane) and trans,trans-dienestrol (trans,trans-3,4-bis(p-hydroxyphenyl)-2,4-hexadiene) were purchased from Nutritional Biochemicals Corporation. Benzestrol (3-ethyl-2,4-bis(p-hydroxyphenyl)hexane) was a gift of Eli Lilly and Company. Other synthetic estrogens and structural analogues were purchased from various commercial sources or obtained from other laboratories. Diethylstilbestrol, hexestrol, dienestrol and benzestrol were dissolved in propylene glycol

with shaking at 30 C and diluted with an equal volume of distilled water for kinetic studies. Solutions of synthetic estrogens were prepared fresh for each experiment and discarded after twenty-four hours. Compounds sparingly soluble in 50 percent (v/v) aqueous propylene glycol were dissolved in dioxane. Water soluble compounds were prepared in aqueous solution.

Ammonium sulfate:

To remove possible traces of metallic contaminants, reagent grade ammonium sulfate was recrystallized from a solution saturated with the salt at 95 C which had been made weakly basic with ammonium hydroxide and contained two grams of ethylene diamine tetraacetate per liter (3). The crystals were air dried and ground to a fine powder with a morter and pestle. Recrystallized ammonium sulfate was used in all enzyme fractionations.

Protamine sulfate:

Protamine sulfate (salmine) was purchased from Nutritional Biochemicals Corporation. A solution saturated at 4 C was prepared in 0.01 M tris(hydroxymethyl)-aminomethane (tris) by adding warm distilled water to 1.0 gram of protamine sulfate and 0.121 grams of tris in a 100 ml volumetric flask. The flask was shaken and, when most of the material was dissolved, the solution was cooled to room temperature, and the pH adjusted to 7.6 by addition of 1.0 N potassium hydroxide. The solution was cooled overnight at 4 C, and the precipitate removed by centrifugation.

Fractionation buffer:

The fractionation buffer, used extensively as the enzyme solvent during the various experiments, consisted of 0.01 M tris hydrochloride

buffer (pH 7.6) which contained 5.0×10^{-4} M 2-mercaptoethanol and 5.0×10^{-4} M disodium ethylenediamine tetraacetate.

Calcium phosphate gel:

Aged calcium phosphate gel prepared by the method of Keilin and Hartree (19) was purchased from Nutritional Biochemicals Corporation. The concentration was adjusted to 50 mg dry weight per ml by addition of distilled water and the gel stored at 4 C.

Distilled water:

Water was twice-distilled from a commercial still and distilled a third time in the presence of basic permanganate in a Pyrex glass apparatus.

Purification of D-glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49):

The enzyme purification was carried out in a cold room at 2 to 4 C. All Pyrex glassware and polyethylene centrifuge tubes had been thoroughly cleaned in a solution of dichromate-sulfuric acid. Centrifugation was done in a Servall Model SS-4 Superspeed Centrifuge at a relative centrifugal force of 25,000 x gravity.

As the source of the enzyme A. aerogenes was cultured on nutrient agar (Difco) for 24 hours at 37 C, harvested and washed twice by centrifugation in 0.01 M tris-HCl buffer (pH 7.0), and suspended in a volume of fractionation buffer such that a 1:80 dilution gave an absorbancy (optical density or $\log_{10} I_o/I$) of 0.5 (measured in a Beckman DU spectrophotometer) at 540 mμ using one cm cuvettes. The cells were ruptured by passage through a chilled French pressure cell (13) at 20,000 p.s.i. at a flow rate of approximately two drops per second. Insoluble cell debris was removed by centrifugation for 30 minutes followed by centrifugation of the supernatant solution for 90 minutes. The clear supernatant solution

was diluted with fractionation buffer to give a protein concentration of 5.0 mg per ml as estimated spectrophotometrically by the method of Kalckar (18) using the formula

$$\text{mg protein/ml} = 1.45\ A_{280} - 0.74\ A_{260}$$

where A is the absorbancy at 280 or 260 mμ. Nucleic acids were precipitated by adding a volume of saturated protamine sulfate solution equal to 0.2 times the volume of the enzyme supernatant fraction with constant mixing. Stirring was continued for 15 minutes and the preparation was centrifuged for 30 minutes. The pale yellow supernatant solution was made 0.5-saturated with ammonium sulfate by gradual addition of the finely ground salt over a 10 minute period, stirring with care to avoid foaming. The amount of solid ammonium sulfate required to yield the desired saturation was calculated by the formula

$$w = \frac{(0.515)(V_1)(S_2-S_1)}{1-(0.272)(S_2)}$$

in which w equals the weight in grams of ammonium sulfate required to increase a certain fraction of volume V_1 in milliliters at saturation S_1 to the desired saturation S_2 at 0 C (29). After stirring, the fraction was allowed to stand 10 minutes and then centrifuged for 30 minutes. In a similar manner the resulting supernatant solution was adjusted to 0.6-saturation with ammonium sulfate and centrifuged. The precipitate was immediately dissolved in a volume (V ml) of fractionation buffer equal to 0.25 times the volume of the corresponding supernatant fraction. This solution, which contained most of the initial activity, was dialyzed approximately 18 hours against two volumes of fractionation buffer of 3 liters each. Ten ml of calcium phosphate gel (50 mg dry weight per ml) were centrifuged and the dialyzed enzyme was added to the pellet with

gentle stirring until an even slurry resulted. Adsorption was continued for 15 minutes followed by centrifugation for 10 minutes. A slurry was immediately made by stirring the gel pellet in V ml of fractionation buffer 0.05-saturated with ammonium sulfate. After standing 15 minutes the slurry was centrifuged for 10 minutes and the pellet was eluted with V ml of fractionation buffer 0.25-saturated with ammonium sulfate. The clear 0.25-saturated eluate (supernatant) was increased to 0.7-saturation by gradual addition of finely ground ammonium sulfate with gentle stirring over a 10 minute interval. The mixture was allowed to stand 15 minutes then centrifuged for 30 minutes. The centrifugal pellet was immediately dissolved in V ml of fractionation buffer and dialyzed 18 hours against two volumes of fractionation buffer of 3 liters each. The partially purified enzyme could be stored at 4 C or at -20 C for several weeks, showing only a slight decline in specific activity.

Enzyme assay:

The enzyme activity of each fraction was assayed by adding 0.1 ml of the fraction to 2.4 ml of a mixture containing 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$ and 10 μmoles D-glucose-6-phosphate in a one cm silica cuvette. The enzymatic reaction was initiated by the addition of 0.2 ml of 0.003 M NADP in a teflon adder-mixer and followed by plotting the increase in absorbancy of the reduced co-substrate at 340 mμ. The time course curves for the various reactions were plotted using a Bausch and Lomb Spectronic 505 recording spectrophotometer equipped with a constant temperature cuvette holder. A temperature of 25±0.2 C was maintained by circulating water through the holder from a thermostatically controlled water bath. The reference cuvette contained distilled water. For the kinetic experiments enzyme activity was expressed

as reaction velocity (v) which is defined as the change in absorbancy at 340 mμ during the interval between 30 and 90 seconds after the addition of NADP under the specified experimental conditions.

A unit of enzyme was defined as that amount which catalyzed the generation of one μmole of reduced NADP per minute in the presence of optimum concentrations of the substrates at a temperature of 25 C and a pH of 7.6 (17). The amount of reduced nucleotide (NADPH) was estimated from the molar extinction coefficient at 340 mμ, $6.22 \times 10^3 \times M^{-1} \times cm^{-1}$ (16). Since one μmole NADPH per 2.7 ml (a concentration of 0.37 mM) has a theoretical absorbancy of 2.3, the increase in the number of μmoles NADPH per 2.7 ml per minute can be calculated by dividing the increase in absorbancy per minute by 2.3. Therefore, from the definitions of the unit of enzyme and of the reaction velocity (v) the number of units of enzyme in the 0.1 ml fraction tested equals v/2.3. Since the concentration of an enzyme is defined as the number of units per one ml (17), the concentration of D-glucose-6-phosphate dehydrogenase is calculated by dividing the reaction velocity by 0.23.

The total activity of a given fraction is expressed as units of enzyme and is determined by multiplying the volume (in milliliters) of the fraction by the concentration of enzyme in the fraction. The over-all percent recovery was calculated by dividing 100 x the total activity of the purified enzyme by the total activity of the initial crude supernatant fraction. Specific activity is expressed as units of enzyme per milligram of protein (17). Unless otherwise specified, protein was determined by the method of Lowry et al. (22) using crystalline bovine serum albumin as the protein standard. The over-all purification value was calculated by dividing the specific activity of the purified enzyme

by the specific activity of the crude supernatant fraction. For a typical enzyme fractionation the over-all purification was 13-fold with 50 percent recovery.

CHAPTER III

EXPERIMENTAL AND RESULTS

Properties of the purified D-glucose-6-phosphate dehydrogenase:

Enzyme activity in the presence of several inorganic salts was measured to determine if certain ions could function as activators. The test system contained: 200 μmoles tris-HCl buffer (pH 7.6), 10 μmoles of the aqueous salt tested (at neutrality) unless otherwise specified, 10 μmoles of D-glucose-6-phosphate, 0.1 ml of the enzyme and 0.6 μmoles NADP in a total volume of 2.7 ml. The control system contained no added salt. NH_4Cl, $(NH_4)_2SO_4$, NaCl, KCl, $NaNO_3$, KI, LiCl, $ZnSO_4$, $CuCl_2$ (3 μmoles) and $FeCl_3$ (0.12 μmoles) did not increase the enzymatic reaction rate above that of the control, while $MgCl_2$, $MnCl_2$, $MnSO_4$ and $CaCl_2$ caused a definite activation. Since other chlorides and sulfates were not activators, the divalent cations Mg^{++}, Mn^{++} and Ca^{++} were assumed to be the ions essential for activation. The reaction velocity in the test system was measured at several concentrations of $MgCl_2$, $MnCl_2$ or $CaCl_2$ to determine the optimum concentration for each of these activators (Figure 1). The optimum concentration in the reaction mixture for $MgCl_2$ or $CaCl_2$ was approximately 0.01 M, while the optimum concentration for $MnCl_2$ was approximately 0.008 M.

Since enzymatic activity was evident in systems to which no activators had been added, one cannot conclude that this enzyme system has an absolute ion requirement. However, it is possible that such a require-

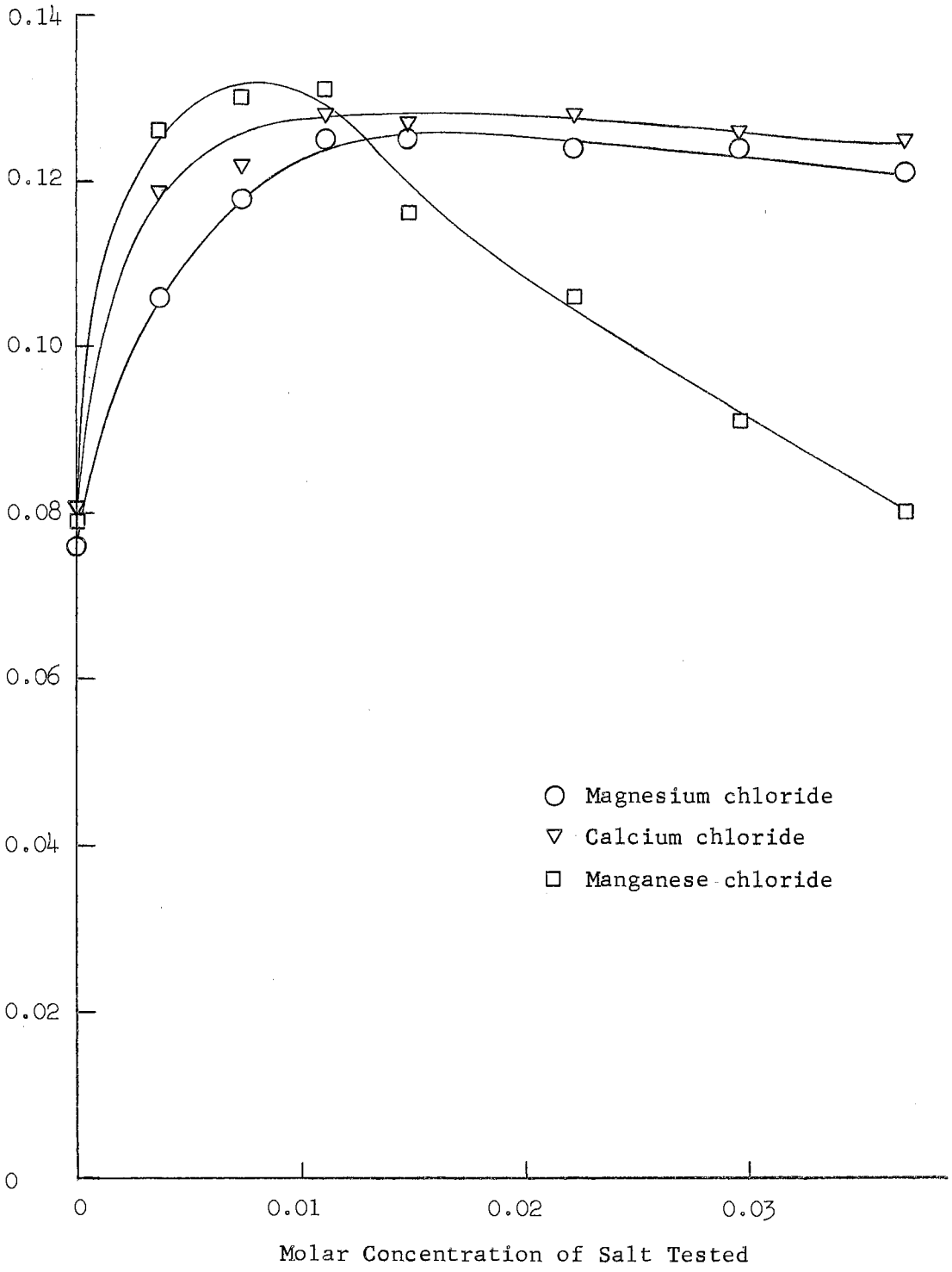


Figure 1: Influence of different concentrations of $MgCl_2$, $MnCl_2$ or $CaCl_2$ on the reaction velocity

ment exists, and that the ions are tightly bound to the apoprotein and could not be removed by the conditions of dialysis and chelation employed in the enzyme purification. Therefore, it was concluded that the activated system containing the divalent cation more nearly represented the physiological state. $MgCl_2$ was chosen as the activator to be used throughout most of the study and incorporated in the various reaction mixtures at a concentration of 30 μmoles per 2.7 ml. This concentration was slightly greater than the optimum concentration for activity.

The optimum pH of the enzyme system was determined by measuring activity in experimental systems in which the pH of the buffer was adjusted to various levels. The system contained 200 μmoles tris-HCl buffer of varying pH, 30 μmoles $MgCl_2$, 10 μmoles D-glucose-6-phosphate, 0.1 ml of the enzyme, and 0.6 μmoles NADP in a total volume of 2.7 ml. The reaction was initiated by addition of NADP. The pH of each reaction mixture was measured 5 minutes after the initiation of the reaction using a Beckman Zeromatic pH Meter. These results are presented in Figure 2 wherein the reaction velocity is plotted against the pH of the reaction mixture. The optimum pH of the system was 7.6. Thus, the experimental system was buffered at pH 7.6 for all experiments.

Studies were conducted to determine if activators or inhibitors were present in the partially purified enzyme preparation. The reaction velocity was measured at several concentrations of the enzyme using a test system which contained 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$, 10 μmoles D-glucose-6-phosphate, 0.1 ml of the enzyme (diluted to several concentrations with fractionation buffer) and 0.6 μmoles NADP in a total volume of 2.7 ml. The reaction velocity is directly proportional to the concentration of the enzyme (Figure 3). Since preliminary

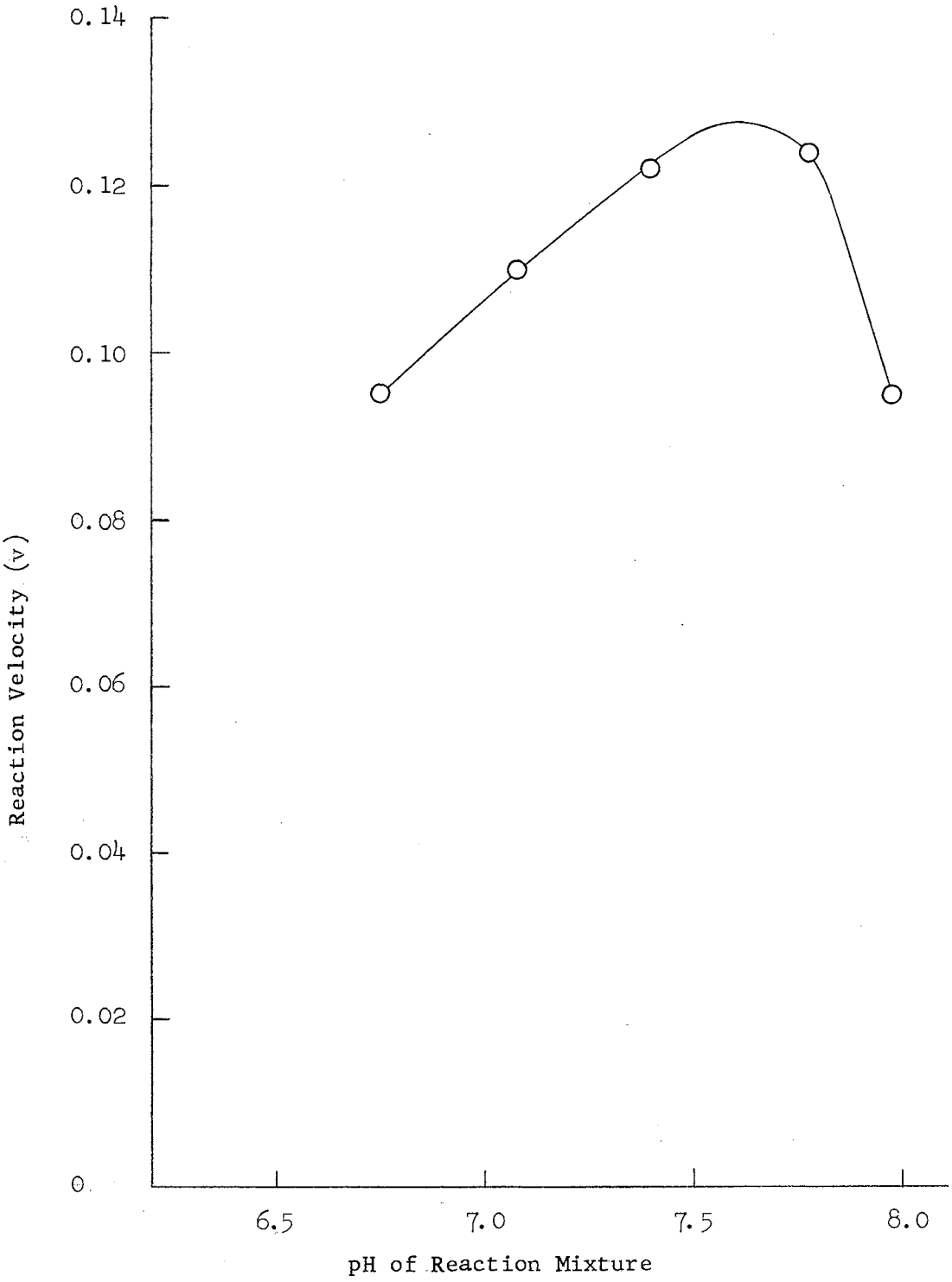


Figure 2. Effect of pH on reaction velocity

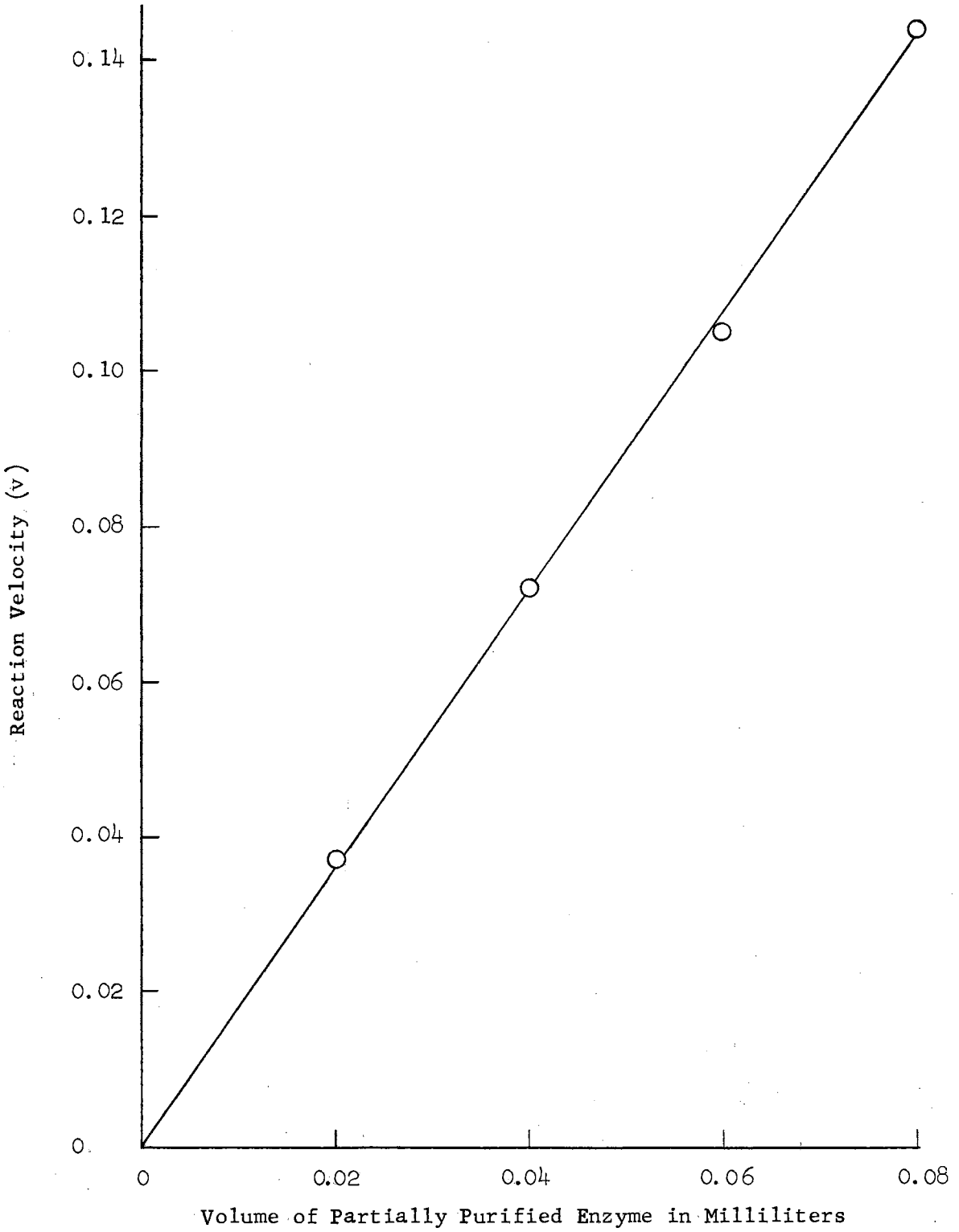


Figure 3. Effect of the concentration of enzyme on reaction velocity

experiments showed that neither substrate (D-glucose-6-phosphate or NADP) nor $MgCl_2$ was rate-limiting at the concentrations used in this study, the partially purified enzyme apparently contained no activators or inhibitors which would interfere with kinetic experiments (9). Also, the results revealed that the test system contained no component(s) which changed in absorbancy at 340 mμ in the absence of the enzyme. This observation lends credence to the spectrophotometric assay.

To ascertain that the partially purified enzyme extract contained no other enzyme(s) which might influence the reaction velocity as determined by the spectrophotmetric assay, a series of experiments was performed. The product of the D-glucose-6-phosphate dehydrogenase reaction is 6-phospho-D-glucono-δ-lactone which can be hydrolyzed to D-gluconate-6-phosphate in the presence of a sufficient amount of gluconolactonase (E.C. 3.1.1.17). Thus, the presence of D-gluconate-6-phosphate dehydrogenase could catalyze the reduction of NADP and interfere with kinetic results based on the rate of reduction of NADP. Studies were conducted to determine if the enzyme extract possessed D-gluconate-6-phosphate dehydrogenase (E.C. 1.1.1.44), an NADP-dependent enzyme in the hexose-monophosphate-shunt pathway. The assay for this enzyme was conducted in an experimental system in which 10 μmoles D-gluconate-6-phosphate was substituted for D-glucose-6-phosphate. No D-gluconate-6-phosphate dehydrogenase activity was found. In experiments in which either D-glucose-6-phosphate or NADP was omitted from the reaction mixture no increase in absorption was observed. The results indicate that other enzymes, which may catalyze reactions yielding products which absorb at or near 340 mμ, were either absent or lacked components essential for activity in the test system. Thus, the partially purified enzyme

preparation was considered to be free from interfering enzymes and, therefore, reliable for kinetic experiments (9).

Tests of stilbenoid compounds and structural analogues for inhibition:

Durham and Leach (10) reported that a D-glucose-6-phosphate dehydrogenase from a partially purified extract of A. aerogenes was inhibited by the synthetic estrogens diethylstilbestrol, hexestrol and dienestrol. Additional studies were conducted to ascertain if other stilbenoid compounds and various structural analogues would affect the enzymatic reaction. The test system contained: 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$, 10 μmoles D-glucose-6-phosphate, 0.1 ml of the enzyme extract, 0.1 ml of a 0.001 M solution of the test compound dissolved in either water, dioxane or 50 percent (v/v) aqueous propylene glycol, 0.6 μmoles NADP, and water to a total volume of 2.7 ml. The reaction velocity in the presence of the test compound was compared with that obtained with the appropriate solvent control. Dioxane depressed the reaction velocity 54 percent when compared with the water control, while only a 9 percent decrease was evident using the 50 percent (v/v) aqueous propylene glycol solvent. Although such "solvent effects" are not desirable, the use of dioxane or propylene glycol was necessitated by the hydrophobic nature of most of the stilbenoid compounds.

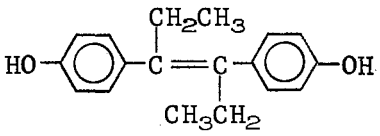
A test compound was considered to be an inhibitor of the enzyme system if it depressed the reaction velocity 5.0 percent or more below that of the control. The structures of the compounds tested are presented in Table I. Only diethylstilbestrol, hexestrol, dienestrol and benzestrol (Compounds 1 — 4) significantly inhibited the enzymatic reaction catalyzed by D-glucose-6-phosphate dehydrogenase.

The structures of the known inhibitors were compared with those of

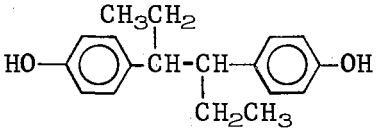
TABLE I

STRUCTURES OF INHIBITORS AND NON-INHIBITORS

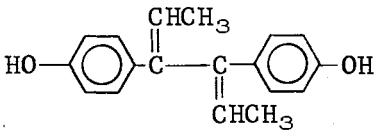
1. Diethylstilbestrol



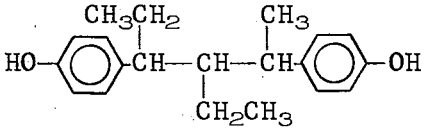
2. Hexestrol



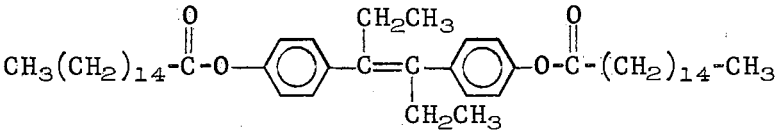
3. Dienestrol



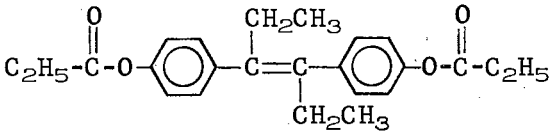
4. Benzestrol



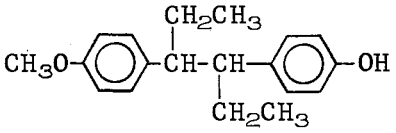
5. Diethylstilbestrol dipalmitate



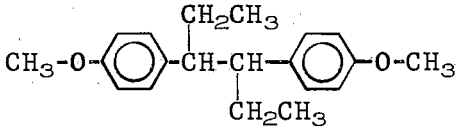
6. Diethylstilbestrol dipropionate



7. Hexestrol monomethyl ether



8. Hexestrol dimethyl ether



9. Diethylstilbestrol dimethyl ether

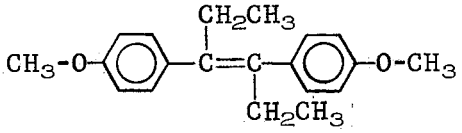


TABLE I (Continued)

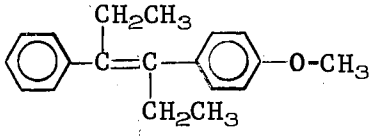
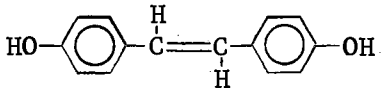
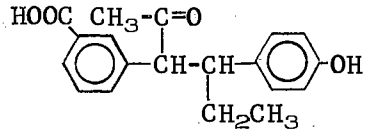
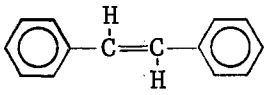
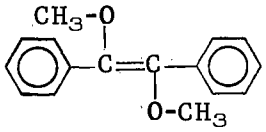
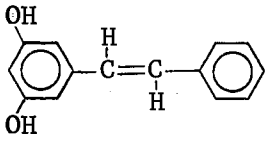
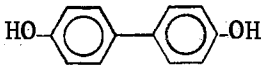
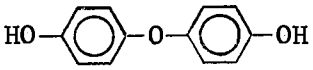
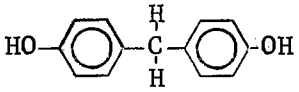
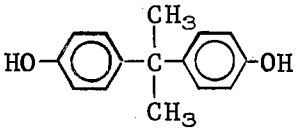
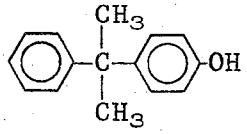
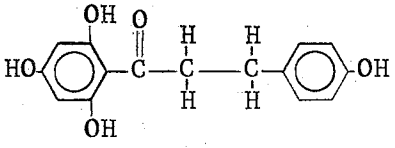
10.	α,α'-Diethyl-4-methoxystilbene	
11.	4,4'-Dihydroxystilbene	
12.	m-(β-Acetyl-α-ethyl-p-hydroxyphenyl)-benzoic acid	
13.	Stilbene	
14.	α-α'-Dimethoxystilbene	
15.	Pinosylvine	
16.	4,4'-Dihydroxy diphenyl	
17.	4,4'-Oxydiphenol	
18.	4,4'-Dihydroxy diphenyl methane	
19.	β,β-Di(4-hydroxyphenyl)propane	

TABLE I (Continued)

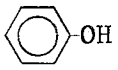
20. β-(4-Hydroxyphenyl)-β-phenylpropane



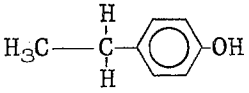
21. Phloretin



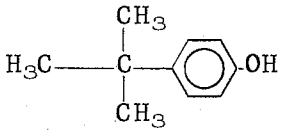
22. Phenol



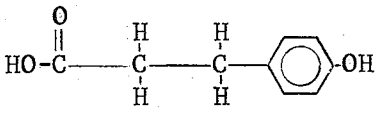
23. p-Ethylphenol



24. p-tert-Butylphenol



25. Phloretic acid



the non-inhibitors (Table I) in an attempt to determine, at least in part, which groups or chemical configurations are essential for inhibition of the enzymic reaction. Two unsubstituted para-hydroxyl groups are featured by each of the four inhibitors (Compounds 1 — 4) and are evidently essential groups. Any substitution (Compounds 5 — 10) or deletion (Compound 10) of one or both of these groups results in a loss of the capacity to inhibit.

The substituents on the central aliphatic carbon chain of the inhibitor molecules also appear to be important. The deletion of the two ethyl groups of diethylstilbestrol (Compound 11) results in the loss of inhibitory potential. Compounds 12 and 21 are non-inhibitory analogues of hexestrol and benzestrol, respectively, which have both altered aliphatic substituents and altered hydroxyl moieties. Compounds 16 - 21 are non-inhibitory analogues of varying central aliphatic structure. Phenol and analogues which contained a single benzene ring (Compounds 22 — 25) were not inhibitory. Other compounds tested and found inactive as inhibitors, not shown on Table I, include α-phenyl-o-cresol, tyrosine, tyramine, p-methyl aminobenzoic acid, p-methoxybenzoic acid, p-nitrophenol.

Comparison of the degree of inhibition derived from equimolar concentrations of each inhibitor:

Experiments were conducted in which the degree of inhibition of each of the four inhibitors was compared to ascertain the most potent inhibitor compound. The test system contained: 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$, 5.0 μmoles D-glucose-6-phosphate, 0.1 ml of the enzyme, 0.1 ml of various concentrations of either diethylstilbestrol, hexestrol, dienestrol, or benzestrol, 0.6 μmoles NADP, and water to a total volume of 2.7 ml. The reciprocals of the reaction velocities obtained

at several concentrations of each inhibitor are plotted in Figure 4. The degree of inhibition, with all inhibitors, is proportional to the concentration of the inhibitor compound. Also, at any given concentration, benzestrol or dienestrol is a more efficient inhibitor than diethylstilbestrol or hexestrol.

Reversibility of the inhibitions:

The kinetic method of Ackermann and Potter (1) was employed to determine if the inhibition by the synthetic estrogens was reversible. The reaction velocity at several concentrations of the enzyme was determined in the presence of two inhibitor concentrations and in the absence of the inhibitor. In analysis of a reversible inhibition the resultant lines intersect at the origin and the slopes of the lines decrease as the inhibitor concentration increases. However, when an inhibitor either does not dissociate from the enzyme or has a dissociation constant that is so small it appears to be non-dissociable, the resultant lines have the same slope (are parallel). The lines obtained in the presence of the apparently non-dissociable inhibitor do not pass through the origin but intersect the abscissa at values directly proportional to the inhibitor concentration, indicating the inhibitor effectively titrates the enzyme (1). The test system in these experiments contained: 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$, 5.0 μmoles D-glucose-6-phosphate, 0.1 ml of enzyme at different concentrations, 0.1 ml of 50 percent (v/v) aqueous propylene glycol or 0.025-0.1 μmole of an inhibitor dissolved in this solvent, 0.6 μmole NADP and water to a total volume of 2.7 ml. The results (Figures 5 and 6) clearly establish that diethylstilbestrol, hexestrol, dienestrol, and benzestrol are reversible inhibitors of this enzyme system.

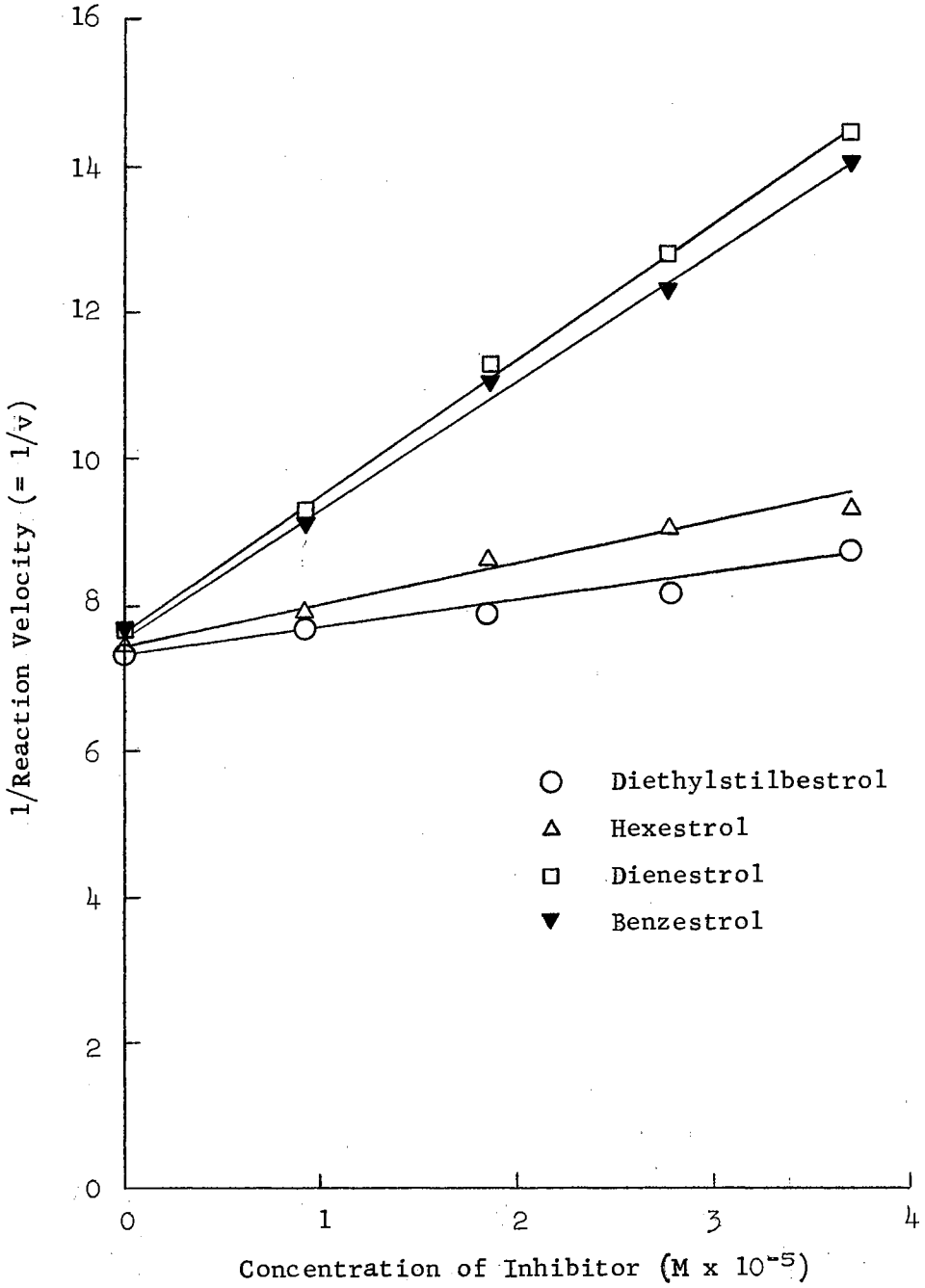


Figure 4. Effect of varied concentrations of the inhibitors on the reaction velocity

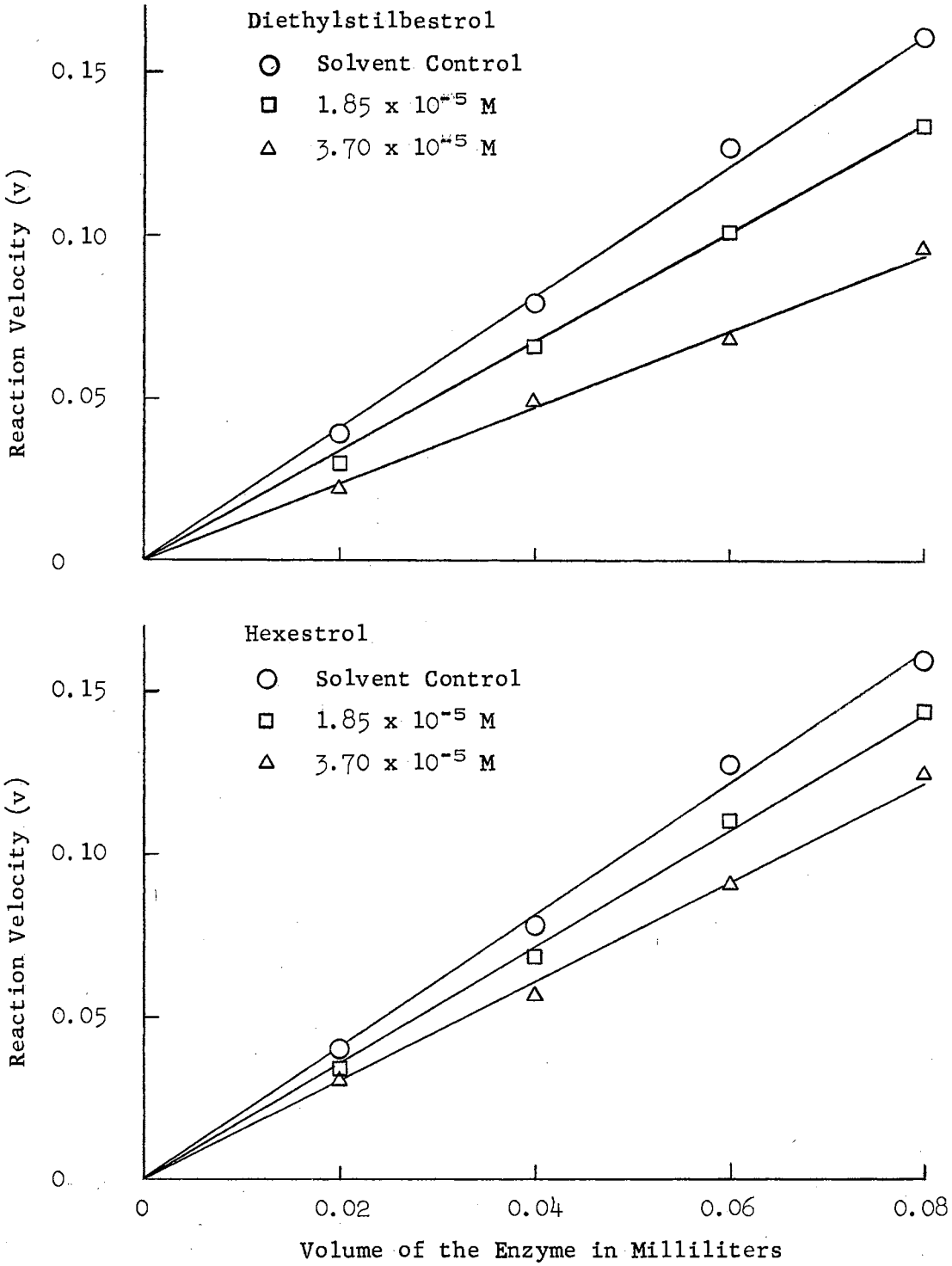


Figure 5. Ackermann-Potter plots of the inhibitions by diethylstilbestrol and hexestrol

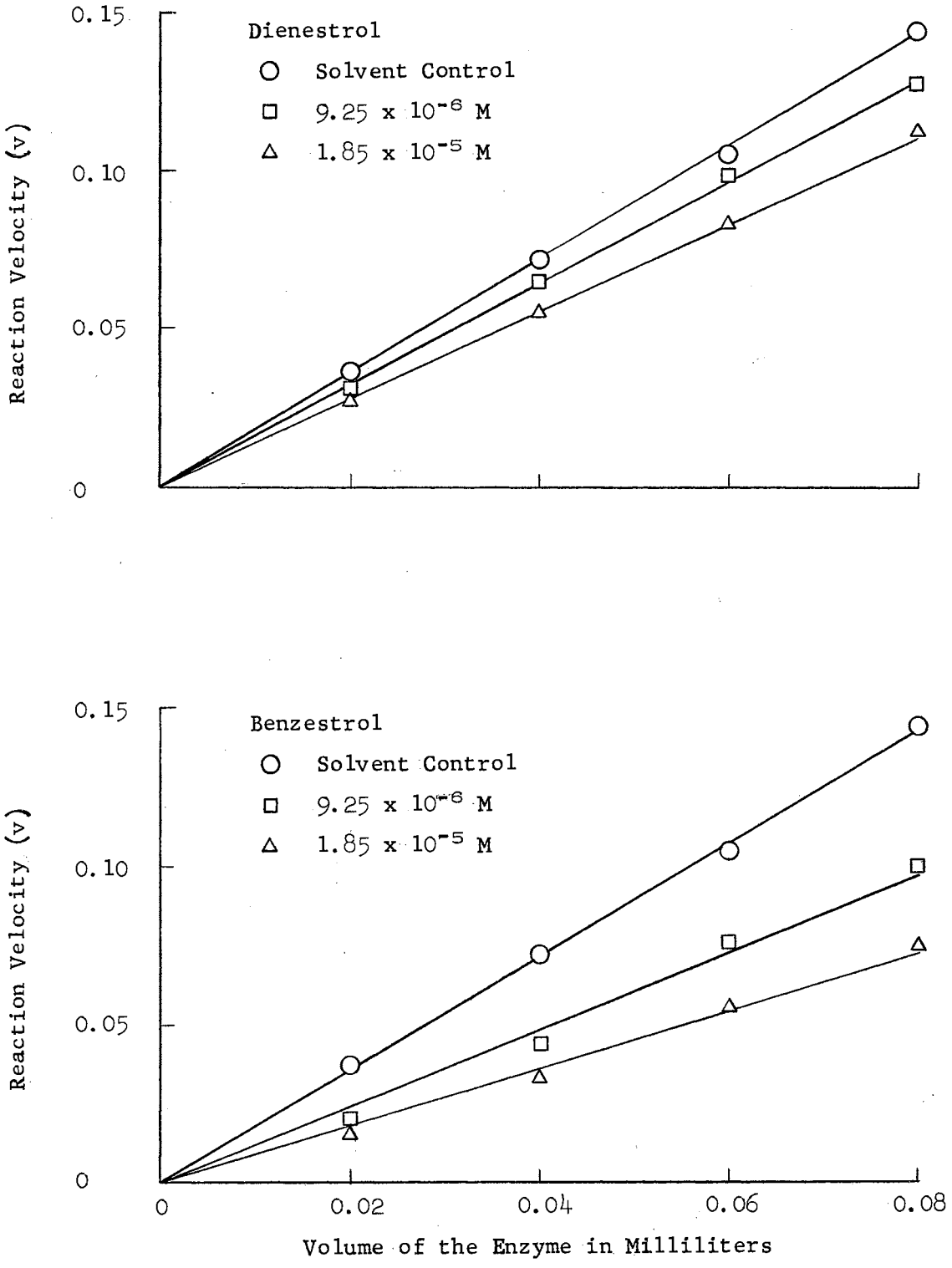


Figure 6. Ackermann-Potter plots of the inhibitions by dienestrol and benzestrol

Studies were conducted to confirm the reversibility of the inhibition by diethylstilbestrol in which an inhibited system was dialyzed in an attempt to remove the dissociated inhibitor molecules from the system and thereby reverse the inhibition. Four solutions (A-D) were prepared. Solutions A and C each contained 600 μmoles tris-HCl buffer (pH 7.6), 90 μmoles $MgCl_2$, 0.3 ml of enzyme, and 0.3 ml of 50 percent (v/v) aqueous propylene glycol, in a total volume of 8.1 ml. Solutions B and D were identical to A and C except that the 0.3 ml of 50 percent (v/v) aqueous propylene glycol portion contained 0.3 μmoles diethylstilbestrol. All solutions were permitted to stand for 20 minutes at 4 C, then solutions C and D were dialyzed seven days against 1500 ml (three portions of 500 ml each) of solution in which fractionation buffer was substituted for enzyme. Constant mixing of the dialysis solutions was accomplished by magnetic stirrers. All four solutions were maintained at 4 C during dialysis. The four solutions were assayed for enzymatic activity by adding 0.1 ml of 0.03 M D-glucose-6-phosphate and 0.2 ml of 0.004 M NADP to 2.7 ml of each solution (A-D). The results are summarized in Table II.

TABLE II

REVERSAL OF THE DIETHYLSTILBESTROL INHIBITION BY DIALYSIS

Solution	Treatment	Diethylstilbestrol Concentration	Reaction Velocity	Percent Inhibition
A	Not Dialyzed	0	.092	
B	Not Dialyzed	3.33×10^{-5} M	.060	34.8
C	Dialyzed	0	.092	
D	Dialyzed	3.33×10^{-5} M	.085	7.6

Since dialysis caused a reduction in the inhibition from 34.8 percent

to 7.6 percent, the results augment the previous findings and establish that the inhibition by diethylstilbestrol is reversible, and the inhibitor-enzyme complex does dissociate. The control systems indicated that dialysis alone did not influence the enzyme activity since the activity in both of the uninhibited systems (A and C) was the same following dialysis.

Kinetic characterization of the inhibitions:

Having established the reversible nature of the inhibitions, kinetic experiments were performed to further characterize the mode of action of the stilbenoid inhibitors. Enzyme reaction velocity was measured at several concentrations of each substrate (D-glucose-6-phosphate or NADP) in the presence and absence of each inhibitor. When the concentration of one substrate was varied, the concentration of the other substrate was held constant and at a high level (2). The results are presented as double reciprocal plots according to the method of Lineweaver and Burk (21) with the modification of Dixon (8). The reciprocal of the reaction velocity is plotted against the reciprocal of the substrate concentration, and the points which correspond to each inhibitor are connected with straight lines which are extrapolated to intersect the abscissa (Figures 7 and 8). The test systems contained: 200 μmoles tris-HCl buffer (pH 7.6), 30 μmoles $MgCl_2$, the substrates incorporated at various concentrations, 0.1 ml enzyme, 0.1 ml of either 0.001 M diethylstilbestrol, 0.001 M hexestrol, 0.00025 M dienestrol or 0.00021 M benzestrol, and water to a total volume of 2.7 ml. In the experiment plotted in Figure 7 each system contained 0.8 μmole NADP, and the D-glucose-6-phosphate varied from 0.5 to 2.0 μmoles. In the experiment plotted in Figure 8 each system contained 20.0 μmoles D-glucose-6-phosphate, and the NADP varied from

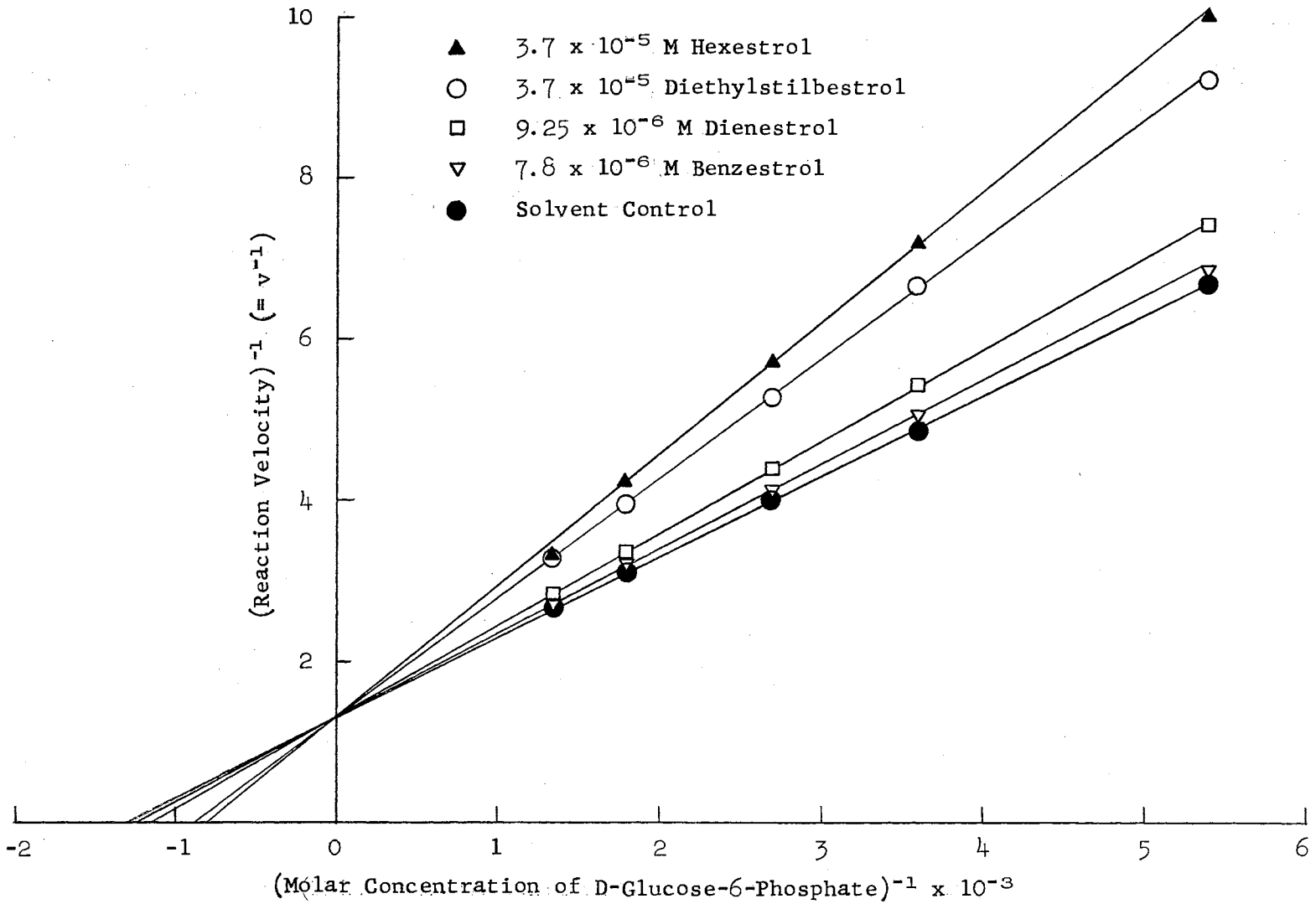


Figure 7. Lineweaver-Burk plots of the inhibitions with respect to D-glucose-6-phosphate

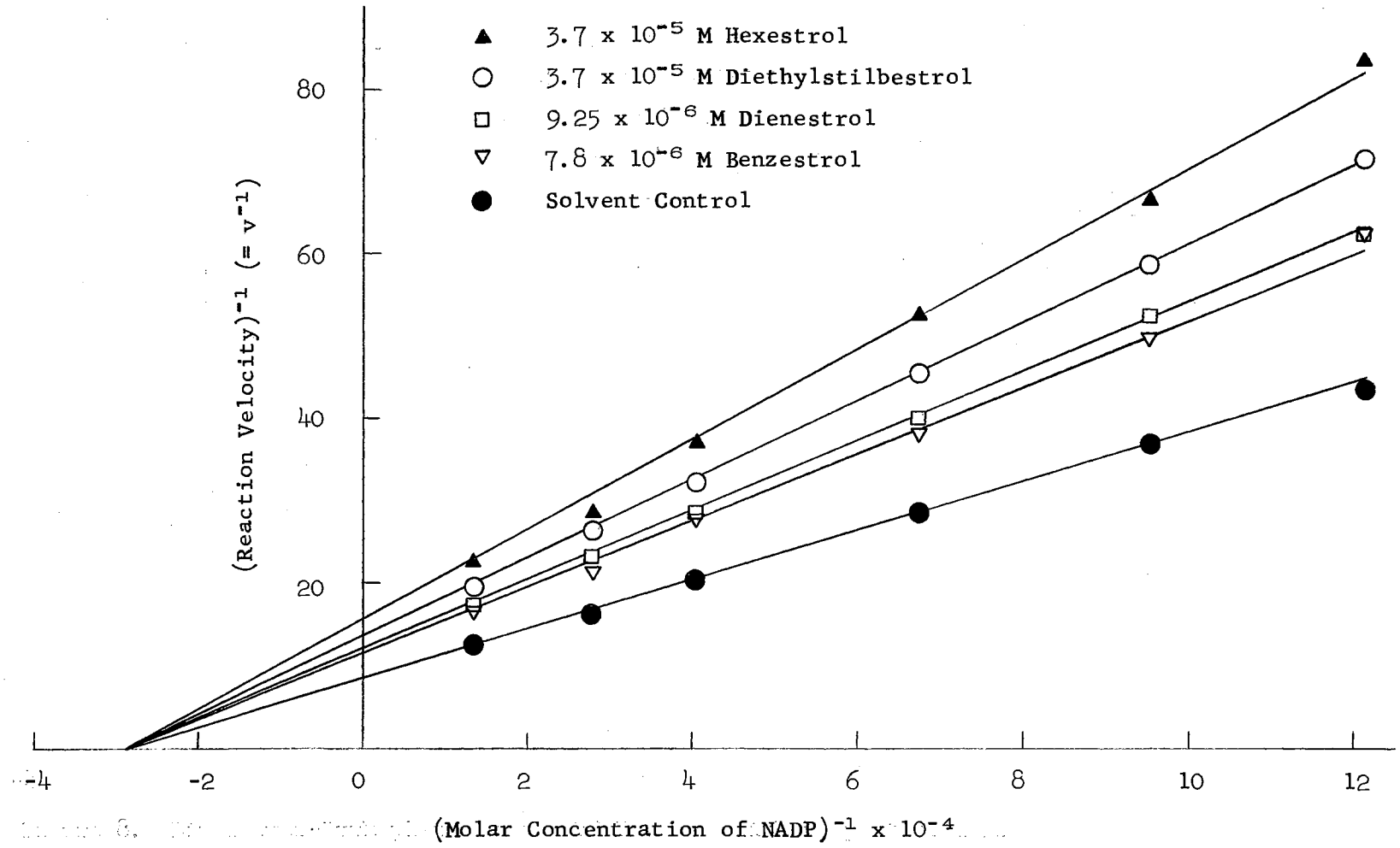


Figure 8. Lineweaver-Burk plots of the inhibitions with respect to NADP

0.02 to 0.08 μmoles.

Analysis of the Lineweaver-Burk-type double reciprocal plot reveals that if the lines intersect at a given point, the velocity and substrate coordinates of that point are the same for each line. Since the lines represent reactions both in the presence and absence of the inhibitors, the point of intersection is the theoretical condition at which the effect of each inhibitor is overcome or reversed. A plot of the inhibition with respect to D-glucose-6-phosphate shows that the lines intersect on the ordinate, where the D-glucose-6-phosphate concentration is theoretically infinite (Figure 7). The kinetic data show that the inhibitions by diethylstilbestrol, hexestrol, dienestrol and benzestrol can be completely reversed in the presence of infinitely high concentrations of the substrate, D-glucose-6-phosphate. Thus, each inhibitor interferes or competes with the binding of D-glucose-6-phosphate to the active enzyme surface and as a result, the inhibitors are conventionally termed competitive with this co-substrate.

A plot of the inhibition with respect to NADP shows that the lines intersect on the abscissa and, since there is no common point of intersection on the ordinate, the theoretical maximum velocity attained at infinitely high concentrations of NADP in the presence of the inhibitors is lower than that attained in their absence. Thus, the inhibitions can not be reversed by high concentrations of NADP at a given concentration of D-glucose-6-phosphate. The kinetic data show that the inhibitors do not interfere with the binding of the NADP co-substrate to the enzyme surface. Therefore, the inhibitors are non-competitive with NADP.

Dixon has demonstrated that the Michaelis constants (K_m) can be conveniently obtained from Lineweaver-Burk-type double reciprocal plots

(8) merely by extrapolating the lines to intersect with the abscissa (1/reaction velocity = 0). At the point of intersection-$1/S = -1/K_m$ where S is the molar concentration of the substrate varied. The Michaelis constants determined in this manner from Figures 7 and 8, respectively, are 7.6×10^{-4} M for D-glucose-6-phosphate and 3.6×10^{-5} M for NADP. It is evident from the data in Figures 7 and 8 that the presence of the inhibitors increases the apparent K_m value for D-glucose-6-phosphate but does not influence the K_m value for NADP.

Comparison of dimensions of molecular models:

Since the results from kinetic studies imply that the substrate D-glucose-6-phosphate and the four stilbenoid inhibitors may be reversibly bound in a mutually exclusive manner at or near the same binding site on the enzyme, the molecular dimensions of these compounds were compared. Scale models (one centimeter equals one angstrom unit) were assembled from a Fisher-Hirschfelder-Taylor Atom kit. Overall lengths were estimated by measuring the longest dimension of the models and are presented in Table III. The three possible free configurations of D-glucose-6-phosphate were measured since the degree of enzyme substrate specificity was not known. The particular isomer cited in Table III for the inhibitors was the form actually used in the experiments, according to the melting points reported by the manufacturer and the review by Grundy (14). The molecular models of hexestrol, dienestrol and benzestrol can be flexed to become considerably shorter than the extended lengths, while the model of diethylstilbestrol is relatively fixed in length by the central double bond. Although the molecular dimensions of D-glucose-6-phosphate and the inhibitors are of the same order, no close correlation exists between the size of the molecules and the efficiency of inhibition.

TABLE III

MOLECULAR DIMENSIONS OBTAINED FROM FISHER-HIRSCHFELDER-TAYLOR MOLECULAR MODELS

Molecule	Overall Length, Å
D-Glucose-6-phosphate	14.3
α-D-Glucopyranose-6-phosphate	12.4
β-D-Glucopyranose-6-phosphate	11.8
Diethylstilbestrol (*trans*)	15.5
Hexestrol (*meso*) --extended	15.1
--flexed	9.2
Dienestrol (*trans*-*trans*) --extended	15.0
--flexed	12.1
Benzestrol --extended	16.4
--flexed	13.9

CHAPTER IV

SUMMARY AND CONCLUSIONS

The mode of action of the inhibition of a partially purified D-glucose-6-phosphate dehydrogenase from A. aerogenes by stilbenoid estrogens has been established. The partially purified enzyme has a pH optimum of 7.6 and is activated by 0.01 M Mg^{++}, Mn^{++} or Ca^{++}. Experiments demonstrated the absence of activators, inhibitors, or interfering enzymes and established that the 13-fold purification obtained was suitable for kinetic experiments. A high degree of structural specificity was exhibited by the enzyme for the inhibitor molecules. Of thirty-two compounds tested which had structures analogous to diethylstilbestrol, only four (diethylstilbestrol, hexestrol, dienestrol and benzestrol) significantly inhibited the D-glucose-6-phosphate dehydrogenase. All inhibitors possess two unsubstituted para-hydroxyl groups and a central aliphatic chain of two or three carbons. The inhibition was a function of the concentration of the inhibitor, and at equimolar concentrations dienestrol or benzestrol were more potent inhibitors than hexestrol or diethylstilbestrol.

The inhibitions were reversible as measured by a kinetic method of Ackermann and Potter (1). The inhibition due to diethylstilbestrol was reversed by dialysis of the enzyme-synthetic estrogen mixture. The method of Lineweaver and Burk (21) showed that the stilbenoid estrogens inhibited the D-glucose-6-phosphate dehydrogenase by competitively interfering with

the binding of the substrate D-glucose-6-phosphate to the enzyme. The stilbenoid compounds did not interfere with the binding of the co-substrate NADP to the enzyme. The mode of action of each of the four inhibitor compounds is the same. Specifically, each of the inhibitors and D-glucose-6-phosphate form mutually exclusive complexes with the enzyme and since the oxidation of D-glucose-6-phosphate is catalyzed only when this substrate is complexed with the enzyme, the effect of the inhibitor is to lower the concentration of free enzyme available for such complexing. Hence, the rate of the observed reaction is inhibited by the stilbenoid estrogens.

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