RELATION OF CHEMICAL STRUCTURE TO THE INDUCTION OF Δ^1-DEHYDROGENASE FROM NOCARDIA RESTRICTUS

by

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(Under the supervision of Professor Charles J. Sih)

The actinomycete, Nocardia restrictus (ATCC 14887) possesses three steroid inducible enzymes capable of introducing double bonds into the C-1(2) and 4(5) positions of steroids possessing either the 5α or 5β configuration. Steroids are rigid molecules of known conformations and thus are attractive model compounds for mapping the binding pocket of the repressor. The interaction between the repressor and inducers can be assessed by comparing the rate of induction of Δ^1-dehydrogenase by a series of alicyclic inducers that bear the same functional group but differ in key positions on the molecule. These studies provide information pertaining to the topochemical pattern of the repressor, complementary to the inducer. Based on this rationale, a systematic correlation of chemical structure of alicyclic compounds to induction of Δ^1-dehydrogenase was made.

The Δ^1-dehydrogenase of N. restrictus is an inducible enzyme and has been found to be coordinately linked with the Δ^4-5β-dehydrogenase. It has been found that the entire steroid nucleus is not needed for induction. Bicyclic compounds such as trans-8-methylhydrindan-1-one is capable of inducing Δ^1-dehydrogenase to high levels. In each case, the

addition of methylene groups to positions corresponding to the steroid nucleus and the presence of electronegative groups at C-17 increase the level of Δ^1-dehydrogenase. Steroids with a 3-keto function are poor inducers because they are metabolized by the organism.

The best inducer is one which has a large hydrophobic group corresponding to the steroid skeleton possessing an electronegative group at C-17 such as 5α-androstan-17-one. It has a K_m of 1.80×10^{-6} M. Since it is not metabolized by the organism it serves as a gratuitous inducer.

Approved: Charles J. Sih

May 8, 1973

Date

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A thesis submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

by

George P. Peruzzotti

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May 19__

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I wish to dedicate this to my parents and my wife Ruth, whose love, understanding and personal sacrifices made the attainment of this goal possible and to whom I am forever indebted.

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I. INTRODUCTION

One of the most striking and significant developments in synthetic organic chemistry is the application of biological systems to effect chemical reactions. Because of their high specificity, biological systems in many instances possess distinct advantages over conventional organic reactions. Industrially, the manufacture of many commercial steroids consists of transforming readily available basic steroids microbiologically to important intermediates, which can then be conveniently converted chemically to the final product, or vice versa.

The ability of enzyme systems from various microorganisms to carry out a great number of different chemical reactions on steroids is now well documented. Steroid hormones and their derivatives were originally studied by means of cells and enzymatic systems of higher vertebrates. The first information concerning their metabolism was also obtained from this source when the administered steroids were estimated after their excretion from the body in the urine. Using a similar approach, transformation of steroids were analyzed after perfusion of organs, notably the liver, or after incubation with tissue slices, cell homogenates or with subcellular particles or fractions. The enzymatic systems of microbial cells are capable of carrying out the same transformation of the steroid skeleton as do enzymes of animal origin. The metabolism of steroids by animal tissues

results in low yields of desired products which not only complicates the isolation and characterization of these compounds but also the attainment of sufficient quantities for pharmacological evaluation. In contrast, microorganisms possess adaptive enzyme systems which can under suitable conditions be raised to very high levels to produce desired products in substantial quantities. For this reason microorganisms have become an invaluable tool in the commercial synthesis of steroid hormones.

II. LITERATURE

The enzymatic introduction of double bonds into steroids comprise a group of reactions widely distributed among microorganisms (1,2). Industrial applications of these reactions have made accessible a variety of highly important analogs of adrenocorticosteroids with proven and interesting properties. Adrenocortical hormones are used clinically not only in replacement therapy, but also in inflammatory diseases where there is no demonstrable abnormality in the corticosteroid metabolism. They alleviate the symptoms of rheumatoid arthritis, such as pain, swelling and stiffness of joints without affecting the course of the disease (3,4).

The introduction of unsaturation into ring A of steroids is of interest in connection with the aromatization involved in the synthesis of estrogens and in the microbiological formation of 1-dehydrosteroids some of which possess interesting physiological properties. Clinically, the 1-dehydro analogs of cortisone and cortisol, prednisone and prednisolone, have 3-5 times greater anti-inflammatory activity than the parent compounds and cause far less retention of salt and water (5,6). In 1953 Fried *et al.* (7) and Vischer and Wettstein (8) demonstrated that double bonds could be introduced at the C-1(2) positions of progesterone with degradation of the side chain. The direct introduction of a 1(2)-double bond into ring A of C_{21}-steroids without any other structural changes was reported by Nobile in 1955 (9). Since

this observation many new organisms, which carry out the introduction of a 1(2)-double bond have been reported (10-17). Other anti-inflammatory steroids have been prepared with additional ring substituents, and such compounds have shown marked improvements in biological activity. However, all such steroids possess the double bond at C-1(2) as a common constituent.

Dehydrogenation of steroids at C-1 and C-4 by cell-free microbial enzymes was first reported by Levy and Talalay (18) using P. testosteroni. They reported that the cell-free extracts contained three separate steroid inducible enzymes capable of promoting the following stereospecific dehydrogenations of ring A of steroids: 1(2)-dehydrogenation, 4(5)-dehydrogenation of steroids in which the A:B ring fusion is cis or trans (19,20). The Δ^1 and Δ^4-5α-dehydrogenase of these preparations were firmly bound to fine particles probably derived from the cell membrane, and these activities sedimented only after prolonged centrifugation at 100,000 X g. The reaction also required the presence of large intracellular particles (sedimentable at 10,000 X g) but this fraction can be replaced by artificial electron acceptors. Phenazine methosulfate was an extremely efficient acceptor for the dehydrogenases of P. testosteroni. Although a partial separation of the Δ^1- and Δ^4-5α-dehydrogenases was reported by Levy and Talalay (2), extensive purification was not accomplished because of lack of success in solubilizing these particulate enzymes. In contrast, the Δ^4-5β-dehydrogenase

was solubilized and purified 100-fold from acetone powders of cells grown on media containing testosterone or progesterone as inducers (21,22).

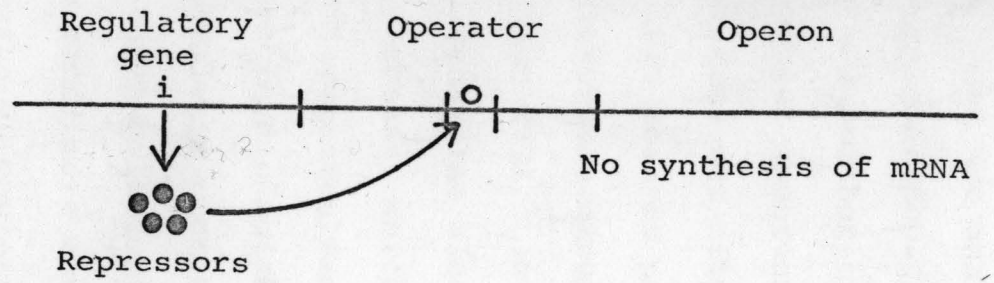
In 1960, Sih and Bennett reported that cell-free extracts of N. restrictus were capable of introducing double bonds into the C-1(2) and C-4(5) positions of steroids possessing either the 5α- or 5β-pregnane nucleus (13). The properties of this Δ^1-dehydrogenase were described in detail by Sih and Bennett (23). The Δ^1-dehydrogenase of P. testosteroni and N. restrictus possess similar properties: they are adaptive enzymes associated with intracellular particles, are sensitive to sulfhydryl inhibitors, capable of utilizing phenazine methosulfate as an electron acceptor and have an alkaline pH optimum. Evidence was also presented that the Δ^1-dehydrogenase of N. restrictus contains a flavin prosthetic group which catalyzes a direct dehydrogenation of the steroid. However the Δ^1-dehydrogenase of N. restrictus differed from that of P. testosteroni in that it was capable of introducing a 1(2)-double bond into 11-oxygenated steroids, utilized oxidation-reduction dyes as electron acceptors and exhibited high activity at pH 7.0, the pH at which the dehydrogenase activity of P. testosteroni was negligible. The bacterial Δ^1-dehydrogenase from Arthrobacter simplex was also shown to have properties similar to that of N. restrictus by Kondo (24).

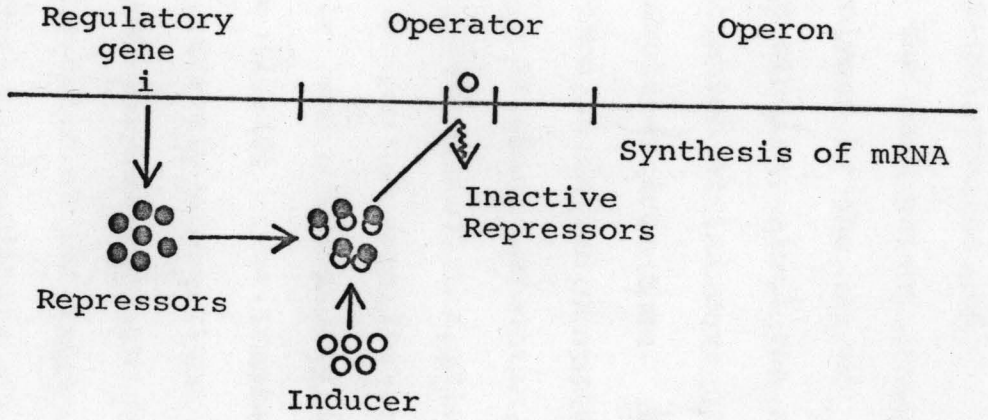
The mechanism of regulation of gene expression is today one of the most actively studied problems in molecular biology, in good part as a result of the pioneering work of

Jacob and Monod on the control of the genes involved in lactose metabolism. In 1961, Jacob and Monod (25,26) presented strong genetic and enzymological evidence for the regulation of protein synthesis at the transcription level. The operator model proposed by Jacob and Monod to explain the regulation of enzyme synthesis has ever since been in the focus of all discussions of regulation. According to this model (Figure 1), the bacterial genome is functionally divided into a number of units of expression, the operon containing the structural genes of one or more enzymes. The transcription of this information into mRNA is under the control of two genetic loci, the regulator gene (i) and the operator gene (o). The regulator gene is the structural determinant of a repressor with 2 functional sites, one interacting with an inducer and the other with steric specificity toward the (o) locus. Attachment of the repressor to the operator locus is believed to block the transcription of the entire operon. With inducible enzymes the addition of inducer is thought to result in a steric modification of the repressor, leading to a decrease in the affinity for the operator locus. This results in a derepression of the operon and a production of mRNA.

The inducible enzymes are found chiefly among those that catalyze the entry into the cell and the breakdown of metabolites supplied in the growth medium. These enzymes are produced in quantity only if the relevant metabolite, or a close analog thereof, is present in the medium. The lactose (lac)

Figure 1. Mechanism of induction and repression according to the operon model proposed by Jacob and Monod (25).


Regulatory
gene
i
Operator
Operon
No synthesis of mRNA
Repressors


Regulatory
gene
i
Operator
Operon
Synthesis of mRNA
Inactive
Repressors
Repressors
Inducer

system of E. coli is the best studied example of gene regulation. Most wild type strains of E. coli can utilize lactose as their main carbon source, and when they do the cells synthesize β-galactosidase. This enzyme acts as a transgalactosidase by splitting lactose and a number of other galactosides. The two analogs methyl-β-D-galactoside and isopropylthio-β-D-galactoside are the most potent inducers known and neither of them is hydrolyzed by the enzyme. This is of great importance for kinetic studies, since the enzyme formed does not affect the concentration of inducers of this kind, nor does it produce new catabolites from them. This very favorable experimental condition is called gratuitous induction (27). In typical E. coli strains, induction of β-galactosidase also releases the synthesis of the galactoside permease, which is responsible for the specific transport of galactosides into the cells, and a transacetylase of unknown physiological significance (28,29). The transacetylase is always produced in amounts strictly proportional to those of β-galactosidase and the two enzymes are said to be under coordinate control (30). The ratio of the rates of synthesis of enzymes that are subject to coordinate control remains constant despite extreme fluctuations in their absolute ratios of synthesis.

Steroids are attractive model compounds for a study of the interaction with the repressor because they are rigid molecules of known conformation. These advantages were pointed out by Ringold and coworkers (31) and utilized by

them in an exhaustive investigation of the "topochemical pattern" of the steroid binding site of 3α-hydroxysteroid dehydrogenase from Pseudomonas testosteroni. In order to determine which segments of the steroid are complementary to the enzyme and important for recognition they studied the rate of reaction, relative to cyclohexanone, and the stereochemistry of reduction of a number of mono-, bi-, tri-, and tetracyclic derivatives of cyclohexanone. In each case the substituents that were added to the basic cyclohexanone nucleus corresponded in their spatial position to part of the steroid nucleus. Their studies with various steroid analogs enabled them to identify the role of individual carbon atoms in allowing the enzyme to discriminate between stereoisomers as well as providing rate enhancement during catalysis.

The importance of hydrophobic interactions has been reviewed by Némethy (32). In recent years the role of hydrophobic bonding in the maintenance of the secondary and tertiary structure of proteins and other macromolecules in aqueous solutions has become increasingly clear. The term hydrophobic bonding describes the tendency of non-polar groups to associate in aqueous solution, thereby reducing the extent of contact with neighboring water molecules. The formation of hydrophobic bonds is favored only because of an entropy effect: the water molecules become more ordered around exposed non-polar solutes. When the hydrophobic bond is formed the order decreases, resulting in favorable entropy

and hence free energy of formation.

The effect of hydrophobic bonding on the binding of inhibitors to enzymes has been treated quantitatively in a paper by Anderson *et al.* (33) who have studied the inhibition of yeast alcohol dehydrogenase by 1-alkyl-3-aminocarbonyl-pyridinium chlorides, as a function of the alkyl chain length over the range C_1 to C_{11}. The effectiveness of the inhibitors, as measured by the binding constant for inhibition, increases linearly with chain length from C_3 to C_{11}.

Although the induction and repression of bacterial enzymes involved in the metabolism of carbohydrates, amino acids, purines and pyrimidines have received some attention, to my knowledge with the exception of the β-galactoside system no systematic examination has been made to date with respect to the relationship of chemical structure to enzyme induction.

N. restrictus (ATCC 14887) possesses a series of inducible enzymes capable of introducing double bonds into C-1(2) and C-4(5) positions of steroids bearing either the 5α or 5β configuration. The objectives of our investigation were to establish whether the enzymes involved in ring A dehydrogenation are coordinately induced; to ascertain whether the inducer for Δ^1-dehydrogenase in *N. restrictus* requires a certain spatial configuration in order to elicit maximal enzyme activity and to determine the affinity constants of some of the inducers with that of the repressor.

III. EXPERIMENTAL PROCEDURE

A. MATERIALS

The following chemicals were purchased from Aldrich Chemical Company: dimethylforamide, phenazine methosulfate, cyclopentanone, cyclohexanone, cycloheptanone, 1-decalone, 2-decalone, 7,7α-dihydro-7α-methyl-1,5-(6H)-indandione (I), 3,4,8,8α-tetrahydro-8α-methyl-1,6-(2H,7H)-naphthalenedione (II), perhydrophenanthrene (IV), 7-keto-1-methoxy-13-methyl-5,6,7,9,10,13-hexahydrophenanthrene (V), 7-keto-13-methyl-5,6,7,9,10,13-hexahydrophenanthrene (VI). Trans-8-methyl hydrindan-1-one (III) was synthesized by Mr. Paul Foss by the method of Johnson (34). Anti-trans-8β-methyl-4,5-(5α-methylperhydrobenzo) hydrindanone (VII) was prepared by Dr. L. Gsell by Wolff-Kishner reduction and Jones oxidation of anti-trans-1β-hydroxy-8β-methyl-4,5-(5α-methyl,4-oxoperhydrobenzo)hydrindane which was supplied by Hoffman-LaRoche, Inc. Trans-1β-hydroxy-8β-methyl-4,5-(4-methoxybenzo)hydrindane (VIII) and retro-trans-1α-hydroxy-8α-methyl-4,5-(4-methoxybenao)hydrindane (IX) were gifts from Roussel UCLAF. Syntex Research kindly made the following two compounds available to us: 4-cyclohexyl-cyclohexanone and 3-methoxy-14β-1,3,5(10)-estratrien-17-one (XX). The following steroids were purchased from Steraloids Inc.: 5α-androstan-3-one (XIII), 5α-androstan-3,17-dione (XIV), 5α-androstan-17-one (XVI), 5β-androstan-17-one (XVII), and 5α-androstane (XVIII).

The following steroids were obtained from Mann Research Laboratories: 5α- and 5β-androst-1-ene-3,17-dione, and 5β-androstane-3,17-dione (XV). 5α-Androstane-4,17-dione (XI) was prepared by Jones oxidation of 5α-androstan-17β-ol-4-one which was supplied by Dr. James Kerwin of Smith Kline and French Laboratories. The following steroids were purchased from G. D. Searle: pregn-4-ene-3,20-dione (XXI), androst-4-ene-3,17-dione (XXV), androst-4-en-17β-ol-3-one (XXVI), androst-5-en-3β-ol-17-one and pregn-5-en-3β-ol-20-one. 1,3, 5(10)-Estratrien-3-ol-17-one (XXIII) was a gift from Ayerst Laboratories. 5α-Pregnan-20-one (XXIV) was prepared by Dr. Bruce Stein from pregn-5-en-3β-ol-20-one by catalytic hydrogenation followed by tosylation, elimination and catalytic hydrogenation. Dr. Yusuf Abul-Hajj prepared the following steroids: 5α-androstan-17β-ol-2-one (X) by liquid ammonia reduction of androst-4-en-17β-ol-3-one followed by the procedures of Butenandt (35) and Clarke (36); 5α-androstane-6,17-dione (XII) from pregn-5-en-3β-ol-17-one using the methods of Nes (37) and Shoppee (38); 17 methylene-5α-androstane (XXII) was prepared from XVI via a Wittig reaction; 5α-androstan-17-oxime (XIX) was synthesized from XVI by reaction with hydroxylamine.

B. METHODS

1. Culture conditions: for Inducer Screen and Kinetic Experiments.

N. restrictus (ATCC 14887) was maintained on slants of nutrient agar supplemented with 1% Yeast extract and 2% Dextrose. Inoculated slants were incubated at 25°C for 2 weeks and stored at 4°C. A cell suspension in sterile distilled water was used to inoculate 100 ml of nutrient broth in a 500 ml Erlenmeyer flask which was incubated at 25°C for 20 hours on rotary shaker at 260 rpm with a 1 inch radius of rotation. After this initial growth period, a 10% (v/v) inoculum was used for a second stage growth in 50 ml of medium containing 0.8% nutrient broth, 2% Dextrose and 1% Yeast extract. The cells were induced 30 hours later with varying concentrations of inducer dissolved in 0.4 ml dimethylformamide.

2. Disruption of Cells and Preparation of Cell-Free Extracts.

After an induction period of 20 hours, the flasks containing the induced cultures, were harvested and cell-free extracts were prepared individually as follows:

a. Harvest: Cells in 50 ml of the induced culture were collected by centrifuging in a Servall superspeed centrifuge with a type SS-1 head at 10,000 X g for 10 min at 0-4°C. The supernatant was decanted and the cell pellet was washed with 10 ml of 0.05 M potassium phosphate buffer, pH 7.5. This was centrifuged again under the same conditions and the cell pellet was suspended in

20 ml of 0.05 M phosphate buffer, pH 7.5 and placed in a chilled 30 ml beaker.

b. Preparation of cell-free extract: The washed cell suspension was sonically disrupted with a Branson Sonifier model S-75 (20 kc) at full power for 2 min intervals with a 1 min cooling period. During sonic disruption, the suspension was kept cool in an ice-salt mixture. The cell-debris was removed by centrifugation for 15 min at 3,800 X g. The supernatant was decanted and used directly as the source of crude enzyme for Δ^1-dehydrogenase activity.

3. Enzyme Assay.

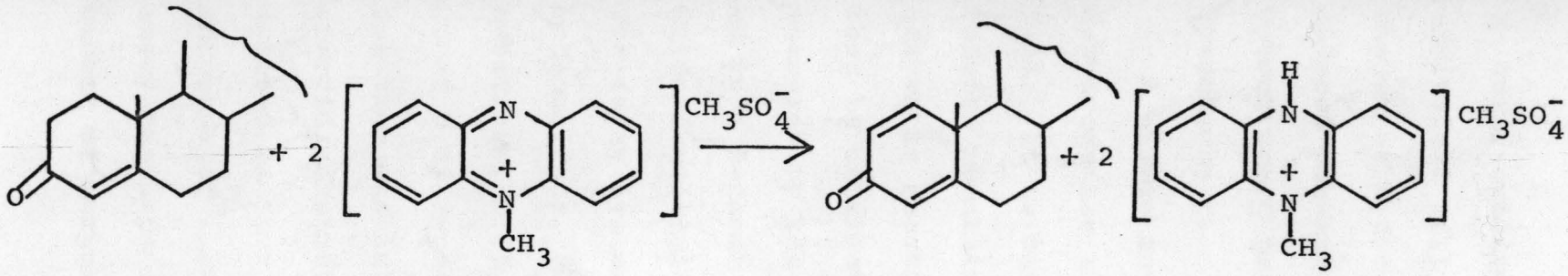
Cell-free extracts of N. restrictus have previously been shown to contain three separate induced enzymes capable of promoting stereospecific dehydrogenation in the 1(2) and 4(5) positions of steroids possessing the 5α- or 5β-configuration. The path of dehydrogenation is as follows: 3-keto 5α or 5β steroids in the presence of the appropriate dehydrogenases are converted to the Δ^4-3 keto products which can be further dehydrogenated to the $\Delta^{1,4}$ keto steroid by the Δ^1-dehydrogenase (Figure 2).

The principle of the assay method can be visualized by the two step reaction illustrated in Figure 3. In the presence of the Δ^1-dehydrogenase, the equivalent of 2 hydrogen atoms is removed from androst-4-ene-3,17-dione.

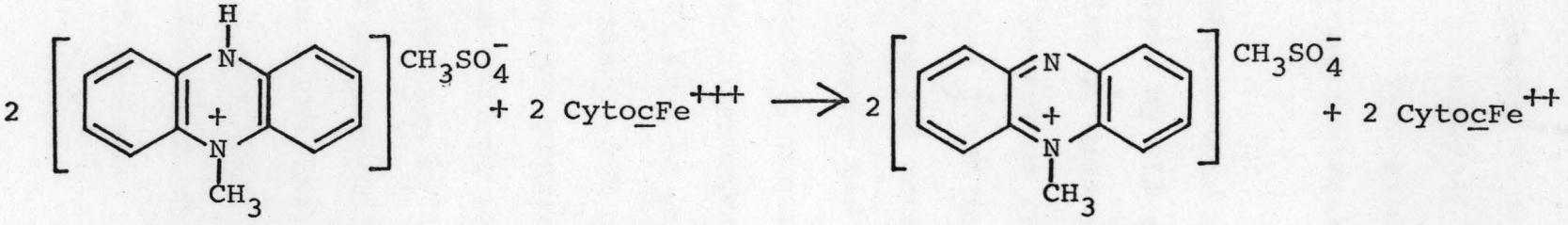
Figure 2. Pathway of dehydrogenation in Nocardia restrictus.

5βΔ 4
5αΔ 4
Δ1
H
H
O
O
O

Figure 3. Mechanism of phenazine methosulfate-cytochrome C assay.



Androst-4-ene-3,17-dione + 2 Phenazine methosulfate → Androst-1,4-diene-3,17-dione + 2 Reduced Phenazine methosulfate



2 Reduced Phenazine methosulfate + 2 Cytoc Fe^{+++} → 2 Phenazine methosulfate + 2 Reduced Cytochrome c

In turn, oxidized phenazine methosulfate accepts the equivalent of 1 electron and 1 proton in becoming reduced, the other hydrogen is released in solution as a proton. Reduced phenazine methosulfate is then oxidized by cytochrome C to give phenazine methosulfate and reduced cytochrome C.

A simple and rapid spectrophotometric assay for Δ^1-dehydrogenase activity was devised by Sih and Bennett (23). It is based on the finding of Massey (39) that reduced phenazine methosulfate undergoes rapid non-enzymatic reaction with cytochrome C and that the reaction is unaffected by air. The enzyme activity was measured by the increase in absorbancy for 1 min at 550 mμ in a Beckman DU spectrophotometer, equipped with a Gilford multiple sample absorbancy recorder model 2000. The assay system consisted of 33.3 μg of phenazine methosulfate, 700 μg of cytochrome C, 150 μg of steroid substrate, 0.05 ml of induced enzyme preparation and 0.7 ml of 0.05 M potassium phosphate buffer, pH 7.5. The reaction was initiated by the addition of the appropriate steroid substrate. One unit of enzyme activity was defined as that amount of enzyme which initiates the increase of 0.10 absorbancy in 1 min under the above assay conditions. Specific activity is expressed as the number of enzyme units per mg of protein.

Steroid Substrates for Enzymes Assayed

Steroid Substrate	Enzyme Assayed
Androst-4-ene-3,17-dione	Δ^1-Dehydrogenase
5α-Androst-1-ene-3,17-dione	Δ^4-5α-Dehydrogenase
5β-Androst-1-ene-3,17-dione	Δ^4-5β-Dehydrogenase

4. Protein Determination.

Protein concentration was determined by the Biuret method (40). Crystalline bovine serum albumin was used as the protein standard.

5. Kinetic Determination.

The reaction was followed by measuring the increase in absorbancy at 550 mμ with a Beckman DU spectrophotometer equipped with a Gilford multiple sample absorbancy recorder model 2000. The kinetic experiments were carried out using a 1 ml reaction mixture in 1 cm silica cuvettes. The cuvettes were filled with solutions containing substrate. The reaction was initiated by the addition of substrate. Initial velocities were determined by measuring the tangent to the recorded curve. Each experimental point was determined in duplicate.

6. Data Processing.

Reciprocal velocities were plotted graphically against the reciprocal of inducer concentrations and any point

which deviated greatly was discarded. All fitting of experimental data were carried out by the least squares method.

IV. RESULTS

A. OPTIMUM INDUCTION PERIOD

Talalay (41) has pointed out that in many instances, the enzymes involved in microbiological transformation of steroids were inducible and may be raised to high levels by exposing the microorganisms to steroid substrates or their analogs. In order to determine the optimum period for induction, testosterone at 1×10^{-3} M was added to cultures of N. restrictus in the early stationary phase of growth. Figure 4 shows the induction pattern, where maximal activities for the dehydrogenases were obtained in about 20 hours after the addition of steroid inducer. In addition, the specific activity of the Δ^1-dehydrogenase was approximately 6 and 12 times that of the Δ^4-5α- and Δ^4-5β-dehydrogenases.

B. EFFECT OF VARIATIONS IN MEDIUM COMPOSITION ON CELL POPULATION

Media of varying composition were tested to determine the medium for optimum cell growth and Δ^1-dehydrogenase activity. Cell population was determined by withdrawing samples periodically and observing the increase in absorbancy at 650 mμ in a Bausch and Lomb Spectronic 20 spectrophotometer. Figure 5 illustrates the growth of N. restrictus in nutrient broth and nutrient broth supplemented with combinations of Yeast extract and/or Dextrose. While nutrient broth alone supported

the growth of this organism albeit poorly, nutrient broth supplemented with 1% Yeast extract and 2% Dextrose gave the highest cell population.

Growth of N. restrictus in supplemented mineral salts media is seen in Figure 6. Mineral salts supplemented with 1% Yeast extract and 2% Sodium succinate yielded the maximum cell population whereas mineral salts with Yeast extract or Yeast extract and Glucose afforded lower levels. Cultures were induced with 1 X 10^{-3} M testosterone at the times indicated by the arrows and were assayed for Δ^1-dehydrogenase activity after an induction period of 20-22 hours.

C. COMPARISON OF Δ^1-DEHYDROGENASE ACTIVITY IN VARIOUS MEDIA

Media containing nutrient broth, 1% Yeast extract and 2% Dextrose or mineral salts with 1% Yeast extract and 2% Sodium succinate gave high cell populations of N. restrictus and in nearly equal amounts (Figures 5 and 6). A comparison of the level of Δ^1-dehydrogenase activities in the various media is given in Table 1. Although the above two media were nearly equal in cell yields only nutrient broth supplemented with 1% Yeast extract and 2% Dextrose produced the maximum yield of enzyme. The production of Δ^1-dehydrogenase in the supplemented mineral salts medium was inhibited.

D. DEMONSTRATION OF COORDINATE INDUCTION BY INDUCERS

Jacob and Monod (25) extended their hypothesis for the

Figure 4. Induction pattern of Δ^1,Δ^4,5α and Δ^4-5β dehydrogenase of N. restrictus (ATCC 14887).

At the indicated time intervals, N. restrictus cells were harvested and ruptured as described in the text. The assay procedure is described in the text.

○—○ Δ^1-dehydrogenase

△—△ Δ^4-5β-dehydrogenase

×—× Δ^4-5α-dehydrogenase

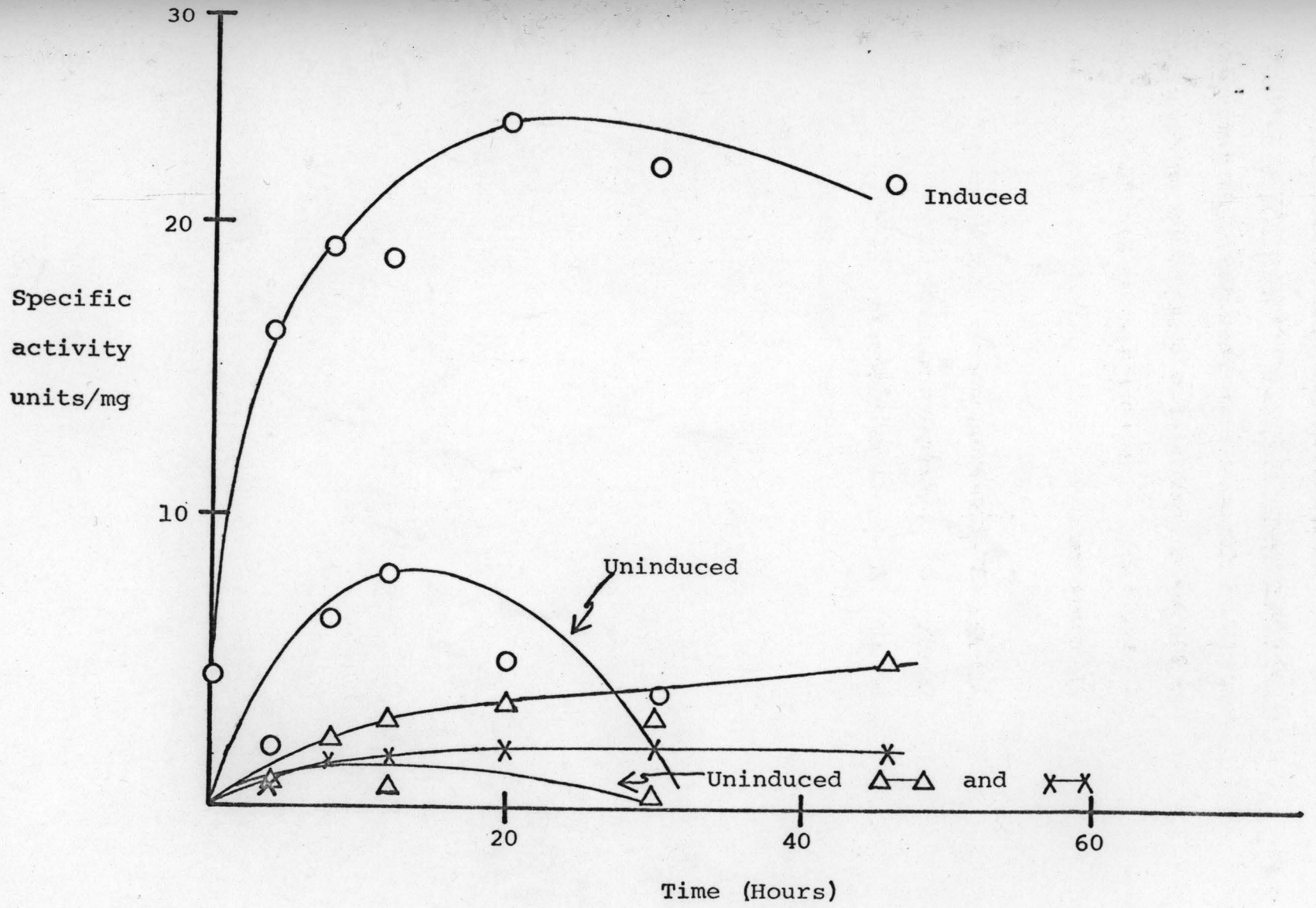
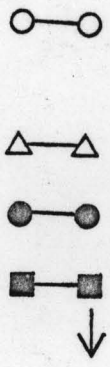

30
20
10
Specific
activity
units/mg
Induced
Uninduced
Uninduced and
20
40
60
Time (Hours)

Figure 5. Growth of N. restrictus in nutrient broth of varying compositions. Culture conditions, induction time and preparation of enzyme for assay are described in the text.



Nutrient broth , 1% Yeast extract, 2% Dextrose

Nutrient broth, 1% Yeast extract

Nutrient broth, 2% Dextrose

Nutrient broth

Indicates point of addition of inducer

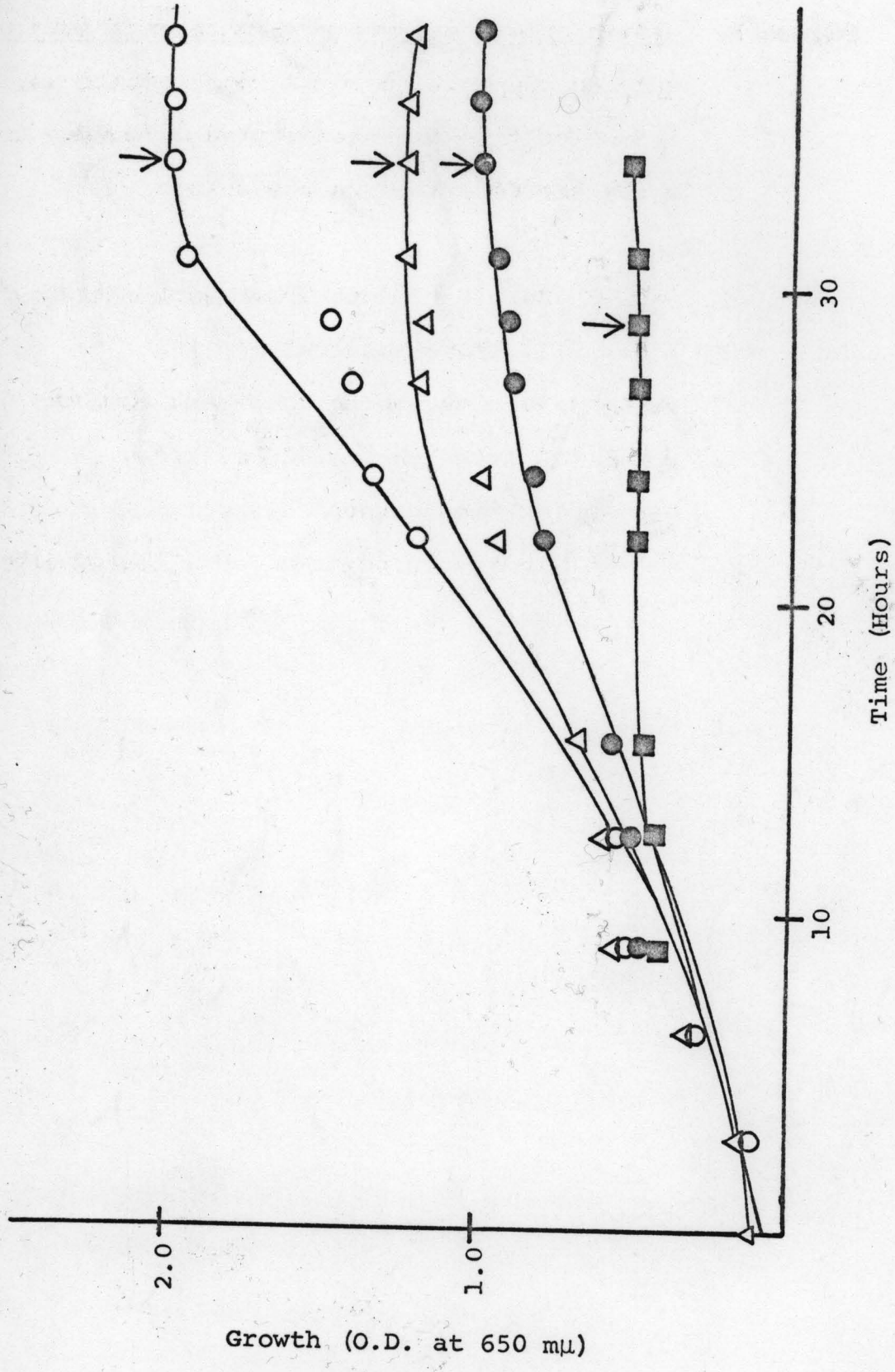

2.0
1.0
10
20
30
Time (Hours)
Growth (O.D. at 650 mμ)

Figure 6. Growth of N. Restrictus in a mineral salts medium. Culture conditions, induction time and preparation of enzyme for assay as described in the text.

○—○ Mineral salts, 1% Yeast extract, 2% Sodium succinate

△—△ Mineral salts, 1% Yeast extract, 2% Dextrose

●—● Mineral salts, 1% Yeast extract

Composition of Mineral salts medium per liter: $(NH_4)_2HPO_4$, 1 gm; $(NH_4)_2H_2PO_4$, 1 gm; KH_2PO_4, 2 gm; and 10 ml of trace element solution.

Composition of trace element solution: $MgSO_4 \cdot 7H_2O$, 20 gm; NaCl, 1 gm; $ZnSO_4 \cdot 7H_2O$, 0.5 gm; $FeSO_4 \cdot 7H_2O$, 0.5 gm; $MnSO_4 \cdot 3H_2O$, 0.5 gm; $CuSO_4 \cdot 5H_2O$, 0.05 gm; 0.1 N H_2SO_4, 10 ml; in 1 L of distilled water.

↓ Indicates point of addition of inducer.

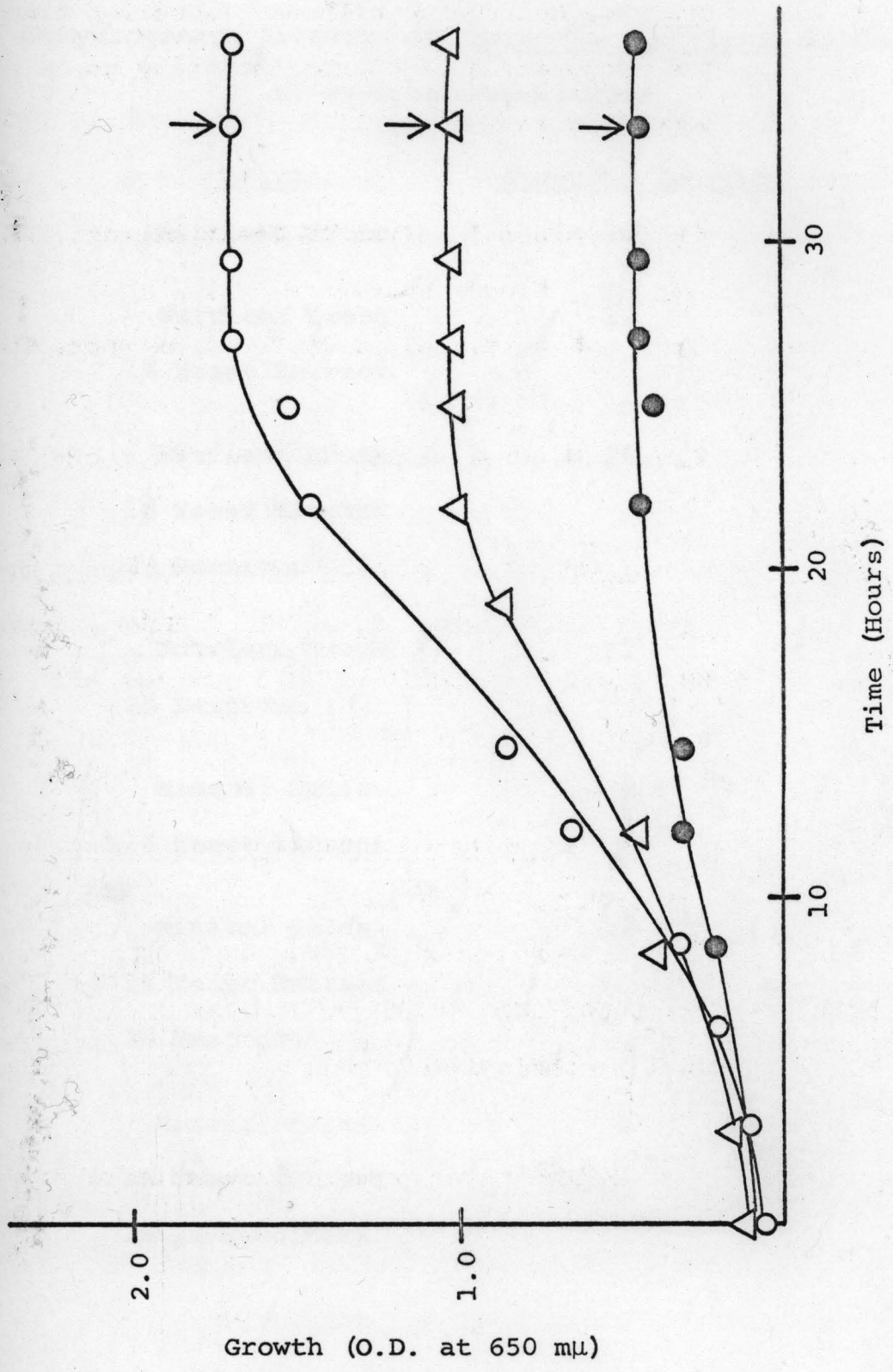

2.0
1.0
10
20
30
Time (Hours)
Growth (O.D. at 650 mμ)

TABLE 1

Δ^1-Dehydrogenase Activity of *Nocardia restrictus* in media of various compositions

Medium	Specific Activity
Nutrient Broth	16
Nutrient Broth 1% Yeast Extract	12
Nutrient Broth 1% Yeast Extract 2% Dextrose	26
Nutrient Broth 2% Dextrose	12
Mineral Salts 1% Yeast Extract	8.5
Mineral Salts 1% Yeast Extract 2% Dextrose	16
Mineral Salts 1% Yeast Extract 2% Na Succinate	1

regulation of protein synthesis to provide mechanisms for coordinate induction, in which a group of enzymes can be induced by a single inducer. In the process of defining the lac operon, they demonstrated coordinate expression of adjoining structural genes coding for three separate proteins: β-galactosidase, lactose permease, and β-galactoside transacetylase.

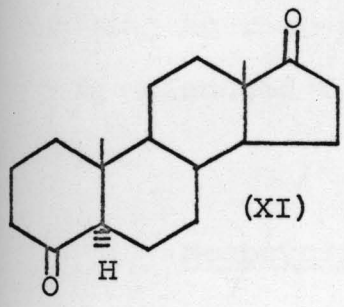
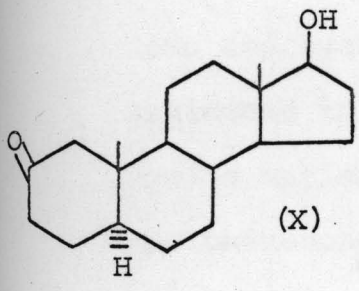
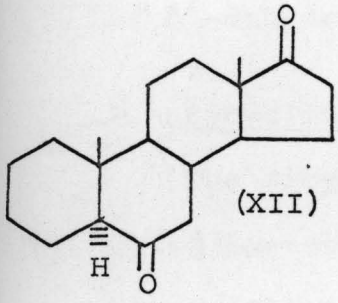
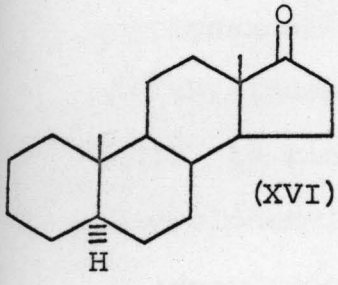
If both the Δ^1-dehydrogenase and the Δ^4-5β-dehydrogenase reside on the same operon, then the ratio of the level of Δ^1-dehydrogenase/Δ^4-5β-dehydrogenase should be constant for the inducers tested. When uninduced enzyme activity was subtracted from the total enzyme activity, the ratio was found to be close to 5 (Table 2). This value appeared to be constant for various inducers indicating that these two enzymes are coordinately regulated.

E. EVALUATION OF INDUCERS

Steroids are particularly attractive molecules for induction studies because they are rigid structures of known conformation. These advantages were discussed by Ringold et al. (31) who utilized them in an elegant investigation of the topochemical pattern of the steroid binding site of 3α-hydroxysteroid dehydrogenase from P. testosteroni. Their studies with various steroid analogs, possessing limited portions of the steroid ring system, enabled them to identify the role of individual carbon atoms in allowing the enzyme to

TABLE 2

Demonstration of Coordinate Induction by Inducers

Inducer	Specific Activity Dehydrogenase		$\frac{\Delta^1\text{-Dehydrogenase}}{\Delta^4\text{-5}\beta\text{-Dehydrogenase}}$
	Δ^1	Δ^4-5β	
 (XI)	60	12	5.0
 (X)	46	10	4.6
 (XII)	63	11	5.7
 (XVI)	69	15	4.6

discriminate between stereoisomers as well as in providing rate enhancement during catalysis. Following this rationale we have attempted to delineate the binding pocket of the repressor for Δ^1-dehydrogenase by studying the binding of alicyclic compounds, which are built up from chair forms of cyclohexanone ring by the addition of carbon atoms corresponding to the steroid skeleton. The enzyme preparations from uninduced flasks had a specific activity of 12.7 enzyme units.

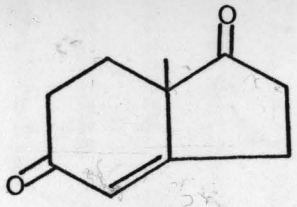
1. Monocyclic structures (Figure 7).

The monocyclic structures cyclopentanone, cyclohexanone and cycloheptanone represent the smallest unit analogous to the D or A ring of steroids. In the monocyclic series 3,4-dimethylcyclohexanol and 3,5-dimethylcyclohexanone represent the A ring with carbons at C-19 and C-6. However, none of these compounds was an inducer of Δ^1-dehydrogenase.

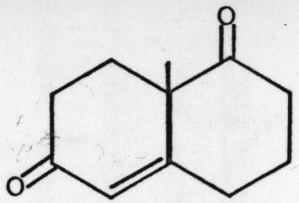
2. Bicyclic structures (Figure 7).

The bicyclic structures 1-decalone, 2-decalone and 3,4,8,8α-tetrahydro-8α-methyl-1,6-(2H,7H)-naphthalenedione (II) are analogous to rings A and B; 4-cyclohexylcyclohexanone to rings A and C; 7,7α-dihydro-7α-methyl-1,5-(6H)-indandione (I) and trans-8-methylhydrindan-1-one (III) to rings C and D. Of the bicyclic compounds tested 4-cyclohexylcyclohexanone is inhibitory to the formation Δ^1-dehydrogenase whereas trans-8-methylhydrindan-1-one

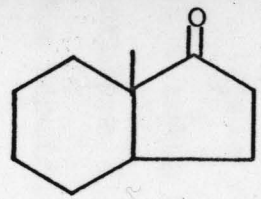
Figure 7. Mono and bicyclic steroid analogs. The arabic number under each structure represents the specific activity of Δ^1-dehydrogenase elicited by the analog.



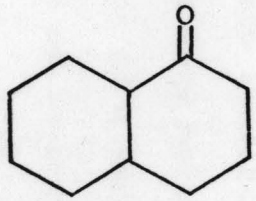
(I) 26



(II) 41



(III) 87



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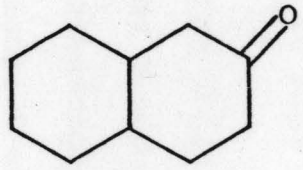

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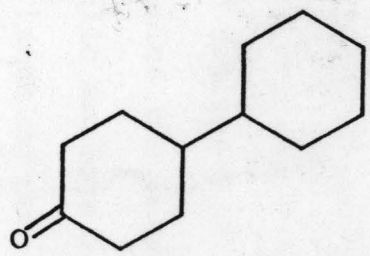

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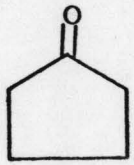
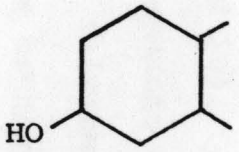


12.4



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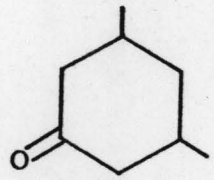


12.9

(III) exhibits high induction levels of Δ^1-dehydrogenase.

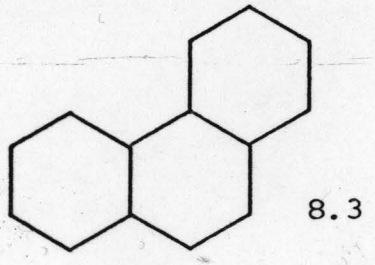
3. Tricyclic structures (Figure 8).

These structures represent the A, B and C rings or B, C and D rings of the steroid skeleton. Perhydrophenanthrene (IV) inhibited the formation of Δ^1-dehydrogenase. The remaining bicyclic analogs in Figure 8 elicited slight to moderate inducing properties. Retro-trans-1α-hydroxy-8α-methyl-4,5-(4-methoxybenzo)-hydrindane (IX) was the only compound among the tricyclic analogs, which raised Δ^1-dehydrogenase to high levels.

4. Tetracyclic structures (Figures 9 and 10).

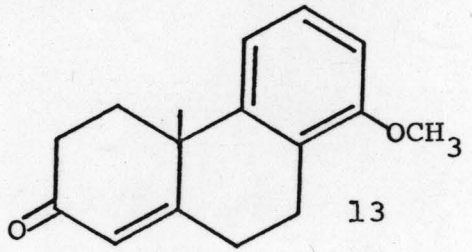
The structures of the tetracyclic compounds tested for inducibility of Δ^1-dehydrogenase are illustrated in Figures 9 and 10. In Figure 9 of all the tetracyclic compounds tested, 5α-androstan-17-one (XVI) was capable of inducing the highest level of Δ^1-dehydrogenase. Structure XVII, the 5β-analog of XVI exhibited somewhat lower activity, while 5α-androstane (XVII) was inhibitory towards the formation of Δ^1-dehydrogenase. Structures XIII, XIV and XV had low specific activities because they were metabolizable and were better substrates than inducers. Introduction of a second keto function at positions C-2, 4 and 6 of 5α-androstan-17-one (XVI) resulted in lower levels of Δ^1-dehydrogenase. In Figure 10, only 5α-androstan-17-oxime (XIX) showed high levels of enzyme induction. 17-Methylene-5α-androstane (XXII)

Figure 8. Tricyclic steroid analogs tested for inducing capability. The arabic number under each structure represents the specific activity of Δ^1-dehydrogenase elicited by the analog.



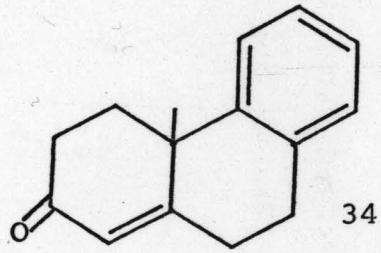
8.3

(IV)



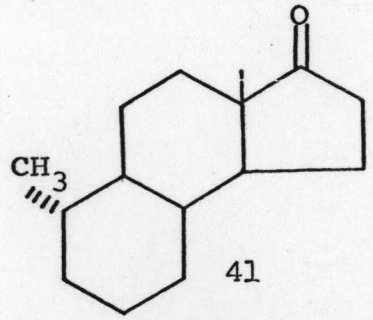
13

(V)



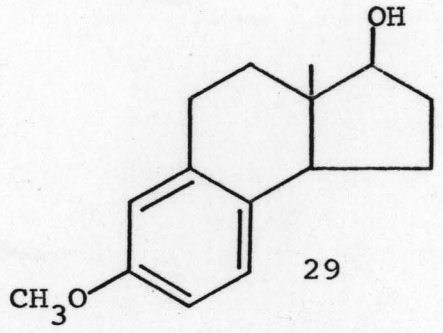
34

(VI)



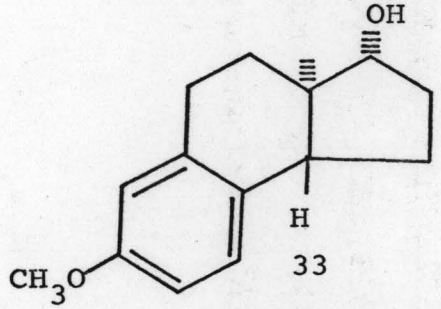
41

(VII)



29

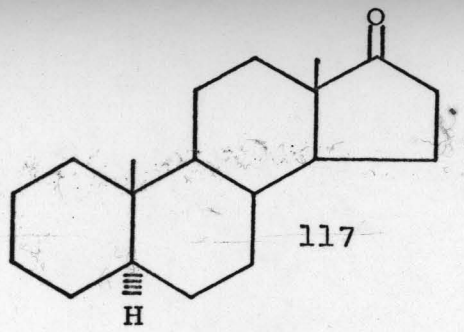
(VIII)



33

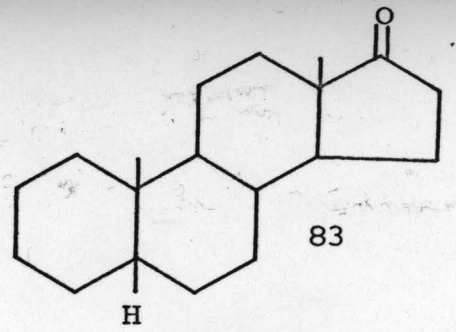
(IX)

Figure 9. Tetracyclic compounds tested for inducing capability. The arabic number under each structure represents the specific activity of Δ^1-dehydrogenase elicited by the compound.



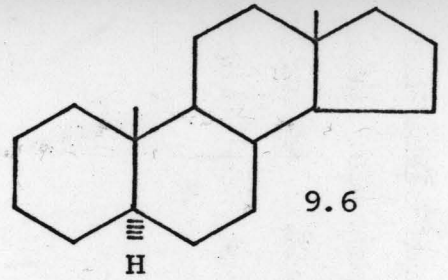
117

(XVI)



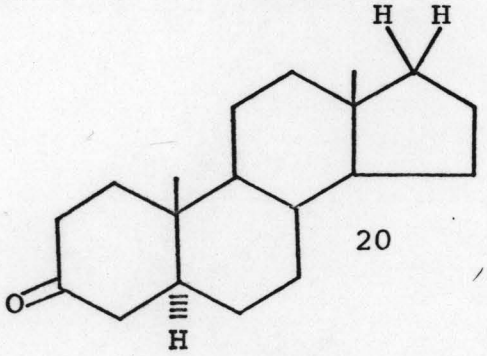
83

(XVII)



9.6

(XVIII)



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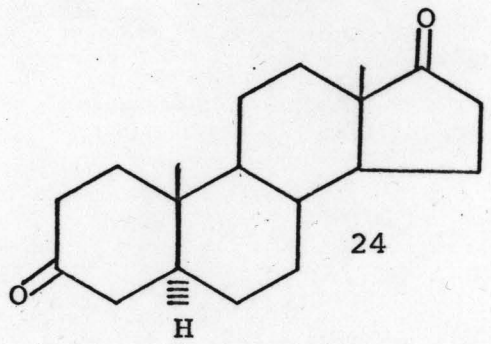
(XIII)



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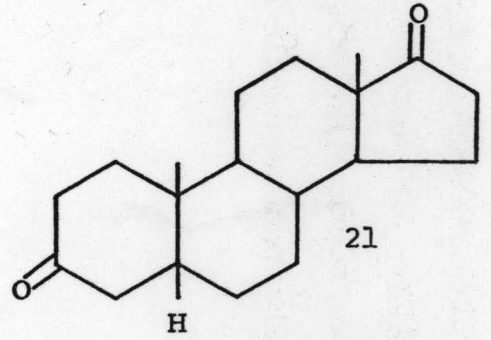
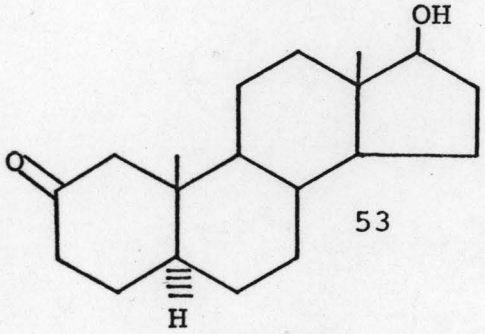
(XIV)



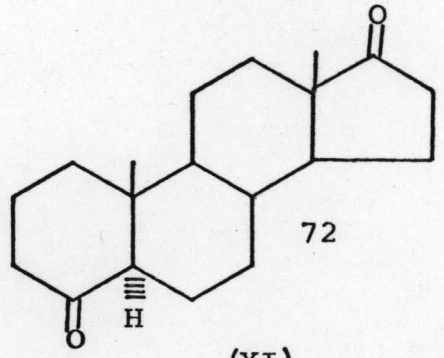
21

(XV)



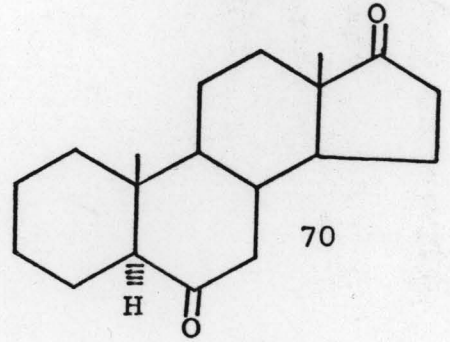
53

(X)



72

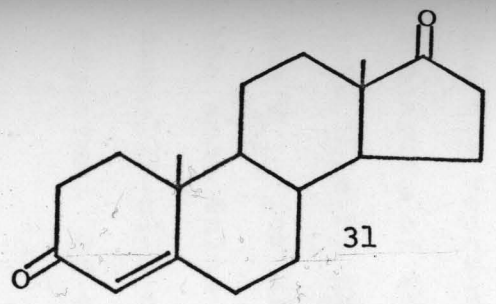
(XI)



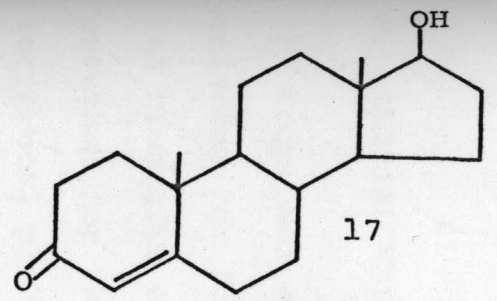
70

(XII)

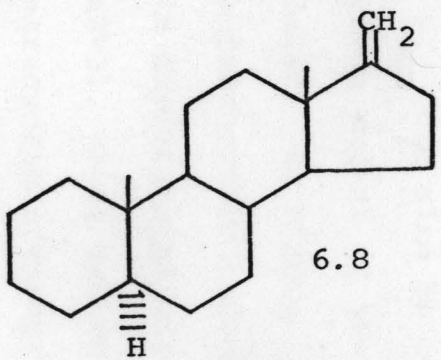
Figure 10. Tetracyclic compounds tested for inducing capability. The arabic number under each structure represents the specific activity of Δ^1-dehydrogenase elicited by the compound.



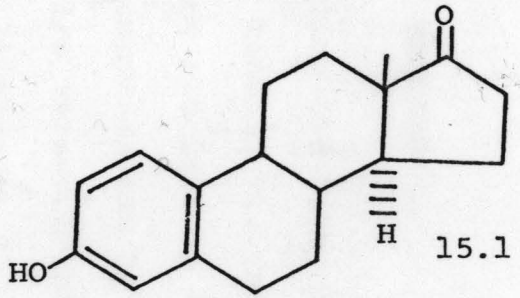
(XXV)



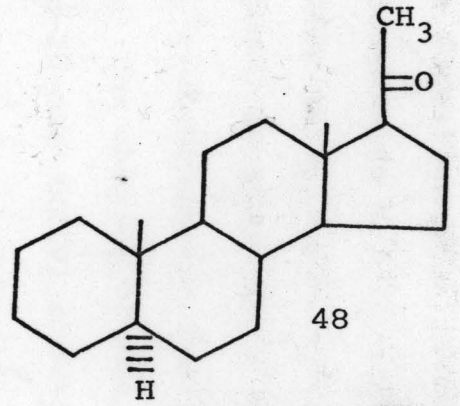
(XXVI)



(XXII)



(XXIII)



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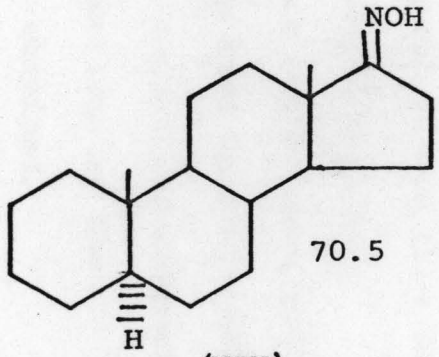
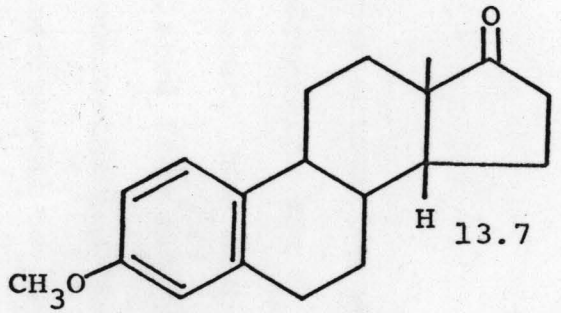
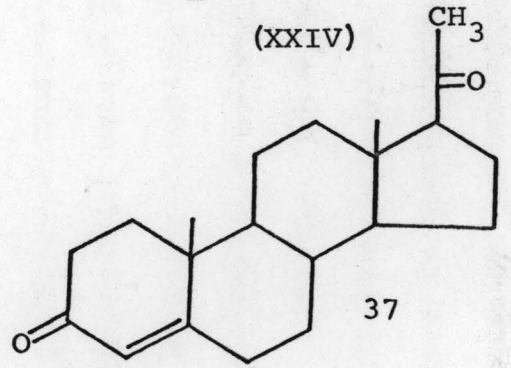


(XIX)



(XX)



(XXI)

inhibited the formation of Δ^1-dehydrogenase. Steroid structures, possessing a 3-keto function such as androst-4-ene-3,17-dione (XXV), androst-4-en-17β-ol-3-one (XXVI) and pregn-4-ene-3,20-dione (XXI) were not efficient inducers of Δ^1-dehydrogenase for they were metabolized by the organism. The absence of the 3-keto-Δ^4-function as in 5α-pregnan-20-one (XXIV) showed slight improvement. The aromatic steroids, 1,3,5-(10)-estratrien-3-ol-17-one (XXIII) and the 3-methoxy-14β-analog of XXIII displayed activities no better than the uninduced control.

F. INITIAL VELOCITY STUDIES

Compounds exhibiting the highest inducer activities were selected for further kinetic analyses to determine the V_{max} and Michaelis constant elicited by the inducer. The Michaelis constant for these compounds can be calculated from double reciprocal plots of 1/v versus 1/inducer concentration and are shown in Figures 11 through 22. The reciprocal of the Michaelis constant is a measure of the affinity of the inducer for the repressor. V_{max} can also be determined and this is a measure of the maximum amount of the enzyme that is inducible by the inducer. In Figure 11 it can be seen that the V_{max} for all the tetracyclic inducers have approximately the same value within experimental error except for androst-4-en-17β-ol-3-one (XXVI), which was metabolized by the organism. Of the tetracyclic structures examined,

5α-androstan-17-one (XVI) possessed the lowest K_m value indicating that it has the highest affinity for the repressor. Changing the configuration at the A-B ring juncture from trans to cis resulted in nearly a 10-fold increase in K_m. Introduction of a keto function at C-4 also resulted in a 10-fold increase in K_m, whereas the presence of a ketone function at C-6 had little effect on the K_m. Replacement of the 17-keto function by an oxime resulted in a considerable increase in the K_m value.

In the tricyclic series of inducers representing rings B-C-D of the steroid nucleus, only retro-trans-1α-hydroxy-8α-methyl-4,5-(4-methoxybenzo)-hydrindane (IX) has a K_m of 8.22 X 10^{-6} M indicating a high affinity for the repressor; however, this compound had a lower V_{max} as compared to 5α-androstan-17-one (XVI). The K_m for the bicyclic structure trans-8-methylhydrindan-1-one (III) representing rings C and D of the steroid nucleus was 2.59 X 10^{-4} M but this compound was capable of inducing higher levels of Δ^1-dehydrogenase than the above tricyclic compound.

G. DETERMINATION OF HYDROPHOBIC BONDING

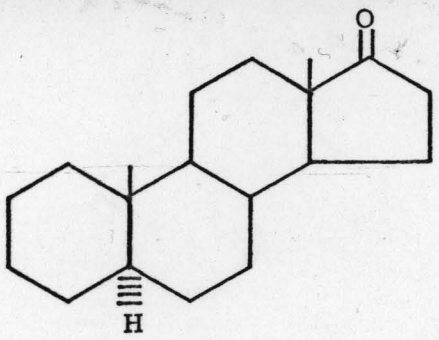
Anderson et al. (33) have prepared and studied the effect of N'-alkylnicotinamide chlorides as inhibitors in the yeast alcohol dehydrogenase catalyzed oxidation of ethanol. The effectiveness of these inhibitors increased with increasing chain length of the alkyl substituent. Free energy changes

associated with the binding of the inhibitors were calculated from the following relationship:

$$\Delta \cdot \Delta F = 2.3\ RT\Delta pK_i$$

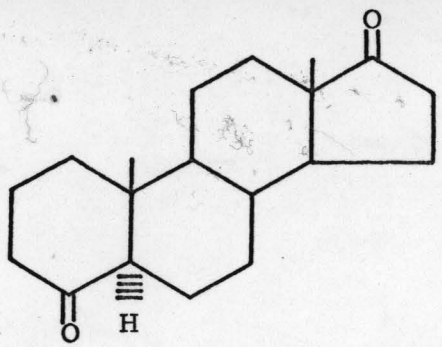
In this manner a free energy change per methylene group of 0.37 kcal/mole was obtained. This value is within the range (0.36 - 0.95 kcal/mole) suggested for interactions through dispersion forces (42). Using the above free-energy relationship a value of 0.327 kcal/mole/—CH_2— was obtained for 5α-androstan-17-one (XVI) (Table 3). This is in good agreement with the theoretically calculated values (42).

Figure 11. K_m and V_{max} for the tetracyclic inducers.



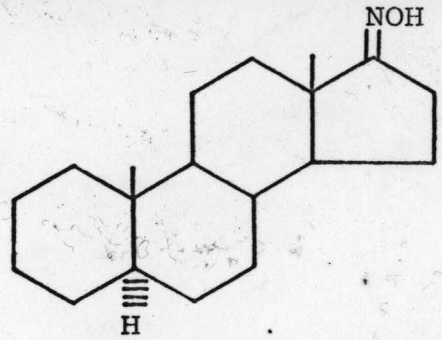
(XVI) V_{max} 1.18×10^{2}

K_m 1.80×10^{-6}



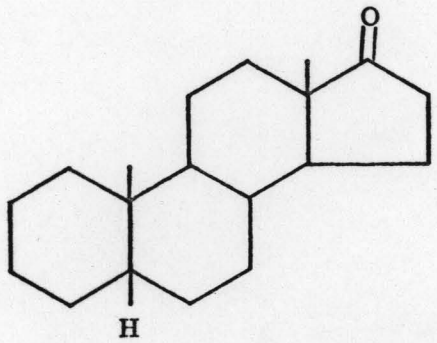
(XI) V_{max} 1.37×10^{2}

K_m 1.79×10^{-5}



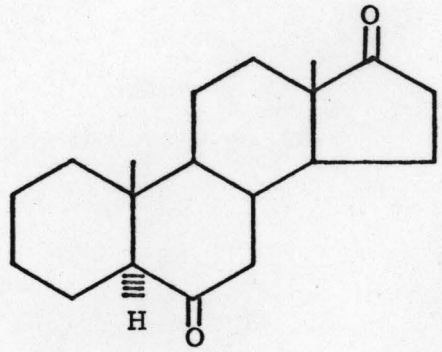
(XIX) V_{max} 1.04×10^{2}

K_m 1.38×10^{-4}



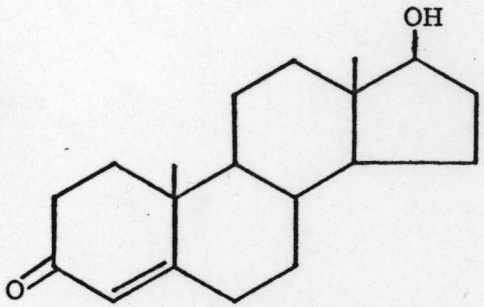
(XVII) V_{max} 1.09×10^{2}

K_m 1.44×10^{-5}



(XII) V_{max} 1.17×10^{2}

K_m 8.75×10^{-6}



(XXVI) V_{max} 3.09×10^{1}

K_m 3.46×10^{-4}

Figure 12. Initial velocity plot for 5α-androstan-17-one (XVI) as inducer. Conditions of the assay are described in the Experimental section.

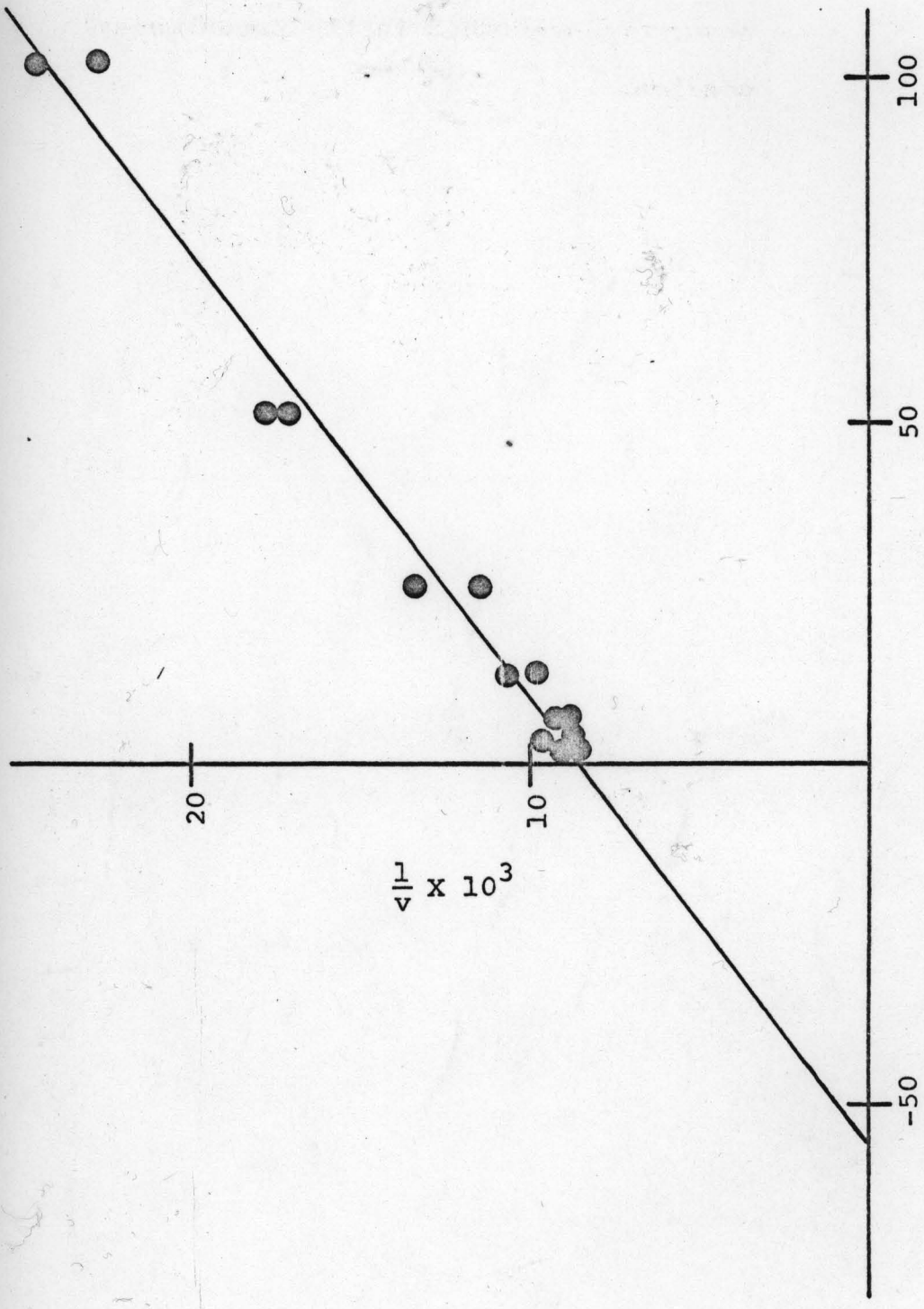

20
10
$\frac{1}{\tau} \times 10^3$
-50
50
100
$\frac{1}{[Ind]} \times 10^{-4}$ м$^{-1}$

Figure 13. Initial velocity plot for 5β-androstan-17-one (XVII) as inducer. Conditions of the assay are described in the Experimental section.

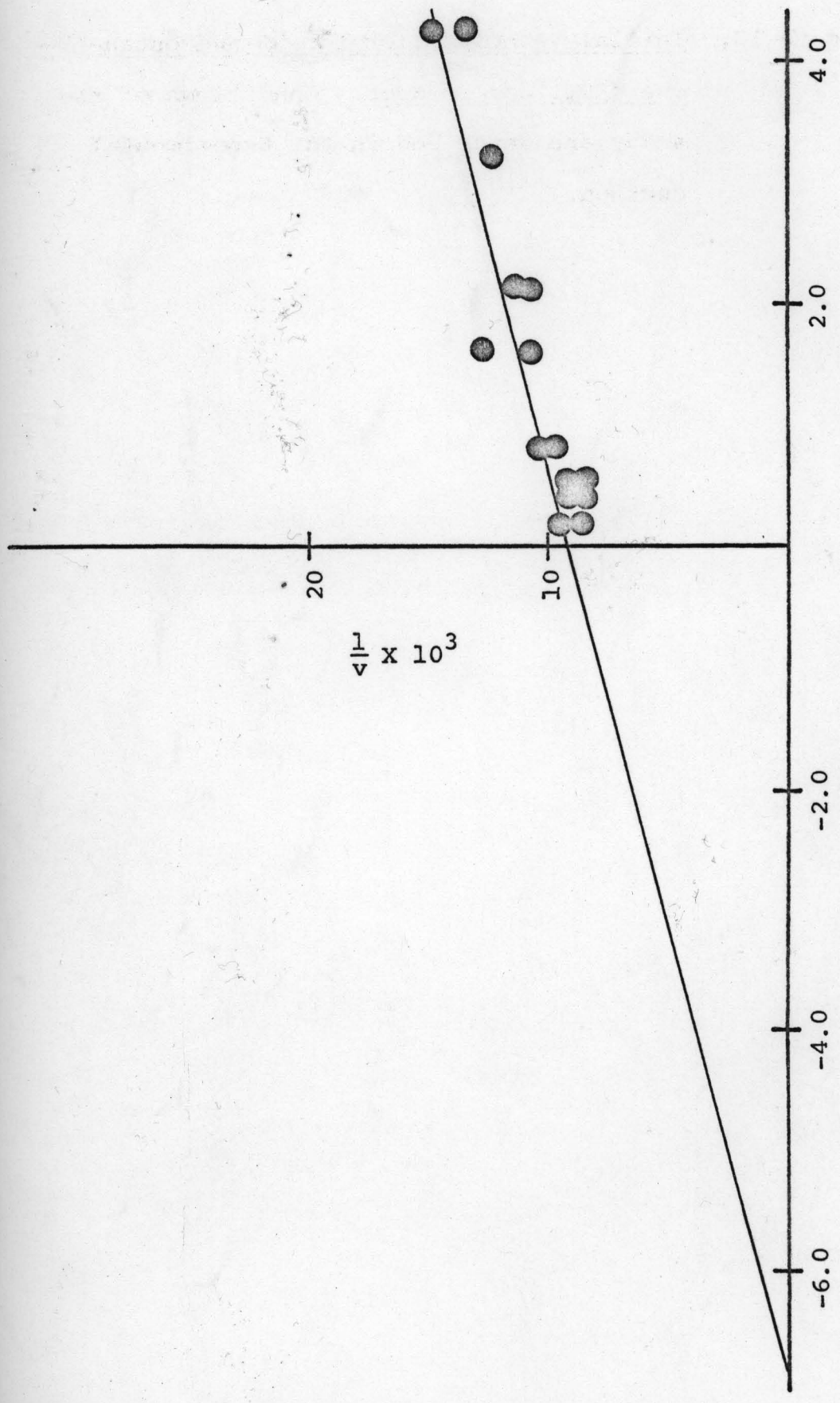

20
10
$\frac{1}{v} \times 10^3$
4.0
2.0
-2.0
-4.0
-6.0
$\frac{1}{[Ind]} \times 10^{-4}$ M^{-1}

Figure 14. Initial velocity plot for 5α-androstan-4,17-dione (XI) as inducer. Conditions of the assay are described in the Experimental section.

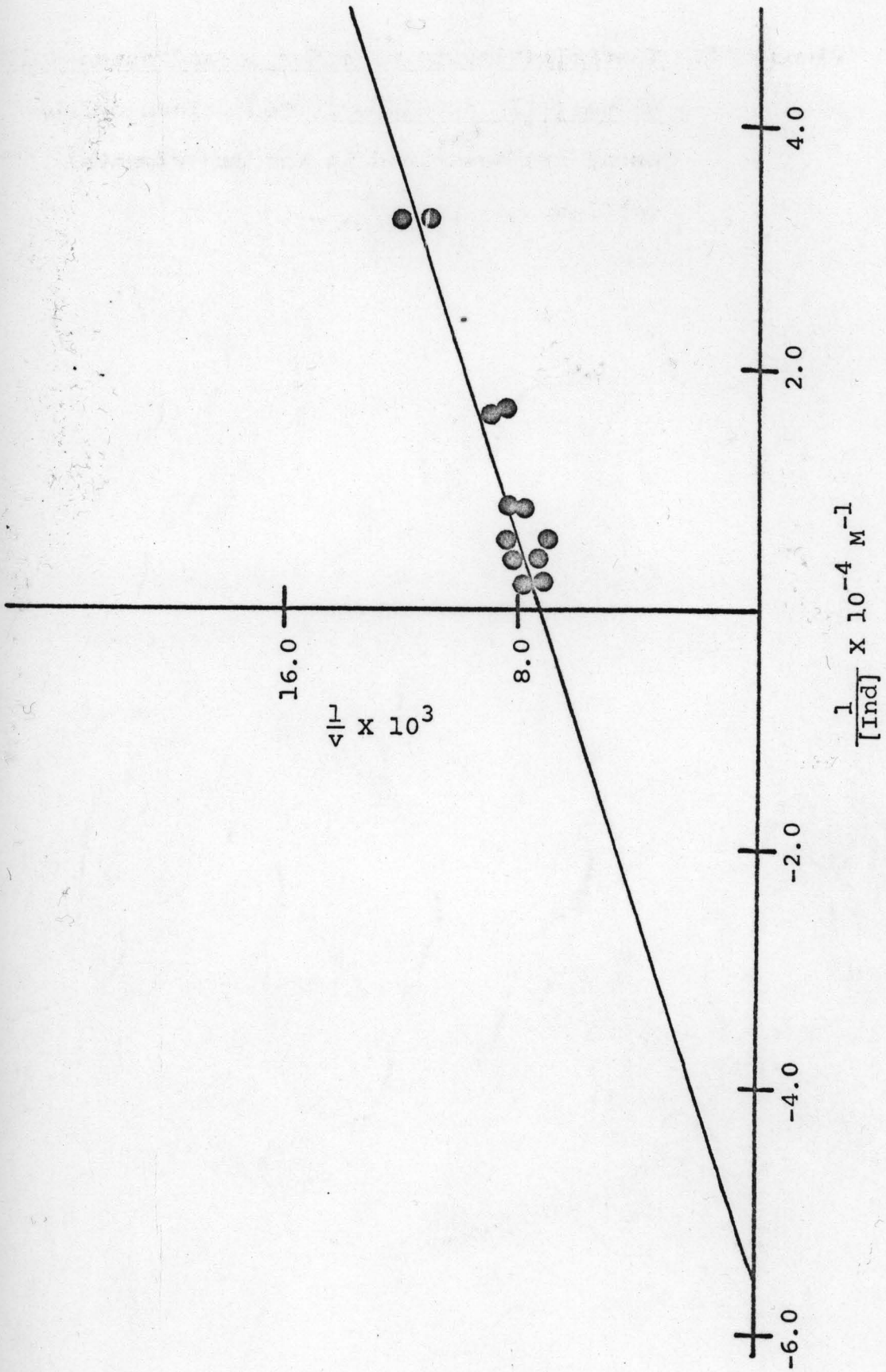

16.0
8.0
$\frac{1}{\tau}$ x 10^3
4.0
2.0
-2.0
-4.0
-6.0
$\frac{1}{[Ind]}$ x 10^{-4} M^{-1}

Figure 15. Initial velocity plot for 5α-androstan-6,17-dione (XII) as inducer. Conditions of the assay are described in the Experimental section.

3.2
1.6
$\langle r \rangle \times 10^2$
-120
-80
-40
40
80
$\frac{1}{[Ind]} \times 10^{-3}\ M^{-1}$

Figure 16. Initial velocity plot for 5α-androstan-17-oxime (XIX) as inducer. Conditions of the assay are described in the Experimental section.

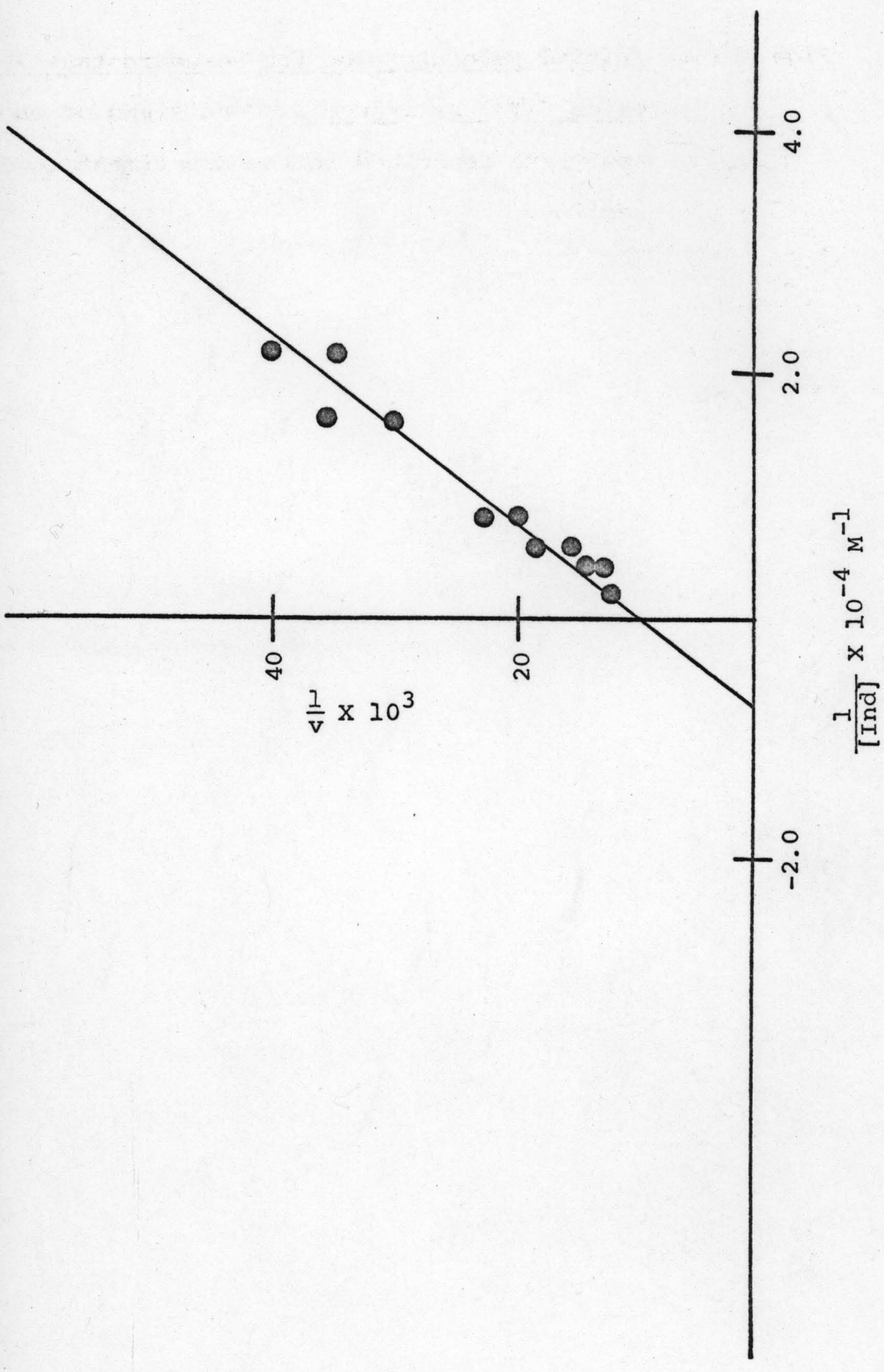

40
20
$\frac{1}{v} \times 10^3$
-2.0
2.0
4.0
$\frac{1}{[Ind]} \times 10^{-4}\ M^{-1}$

Figure 17. Initial velocity plot for androst-4-en-17β-ol-3-one (XXVI) as inducer. Conditions of the assay are described in the Experimental section.

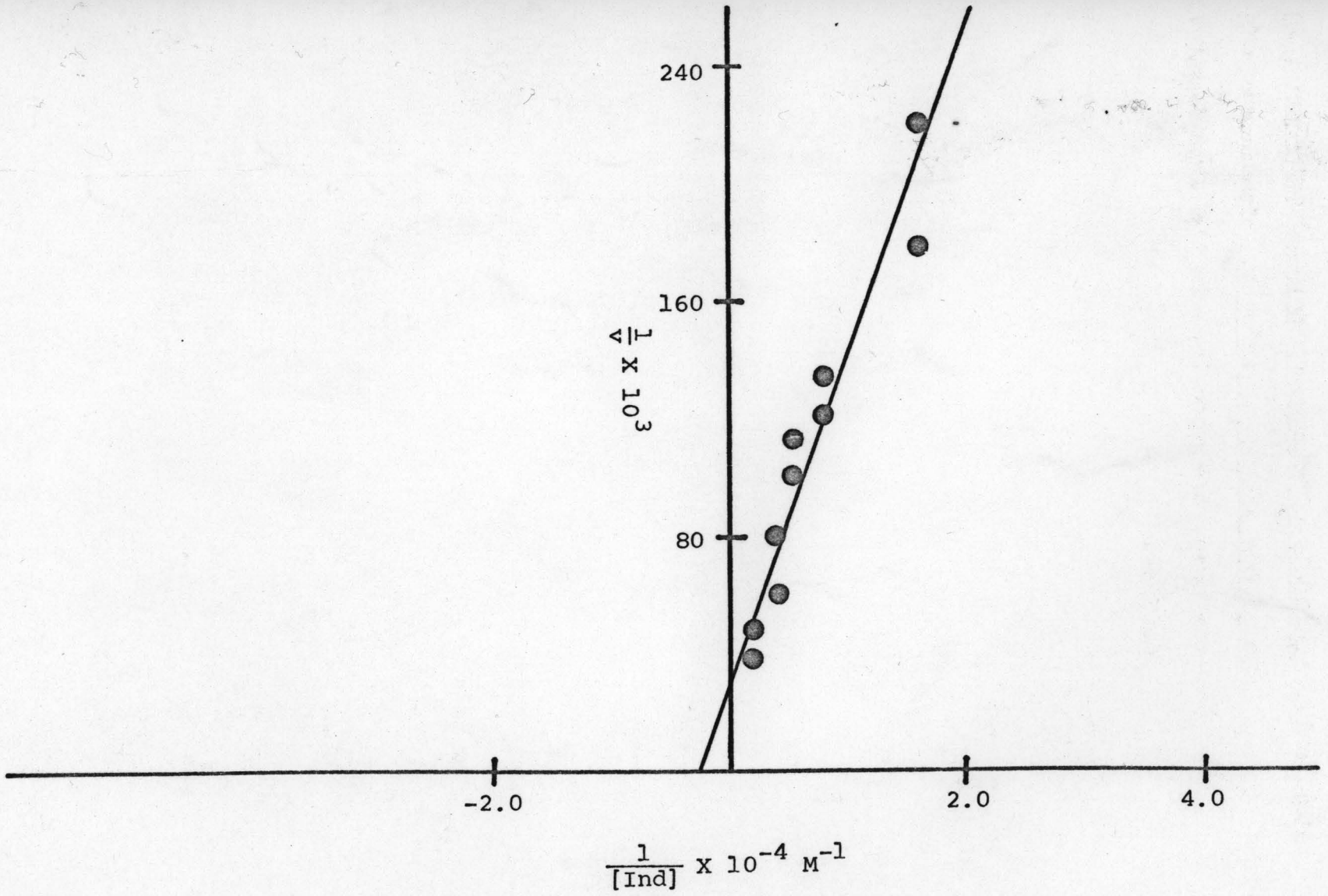
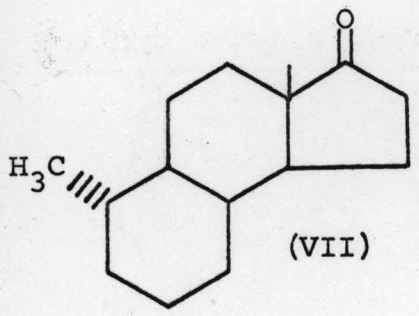

240
160
80
$\frac{1}{v} \times 10^3$
-2.0
2.0
4.0
$\frac{1}{[Ind]} \times 10^{-4}\ M^{-1}$

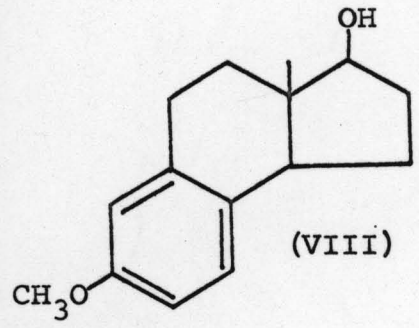
Figure 18. K_m and V_{max} for tricyclic and bicyclic inducers.



(VII)

V_{max} 4.76×10^{1}

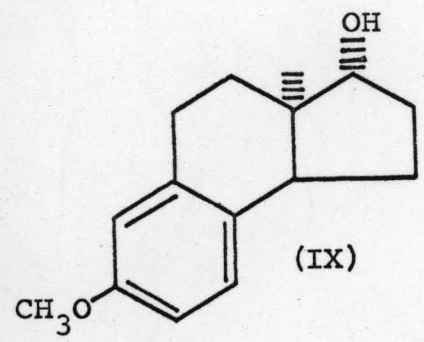
K_m 1.07×10^{-4}



(VIII)

V_{max} 2.0×10^{1}

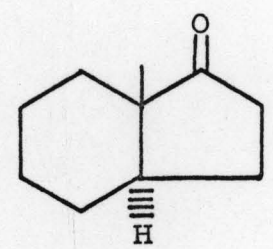
K_m 1.08×10^{-4}



(IX)

V_{max} 3.21×10^{1}

K_m 8.22×10^{-6}



(III)

V_{max} 8.47×10^{1}

K_m 2.59×10^{-4}

Figure 19. Initial velocity plot for anti-trans-8β-methyl-4,5-(5α-methyl-perhydrobenzo)-hydrindanone (VII) as inducer. Conditions of the assay are as described in the Experimental section.

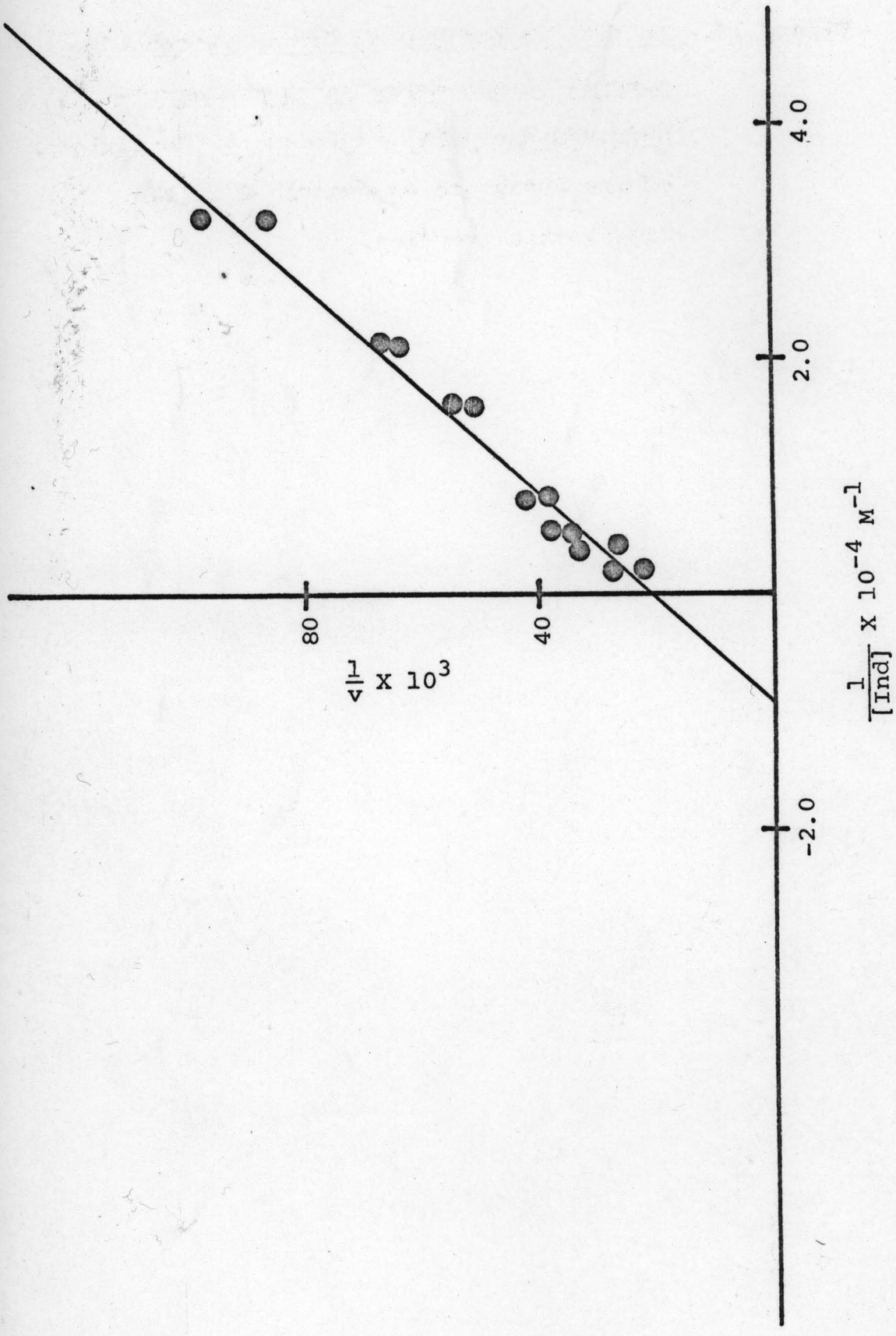

80
40
$\frac{1}{v} \times 10^3$
-2.0
2.0
4.0
$\frac{1}{[Ind]} \times 10^{-4}$ M^{-1}

Figure 20. Initial velocity plot for trans-1β-hydroxy-8β-methyl-4,5-(4-methoxybenzo)-hydrindane (VIII) as inducer. Conditions of the assay are described in the Experimental section.

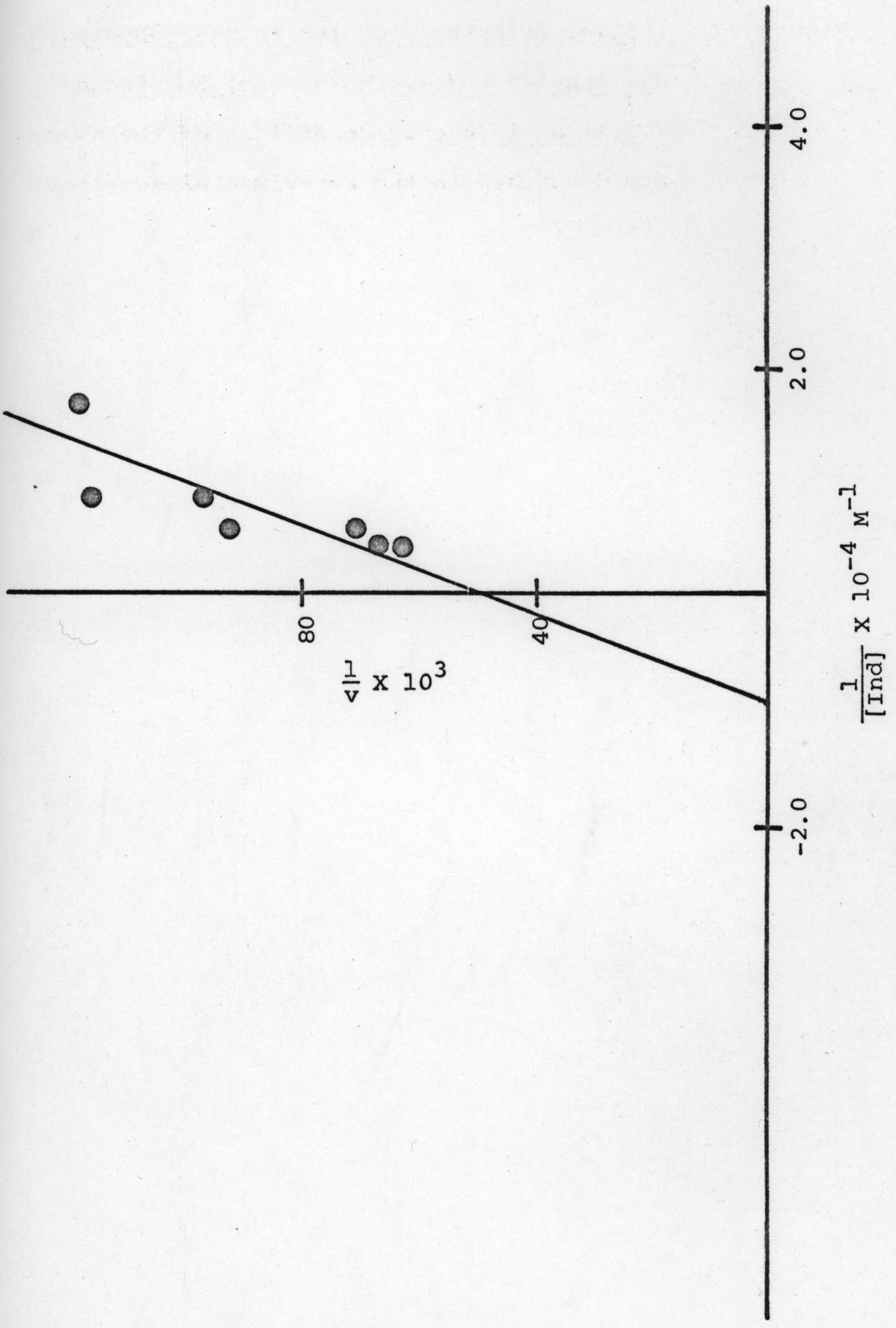

80
40
$\frac{1}{v} \times 10^3$
4.0
2.0
-2.0
$\frac{1}{[Ind]} \times 10^{-4}\ M^{-1}$

Figure 21. Initial velocity plot for retro-trans-1α-hydroxy-8α-methyl-4,5-(4-methoxybenzo)-hydrindane (IX) as inducer. Conditions of the assay are described in the Experimental section.

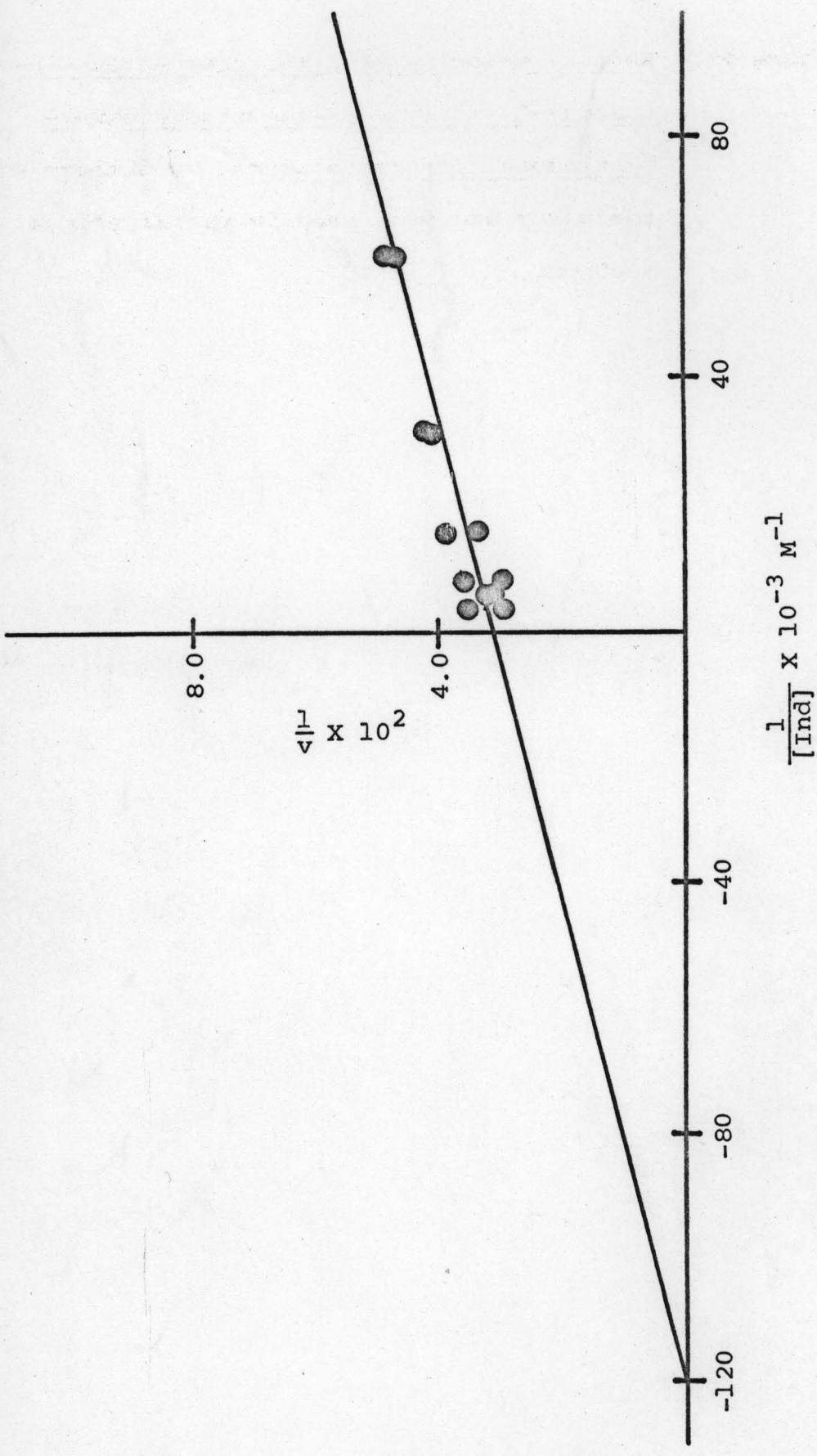

8.0
4.0
$\frac{1}{\tau} \times 10^2$
80
40
-40
-80
-120
$\frac{1}{[Ind]} \times 10^{-3}$ M^{-1}

Figure 22. Initial velocity plot for trans-8-methylhydrindan-1-one (III) as inducer. Conditions of the assay are described in the Experimental section.

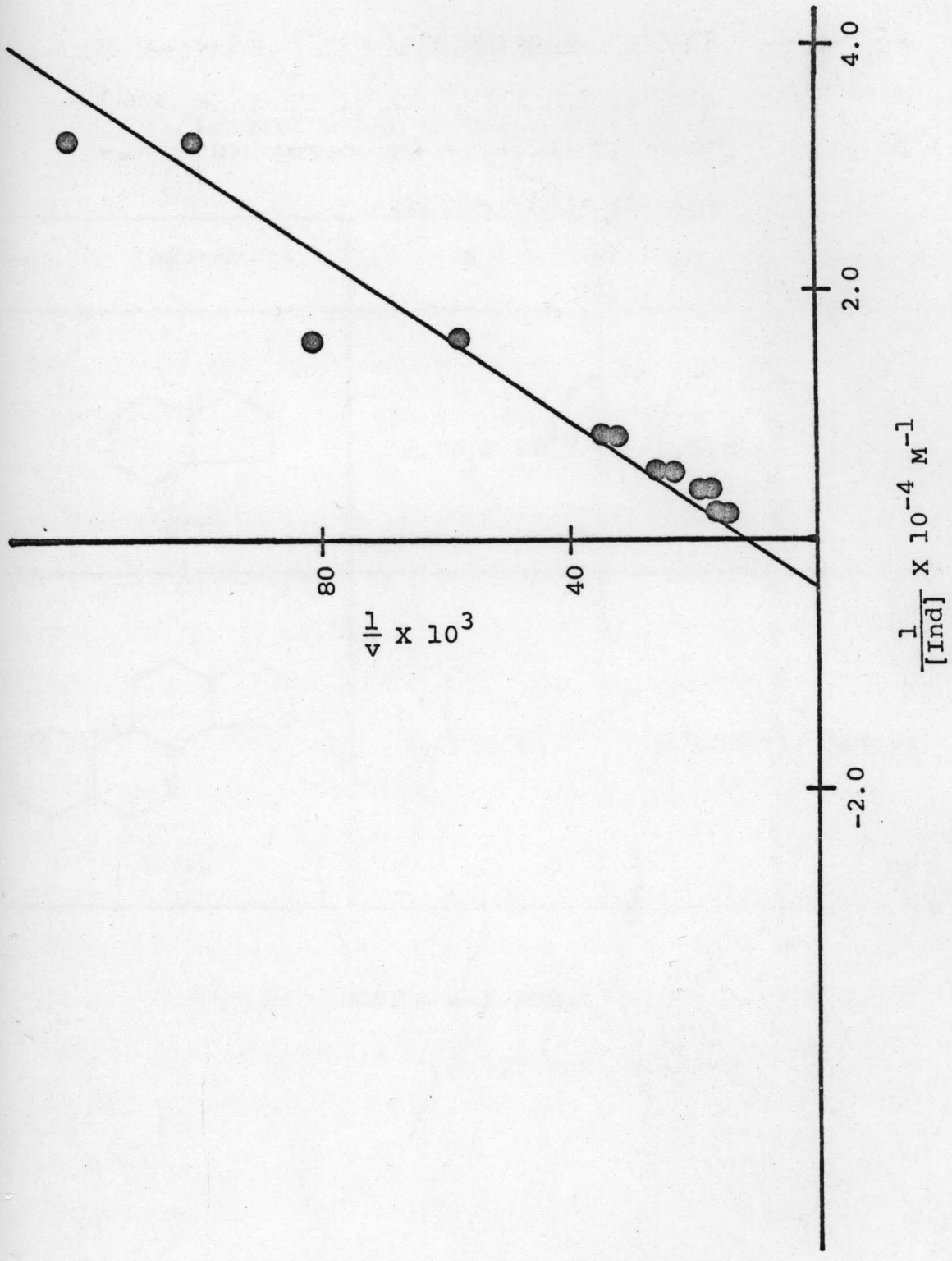
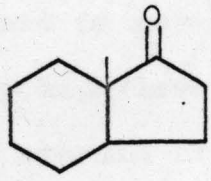
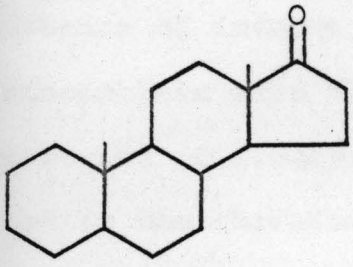

80
40
$\frac{1}{<I>} \times 10^3$
4.0
2.0
-2.0
$\frac{1}{[Ind]} \times 10^{-4}$ M^{-1}

TABLE 3

Determination of Hydrophobic Bonding

Inducer	K_m	pK_m	ΔpK_m
 (III)	2.59×10^{-4}	3.5867	—
 (XVI)	1.80×10^{-6}	5.7442	2.1580

$$\Delta\Delta F = 2.3\ RT\Delta pK_m$$

$$= 0.327 \text{ kcal/mole/}-CH_2-$$

V. DISCUSSION

Cells of N. restrictus (ATCC 14885) possess three enzymes, capable of introducing double bonds into 1,2 and 4,5 positions of steroids possessing either the 5α or 5β configuration. The dehydrogenases of this microorganism are adaptive, and in particular, the Δ^1-dehydrogenase can be raised to very high levels by exposing the organism to inducers having steroid or steroid-like configurations. In 1961 Jacob and Monod (25) proposed that the lactose operon of E. coli was governed by a macromolecular repressor which, in the absence of inducer, prevented the synthesis of messenger ribonucleic acid that coded for β-galactosidase. Since then a series of elegant genetic and biochemical experiments have led to the characterization of the messenger RNA as well as to the isolation of crystalline β-galactosidase (43), a homogeneous preparation of the repressor (44,45) and the deoxyribonucleic acid of the lac operon itself (46). Although many investigations have been carried out concerning regulation in inducible systems (47), none has been performed with the intent of defining the specificity of the inducer for the repressor.

The importance of medium composition on induction of Δ^1-dehydrogenase can be seen in Figures 5 and 6 and Table 1. Both nutrient broth, 1% Yeast extract and 2% Glucose and mineral salts, 1% Yeast extract and 2% Sodium succinate were capable of promoting high cell populations of N. restrictus.

However, only nutrient broth, 1% Yeast extract and 2% Glucose was capable of yielding high levels of Δ^1-dehydrogenase. Variation of the compositions of nutrient broth or mineral salts by deleting an ingredient one at a time resulted in lowered cell populations and Δ^1-dehydrogenase levels.

Monocyclic steroid analogs such as cyclopentanone, which represent ring D, and cyclohexanone and its derivatives, representing ring A of the steroid nucleus, have little or no effect on induction of the Δ^1-dehydrogenase in this organism. In the bicyclic series, which represents the A and B rings, or C and D rings of a steroid, only trans-8-methyl-hydrindan-1-one (III) gave rise to high levels of the enzyme. A possible explanation of this result may be that two molecules of this compound orient themselves in such a manner resembling a tetracyclic-like structure thus creating a space filling molecule. A similar explanation may be used to describe the slightly elevated level of Δ^1-dehydrogenase induced by 2-decalone, in that a tetracyclic structure is formed bearing a keto function at C-3 and the equivalent of C-16. 4-Cyclohexylcyclohexanone formally representing rings A and C was found to be inhibitory to the formation of the Δ^1-dehydrogenase of N. restrictus but it has been shown to be the most potent inducer of Δ^1-dehydrogenase of P. testosteroni (31). Also, this compound was reported by Ringold (31) to be recognized and reduced by the 3α-hydroxy-steroid dehydrogenase of P. testosteroni to give 99% axial alcohol. Addition of a methylene group equivalent to C-6 of ring B of a steroid

enhances the inducing properties of 3,4,8,8α-tetrahydro-8α-methyl-1,6-(2H,7H)-naphthalenedione (II) approximately 1.6 times when compared to 7,7α-dihydro-7α-methyl-1,5-(6H)-indandione (I).

The structures in the tricyclic series formally represent the A-B-C rings or B-C-D rings of steroids. Perhydrophenanthrene (IV), was found to be markedly inhibitory towards induction of the Δ^1-dehydrogenase. Addition of the third ring to bicyclic trans-8-methyl-hydrindan-1-one (III) noticeably lowers the inducing capability of this compound. It was interesting to note that of the two compounds having an aromatic B ring (Figure 18) the one with a retro D ring (IX) had higher affinity for the repressor. No definite explanation can be given for this increase in inducibility.

The structural requirements for induction by tetracyclic molecules may be seen by comparison of the various steroid features to 5α-androstan-17-one (XVI), the most effective inducer tested. 5α-Androstan-17-one (XVI) contains no other functional groups besides the ketone at C-17. A striking feature of the molecule is its flat surface on the α-side. Our data suggest that this planar surface plays a critical role in binding to the repressor presumably through hydrophobic bonding between this planar surface and a corresponding apolar region of the repressor. Models of 5α-androstan-17-one (XVI) reveal that seven hydrophobic bonds might participate in this binding. In addition, a hydrogen bond could form

between the C-17 keto group and a donor grouping on the repressor. In steroids possessing the A/B cis configuration, such as 5β-androstan-17-one (XVII) the A ring projects approximately 90^0 from the planar surface and lowered the inducing ability of the molecule. Removal of the 17-keto function or replacing it with a methylene group decreased the effectiveness of the compound as an inducer. Substitution at C-17 with an electronegative function such as an oxime restored the effectiveness of 5α-androstane (XVIII) as an inducer, perhaps by again being able to hydrogen bond with a donor grouping on the repressor. Steroids with a 3-keto function or a ketone at C-3 and C-17 were poor inducers because they were readily metabolized by the organism, whereas steroids with ketones at C-2, C-4 and C-6 were resistant to metabolism and thereby increased their effectiveness as inducers.

Gilbert and Müller-Hill (39) reported the isolation of the product of the control gene of the lactose operon, i.e. the lac repressor and have shown that it is a protein. They have also shown that isopropyl-β-D thiogalactoside, an inducer, binds with the repressor with a K_m of $\sim 5.7 \times 10^{-7}$ for i^t (tight-binding) mutant and for i^{wt} (wild-type) mutant, a K_m of $\sim 1.3 \times 10^{-6}$. Reciprocal plots of rate of enzyme synthesis versus inducer concentration showed that 5α-androstan-17-one (XVI), the best inducer for the Δ^1-dehydrogenase, had a K_m of 1.8×10^{-6} M. The second best inducer was 5α-androstan-6,17-dione (XII). The K_m for this compound as an

inducer was 8.75 X 10^{-6} M. The additional keto group in the ring caused a slight distortion from planarity and thus resulting in a less favorable binding of the inducer with the repressor. These are followed by, in decreasing order of affinity for the repressor, 5β-androstan-17-one (XVII), 5α-androstane-4,17-dione (XI) and 5α-androstan-17-oxime (XIX). All of the above named steroids have approximately the same V_{max} within experimental error. Androstan-4-en-17β-ol-3-one (XXVI), however, was metabolized by this organism and thus had a lower affinity for the repressor.

Kinetic data in the tricyclic series, analogous to steroid rings B, C and D, showed that retro-trans-1α-hydroxy-8α-methyl-4,5-(4-methoxybenzo)hydrindanone (IX) has a K_m of 8.22 X 10^{-6} M indicating a high affinity for the repressor, as compared to trans-1β-hydroxy-8β-methyl-4,5-(4-methoxybenzo) hydrindane (VIII) and anti-trans-8β-methyl-4,5-(5α-methyl-perhydrobenzo)hydrindanone (VII). Unfortunately, no satisfactory explanation can be given for this. Although compound IX has a high affinity for the repressor it induced low levels of the Δ^1-dehydrogenase. The bicyclic steroid analog, trans-8-methyl-hydrindan-1-one (III) had a K_m of 2.6 X 10^{-4} M and exhibited a low affinity for the repressor, yet it induced high levels of the Δ^1-dehydrogenase. It is possible that two molecules may be required for induction. As to the low affinity of these compounds for the repressor with the exception of compound IX, it is likely that for induction of high levels of Δ^1-dehydrogenase a large hydrophobic group is needed

in addition to a ketone function at C-17.

In summary, the Δ^1-dehydrogenase of N. restrictus is an adaptive enzyme which can be raised to high levels by certain steroids and steroid analogs. The effects of altering the steroid structure on induction was employed in delineating features of the repressor. The best inhibitor tested, 5α-androstan-17-one (XVI), contains no other substituents and is characterized by a large, planar surface which appears to play a dominant role in binding the steroid to the repressor. Presumably the binding occurs through hydrophobic interactions. Essentially all structural alterations, which disrupt the planarity of the ring system, diminish induction. Studies with steroid analogs possessing various portions of the ring system suggest that the entire steroid skeleton is not needed for induction. Compounds as simple as the bicyclic structure, trans-8-methyl-hydrindan-1-one (III), are capable of inducing high levels of Δ^1-dehydrogenase. Removal of electronegative substituents from C-17 diminishes its capacity as an inducer while addition of electronegative groups to this position restores its inducing capabilities. Addition of methylene groups to positions corresponding to the steroid nucleus increases the hydrophobic bonding and induction capacity of the molecule. The K_m for 5α-androstan-17-one (XVI) was determined to be 1.80×10^{-6} M indicating a high affinity for the repressor and that very few molecules are needed for induction. This is in good agreement with data reported by Gilbert and Müller-Hill for the lac repressor.

5α-Androstan-17-one (XVI) is not metabolized by the cell and acts as a gratuitous inducer.

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VII. TOTAL SYNTHESIS OF PROSTAGLANDINS