NAD<sup>+</sup>-DEPENDENT 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE FROM SWINE KIDNEY:
CHARACTERIZATION AND KINETIC MECHANISM

THESIS

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Cytoplasmic 15-hydroxyprostaglandin dehydrogenase from swine kidney was purified to specific activity of 1.2 U per mg protein, by chromatographic techniques.

Native molecular weight of enzyme was estimated at 45,000. Enzyme was inhibited by sulfhydryls, diuretics, and various fatty acids. Substrate studies indicated NAD+ specificity and ability to catabolize prostaglandins, except prostaglandin B and thromboxane B.

Initial velocity studies gave intersecting plots conforming to a sequential mechanism. 15-keto-prostaglandin exhibited linear noncompetitive production inhibition with respect to either prostaglandin or NAD+; NAD yielded linear competitive production inhibition with respect to NADH. Results, and those of dead-end inhibition and alternated substrate studies, are consistent with an ordered Bi-Bi mechanism: NAD+ is added first, then prostaglandin; then 15-keto-rostaglandin is released, then NADH.
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INTRODUCTION

Goldblatt (1933) and von Euler (1934) independently demonstrated that extracts of human seminal plasma and vesicular gland of sheep produced a fall in blood pressure and stimulated a variety of smooth muscle organs. The active principle was named prostaglandin (PG) by von Euler (1936), who also showed that it was lipid soluble and had acidic properties. Further elucidation of the chemical structure was achieved by Bergström and Sjövall (1957), who isolated and identified this active principle as a family of C_{20}-unsaturated fatty acids having a cyclopentane ring with functional keto and hydroxyl groups. Subsequent studies by Swedish and Dutch workers led to the important discovery that prostaglandins were derived from certain polyunsaturated essential fatty acids via the formation of short-lived prostaglandin endoperoxides (PGG_{2} and PGH_{2}) (Bergström et al., 1964; van Dorp et al., 1964; Hamberg et al., 1975). Three of these fatty acids, dihomo-γ-linolenic acid (C_{20}^{Δ8,11,14}), arachidonic acid (C_{20}^{Δ5,8,11,14}), and all-cis-5,8,11,14,17-eicosapentaenoic acid (C_{20}^{Δ5,8,11,14,17}), are found to be the precursors of prostaglandins of the 1, 2, and 3 family having 1, 2, and 3 double bonds respectively in the fatty acid chain. Each of these families consists of 3 different types of prostaglandins. The biosynthesis of prostaglandins of the 2 family, which is the most common one in the mammalian tissues, is shown in Figure 1.
Figure 1. Biosynthesis of prostaglandins of the 2 family.
Prostaglandins of the E type can be further dehydrated to prostaglandins of the A type, which can be isomerized to prostaglandins of the C type and eventually to prostaglandins of the B type, as depicted in Figure 2.

![Chemical diagram](image)

**Figure 2.** Biosynthesis of PGA, PGB and PGC.

Prostaglandins have been found in almost all tissues investigated, at least in low concentration, suggesting that these compounds have a fundamental role to many, if not all, animal cells. Although the physiological role of prostaglandins has not been clearly established, the pharmacological actions of these compounds are numerous. PGEs stimulate smooth muscle activity, lower blood pressure, and inhibit lipolysis and platelet aggregation. PGFs contract smooth muscle, and are vasopressors. PGDs are inhibitors of platelet aggregation with little vascular activity. PGAs are vasodepressors without smooth muscle activity. PGCs behave similar to PGAs but are short-lived. PGBs do not appear to have any prominent biological activity.

The diversity of prostaglandins is further complicated by the recent discovery of two potent novel substances, thromboxane A$_2$ (TXA$_2$) (Hamberg et al., 1975) and prostacyclin (PGI$_2$) (Moncada et al., 1976), both of which are derived from arachidonic acid via prostaglandin endoperoxides, as shown in Figure 3.
Figure 3: Biosynthesis of Thromboxane A$_2$ and Prostacyclin.
Both TXA\textsubscript{2} and PGI\textsubscript{2} are very unstable and are readily hydrolyzed to TXB\textsubscript{2} and 6-keto-PGF\textsubscript{1\alpha} respectively. TXA\textsubscript{2} induces platelet aggregation and contracts smooth muscle, while PGI\textsubscript{2} inhibits platelet aggregation and relaxes smooth muscle. The biological potency of TXA\textsubscript{2} and PGI\textsubscript{2} surpasses that of the classical prostaglandins. The potent and opposing actions of TXA\textsubscript{2} and PGI\textsubscript{2} have led to the speculation that the control of the balance of these two elements may be of vital importance in cardiovascular functions and diseases.

Prostaglandins and the related compounds are rapidly metabolized and inactivated \textit{in vivo}. It was estimated that more than 90\% of bioassayable activity of PGE or PGF (except PGA) is lost on one passage through the lung (Piper et al., 1970). Similarly, circulation through the liver or kidney results in rapid inactivation. It has therefore been proposed that prostaglandins may be acting like local hormones. The initial steps of the catabolism of these biologically active compounds involves the oxidation of 15(S)-hydroxyl group followed by the reduction of \(\Delta^{13}\)-double bond, forming 15-keto-13,14-dihydroprostaglandins (Anggård et al., 1965). Further catabolism includes \(\beta\)-oxidation from the carboxyl end and \(\omega\)-oxidation of the terminal carbon, resulting in the formation of \(C_{16}\)-dicarboxylic acids, which are excreted in the urine (Hamberg and Samuelsson, 1971). A representative catabolic cascade of PGE\textsubscript{2} is shown in Figure 4.
15-Hydroxyprostaglandin dehydrogenase

\[
\text{PGE}_2 \rightarrow 15\text{-Keto-PGE}_2 \rightarrow 15\text{-Keto-13,14-Dihydro-PGE}_2 \rightarrow \text{C}_{15}\text{-Major Urinary Metabolite}
\]

**Figure 4.** Catabolism of PGE$_2$ to major urinary metabolite.
The enzyme involved in the first step of catabolic degradation of these naturally occurring prostaglandins and the related compounds is termed 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH) (EC.1.1.1.141) and was detected in guinea pig lung homogenates by Anggard and Samuelsson (1964). This enzyme appears to be the key enzyme in the control of the biological inactivation of prostaglandins, since the 15-keto-prostaglandins have about one-tenth or less of the parent compounds' biological activity (Anggard, 1966). Two types of 15-OH-PGDH have been described. Type I is NAD⁺-dependent, while Type II is NADP⁺-dependent (Lee and Levine, 1975). In addition to coenzyme requirements, two types of enzymes could be easily distinguished by chromatographic properties and relative affinities for prostaglandins (Lee et al., 1975). In view of the much lower \( K_m \) exhibited for prostaglandins, and the fact that most of the cytoplasmic NAD⁺ occurs in the oxidized form, Type I 15-OH-PGDH may be primarily responsible for the in vivo formation of 15-keto-prostaglandins. The following description of 15-OH-PGDH refers to Type I.

15-OH-PGDH has been found to be present in the cytosolic fraction of almost all mammalian tissues of various species (Ånggard et al., 1971; Sun et al., 1976; Tai, 1976; Moore and Hoult, 1978). The isolation and purification of 15-OH-PGDH from various tissues have been of great interest to several investigators. Ånggard and Samuelsson (1966) purified it from swine lung; Saeed and Roy (1972), Matschinsky et al. (1974), and Nagasawa et al. (1974) from bovine lung; Limas and Cohn (1973) from canine heart; Tai et al. (1974), and Oliw et al. (1976) from swine kidney; Sun et al. (1976) from monkey lung; Thaler-Dao et al. (1974), Schlegel et al. (1974), Braithwaite and Jarabak (1975), Jung et al. (1975), and
Bardsley and Crabbe (1976) from human placenta. Most of these studies were related to the development of purification techniques, the modulation of enzyme activity, and kinetic studies. In only one case has the enzyme been purified to homogeneity (from human placenta; Braithwaite and Jarabak, 1975) and rigorous kinetic studies carried out (Jarabak and Braithwaite, 1976).

Purification of 15-OH-PGDH has been hampered by its instability, although addition of glycerol in the buffer was reported to be effective in protecting the enzyme from inactivation in some cases (Jarabak, 1972; Rückrich et al., 1976; Bardsley and Crabbe, 1976). Classical fractionation techniques including salt precipitation, organic solvent fractionation, ion exchange chromatography, adsorption chromatography, and Sephadex gel filtration have been used in the past; however, significant purification could only be achieved by using affinity chromatography. Braithwaite and Jarabak (1975) employed NAD\textsuperscript{+}-hexane-agarose affinity chromatography to purify human placental 15-OH-PGDH with the final preparation had a specific activity of 1.7 U per mg protein and was claimed to be homogeneous, as judged by disc gel electrophoresis. The molecular weight of the enzyme determined by gel filtration was 51,000. No evidence was obtained for the existence of multiple forms of the enzyme or for subunits. 15-OH-PGDH from human placenta, as well as from other tissues and species, showed a broad substrate specificity. Most of the prostaglandins were found to be oxidized by this enzyme except PGBs (Anggard and Samuelsson, 1966; Nakano et al., 1969; Thaler-Dao et al., 1974). PGEs have the lowest \( K_m \) values which are in the order of \( 10^{-6} \) M, whereas the other prostaglandins have \( K_m \) values in the range of \( 10^{-5} \) M.
Modulation of 15-OH-PGDH activity by endogenous physiological substances and by pharmacological agents has been extensively studied. This is probably attributed to the view that 15-OH-PGDH is a key enzyme in controlling the levels and the biological activity of prostaglandins. Factors that may either activate or inhibit the enzyme are of potential value in regulating the cellular levels of prostaglandins. Thus fatty acids were reported to be potent inhibitors of 15-OH-PGDH from swine lung (Marrazzi and Matschinsky, 1972) and from human placenta (Jarabak and Braithwaite, 1976), while thyroid hormones were found to be highly inhibitory to 15-OH-PGDH from swine kidney (Tai et al., 1974) and from chicken heart (Lee and Levine, 1975). Nucleotides and nucleosides were also found to be inhibitory to 15-OH-PGDH isolated from swine lung (Marrazzi and Matschinsky, 1972). However, the significance of the inhibitory effects produced by these endogenous substances remains to be established.

15-OH-PGDH has also been found to be inhibited by a variety of pharmacological agents. Indomethacin was found to inhibit 15-OH-PGDH from dog spleen, rabbit lung (Flower, 1974), and bovine lung (Hansen, 1974). Theophylline was a competitive inhibitor of 15-OH-PGDH versus NAD$^+$ from swine lung (Marrazzi and Matschinsky, 1972). A variety of diuretic drugs such as furosemide and ethacrynic acid were shown to be inhibitors of 15-OH-PGDH from human placenta (Paulsrud et al., 1974) and bovine lung (Oien et al., 1976). The latter finding is of particular interest, since these drugs by acting on renal 15-OH-PGDH will prolong the half life and the effectiveness of endogenous PGE$_2$ and PGA$_2$, which can also induce diuresis. As of this date, only psychototropic drugs such as chlorpromazine
and imipramine have been found to activate 15-OH-PGDH (Tai and Hollander, 1976a).

Kinetic studies on 15-OH-PGDH have only been carried out with the human placental enzyme (Schlegel and Greep, 1975; Rückrich et al., 1975; Bardsley and Crabbe, 1976; Jarabak and Braithwaite, 1976). Using initial velocity product inhibition and alternate substrate studies, these investigators proposed that human placental 15-OH-PGDH proceeds by an ordered Bi-Bi mechanism, in which NAD\(^+\) is added first, followed by PGE, with 15-keto-PGE being released, followed by NADH.

Although many studies have been conducted with 15-OH-PGDH from various tissues, particularly, with human placenta, little is known about the nature and the mechanism of 15-OH-PGDH from kidney, and about the significance of this enzyme in renal function. The kidney is one of the richest sources of this enzyme in all species investigated. Studies on the distribution of 15-OH-PGDH in kidney indicated that most of the enzyme activity is localized in the renal cortex (Ånggård et al., 1971). This is in contrast to prostaglandin synthetic activity, which is highest in the renomedullary region (Ånggård et al., 1972). The fact that prostaglandins are actively synthesized and catabolized in kidney suggests that prostaglandins may have an important role in renal function.

It has long been appreciated that the kidney has a central role in fluid and electrolyte homeostasis, regulation of systemic arterial blood pressure, and maintenance of plasma volume. When the kidney functions improperly, it leads to edema in pathological states, such as congestive heart failure, cirrhosis, and nephrosis. Similarly, abnormalities in renal blood flow and metabolism are believed to be involved in the development
of renovascular and essential hypertension. The biosynthesis and metabolism of prostaglandins in the kidney are of particular interest in view of the evidence that PGA₂ and PGE₂ are normally present in the renal medulla. They are the most potent renal cortical vasodilators and can cause appreciable increases in renal blood flow. Secondly, PGA₂ and PGE₂ are antagonists of angiotensin and norepinephrine, pressor systems widely regarded as being intimately involved in blood pressure elevation. Thirdly, PGA₂ and PGE₂ increase sodium and potassium excretion, and may play a role in regulating diuresis and natriuresis.

Convincing evidence that prostaglandins may play a physiological role in renal function is the finding that genetic hypertension in rats is accompanied by a defect in renal prostaglandin metabolism. A significantly lower 15-OH-PGDH activity was found in kidneys of genetically hypertensive New Zealand rats (Armstrong et al., 1976) and of spontaneously hypertensive Japanese rats (Pace-Asciak, 1976; Limas and Limas, 1977; Tai et al., 1979), as compared to the normal controls. Furthermore, a significantly higher prostaglandin synthetic activity was observed in kidneys of spontaneously hypertensive Japanese rats (Dunn, 1976; Limas and Limas, 1977). Consequently, elevated renal levels of prostaglandins are expected in genetically hypertensive rats.

Owing to the possibly significant role that 15-OH-PGDH may play in regulating renal function, this study was initiated to purify 15-OH-PGDH from swine kidney and to undertake the following investigations: (A) characterization of the enzyme with respect to molecular weight determination, coenzyme and substrate specificity and sulfhydryl sensitivity; (B) modulation of the enzyme activity by diuretic drugs and fatty acids; and (C) elucidation of the kinetic mechanism.
MATERIALS AND METHODS

Materials

Dye-reagent concentrate (protein assay), and Affi-Gel Blue (100-200 mesh, wet, albumin capacity 13.6 mg/ml) were obtained from Bio-Rad Laboratories. Ammonium chloride, and 2,5-diphenyloxazole (PPO) were obtained from the Fisher Scientific Co. Furosemide, acetazolamide, N-ethylmaleimide, meralluride, and spironolactone were respectively given by Hoechst Pharmaceuticals, Inc., Lederle Laboratories (American Cyanamid Co.), Mann Research, Lakeside Laboratories, and Searle Co. Chlorothiazide, hydrochlorothiazide, and ethacrynic acid were obtained from Merck, Sharp and Dohme Research Laboratories. D-[\textsuperscript{1-}\textsuperscript{3}H]galactose (14.2 Ci/m mol) was obtained from the New England Nuclear Chemical Corp. 15-(S)-[\textsuperscript{15-}\textsuperscript{3}H]-PGE\textsubscript{2} was prepared according to Tai (1976). 5'-AMP-Sephrose was obtained from the Pharmacia Fine Chemicals. NAD\textsuperscript{+}-Agarose column (AGNAD Type 1) was from the P. L. Biochemicals. Triton X-100 was obtained from the Research Products International Co. Charcoal was purchased from the Amend Drug and Chemical Co. P-Hydroxy-mercuribenzoate (PHMB), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), iodoacetic acid (recrystallized from petroleum ether prior to use), sodium mersalyl, dextran, blue dextran, horse heart cytochrome C, rabbit muscle lactate dehydrogenase (LDH) (750 U/mg), horse liver alcohol dehydrogenase (ADH) (1.61 U/mg), bovine liver L-glutamic dehydrogenase (0.5 U/mg), bovine serum albumin (BSA), soybean lipoxidase (165,000 U/mg), adenosine-5'-diphosphoribose, 3-acetylpyridine adenine dinucleotide, thionicotinamide-NAD\textsuperscript{+}, nicotinamide hypoxanthine dinucleotide,
α-ketoglutarate monosodium salt, 3-pyridinealdehyde-NAD⁺, deamine-NAD⁺, arachidonic acid, linoleic acid, oleic acid, POPOP, NAD⁺, NADH, DL-dithiothreitol (DTT), DEAE-cellulose (exchange capacity 0.9 meq/gm), Sephadex G-25 and G-100 were obtained from the Sigma Chemical Co. Ultrapure ammonium sulfate was obtained from Schwarz/Mann Co. Stearic acid and palmitic acid were obtained from Supelco, Inc. Prostaglandin E₁ (PGE₁), prostaglandin E₂ (PGE₂), prostaglandin A₁ (PGA₁), prostaglandin A₂ (PGA₂), prostaglandin F₁α (PGF₁α), prostaglandin F₂α (PGF₂α), prostaglandin I₂ (PGI₂), prostaglandin B₂ (PGB₂), 15-keto-prostaglandin E₂ (15-keto-PGE₂), 6-keto-prostaglandin F₁α (6-keto-PGF₁α), and thromboxane B₂ (TXB₂) were kind gifts from Dr. John Pike of the Upjohn Co.

Methods

Enzyme Assays

Method A. --The assay was based on the development of a strong and transient chromophore at 500 nm following alkali treatment of the reaction product 15-keto-PGE₂ as described by Ånggård and Samuelsson (1966). The assay mixture contained NAD⁺, 0.5 μmole; PGE₂, 28 nmole; and enzyme in a final volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.5. The reaction was carried out at 37°C and terminated by the addition of 0.1 ml of 2 N NaOH. The absorbance of the chromophore was determined at 500 nm, and the concentration of 15-keto-PGE₂ was estimated using a molar extinction coefficient of 27,000 (Oliw et al., 1976). This assay was used for rapid analysis of chromatographic fractions. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmole of 15-keto-PGE₂ from PGE₂ in 1 minute under the above conditions.
Method B.--Enzyme activity was determined by measuring the transfer of tritium from 15(S)-[15-3H]-PGE₂ to glutamate by coupling with glutamate dehydrogenase according to Tai (1976). The incubation mixture contained NH₄Cl (5 μmole), monosodium α-keto-glutarate (1 μmole), NAD⁺ (1 μmole), 15(S)-[15-3H]-PGE₂ (1 nmole, 20,000 cpm), excess glutamate dehydrogenase (100 μg) and appropriate amount of 15-OH-PGDH in a final volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.5. The reaction was initiated by the addition of the enzyme and allowed to proceed for 5 minutes at 37°C. The reaction was terminated by the addition of 0.2 ml of 10% charcoal suspension in 1% dextran solution. The reaction mixture was centrifuged at 1,000 xg for 8 minutes, standing for 10 minutes at room temperature. The supernatant was decanted and the radioactivity was determined by liquid scintillation counting. All the initial velocity, product inhibition (except NADH as a product), dead-end inhibition, and alternate substrate studies were carried out by this method. The amount of substrate oxidized was calculated on the assumption that no kinetic isotopic effect was involved in the removal of 15(S)-tritium.

Method C.--Enzyme activity was determined by following the formation of NADH fluorometrically. The reaction mixture contained NAD⁺, 0.5 μmole; PGE₁, 28 nmole; and enzyme in a final volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 0.02% BSA and 1 mM EDTA. The reaction was initiated by the addition of enzyme and allowed to proceed at room temperature. The NADH formed in the reaction mixture was recorded by the increase in fluorescence at 460 nm with excitation at 340 nm, using Aminco SPF-500 coupled to a Aminco X-Y recorder. This instrument was standardized using different amounts of NADH determined by direct measurement.
of the absorbance at 340 nm, using a molar extinction coefficient of 
6.22 x 10^3 M\(^{-1}\) cm\(^{-1}\) (Horecker and Kornberg, 1947). All the substrate 
specificity studies and the effect of sulfhydryl reagents and diuretic 
drugs were carried out by this method.

**Method D.** -- Enzyme activity was determined by following the formation 
of NADH spectrophotometrically. The reaction mixture has the same content 
as that described in Method C. The reaction mixture was incubated at 
25\(^{\circ}\)C and the NADH formed was recorded by the increase in absorbance at 
340 nm, using a Gilford 250 spectrophotometer attached to a recorder. 
The assay method was only employed in product inhibition studies where 
NADH was used as a product.

**Protein Determination**

Protein was determined by the dye-binding assay of Bradford (1976), 
using bovine serum albumin as a standard.

**Molecular Weight Determination**

Sephadex G-100 gel was swollen and equilibrated in 50 mM potassium 
phosphate buffer, pH 7.5 containing 1 mM EDTA, 0.2 mM DTT, and 0.25% 
sucrose, and packed under gravity. Even packing of each column was 
checked by watching the passage through it of a band of colored substance 
(e.g. blue dextran).

The Sephadex G-100 column (2.7 x 38.5 cm) was washed with equilibrating 
buffer overnight at 4\(^{\circ}\)C until stabilization of bed height and a 
constant flow rate of 30 ml per hour were achieved. The void volume (Vo) 
was determined with blue dextran (M.W. 200,000). Proteins of known 
molecular weights, namely cytochrome C, trypsin inhibitor, bovine serum
albumin (BSA), horse liver alcohol dehydrogenase, soybean lipoxidase, and rabbit muscle lactic dehydrogenase were used to calibrate the column.

BSA was detected by absorbance at 280 nm. Trypsin inhibitor was measured by absorbance at 215 nm. Cytochrome C was estimated at 412 nm. For alcohol dehydrogenase, assay mixture contained 2 M ethanol, 0.5 mM NAD\(^+\) in 0.1 M Tris-HCl, pH 8.0 containing 1 mM EDTA, and sufficient alcohol dehydrogenase to effect an absorbance change at 340 nm. For lactate dehydrogenase, assay mixture contained 2 mM NAD\(^+\), 5 mM sodium pyruvate in 0.1 M potassium phosphate buffer, pH 7.5 containing 1 mM EDTA, and enzyme. For lipoxidase, assay mixture contained diluted linoleic acid and enzyme in 0.2 M borate buffer, pH 9.0. The increased absorption accompanying linoleate oxidation was measured at 234 nm at 25\(^{\circ}\)C.

**Kinetic Studies**

All kinetic measurements such as initial velocity study, product inhibition, alternate substrate study, and dead-end inhibition were performed using tritium transfer Method B. Each reaction was initiated by the addition of enzyme to the reaction mixture containing varying amounts of substrate with or without inhibitors in a total volume of 1 ml of 50 mM potassium phosphate buffer, pH 7.5. One to two \(\mu\)g of partially purified enzyme (Affi-Gel Blue fraction) were used.

**Data Processing**

The nomenclature used herein is that of Cleland (1963 a & b). Reciprocal velocities were plotted graphically against the reciprocal of substrate concentrations. Reciprocal plots of initial velocity, product inhibition, and dead-end inhibition data were examined to determine the
pattern (i.e. intersecting, competitive inhibition, etc.), and the slope and intercepts were plotted graphically against either the reciprocal of the non-varied substrate concentration (for initial velocity experiments) or the inhibitor concentration (for inhibition experiments), to determine the linearity of these replots. Data conforming to a sequential initial velocity pattern, a linear competitive inhibition pattern, a linear uncompetitive, and a linear non-competitive inhibition pattern were fitted to Equations 1, 2, 3 and 4, respectively.

\[
v = \frac{V_{AB}}{K_i a K_b + K_a B + K_b A + AB}
\]  

(1)

\[
v = \frac{V_A}{K(1 + I/K_{iS}) + A}
\]  

(2)

\[
v = \frac{V_A}{K + A(1 + I/K_{ii})}
\]  

(3)

\[
v = \frac{V_A}{K(1 + I/K_{iS}) + A(1 + I/K_{ii})}
\]  

(4)

\[
F = \sum_{i=1}^{N} \left[ \frac{Y_{i, \text{exp}} - Y_{i, \text{calcd}}}{\sigma_i} \right]^2
\]  

(5)

In Equation 1, \(K_a\) and \(K_b\) are Michaelis constants of substrates A and B, respectively, and \(K_{ia}\) is the dissociation constant for substrate A. In Equations 2 through 4, \(K_{iS}\) and \(K_{ii}\) are apparent inhibition constants for slope and intercept. Curve fitting to these equations was done by a Fortran program package VINIT, which utilizes the nonlinear regression subroutine STEPIT (Chandler, 1971) to minimize a weighted sum of squares \(F\) defined by Equation 5, where \(Y_{i, \text{exp}}\) is the measured velocity, \(Y_{i, \text{calcd}}\) is the computed velocity, \(N\) is the number of points and \(\sigma_i\) is the standard deviation in measured velocity (Thompson et al., 1976).
RESULTS

Purification of NAD$^+$-Dependent 15-Hydroxyprostaglandin Dehydrogenase

All steps of purification were carried out at 0° - 4°C. Swine kidneys were obtained fresh from a local slaughterhouse and transported to the laboratory in ice. Kidneys were either used immediately or frozen at -80°C for later use. All the buffers included 0.2 mM DTT to protect the enzyme from inactivation. A typical purification procedure requires one week of operation.

Preparation of Crude Extract

The 15-OH-PGDH from swine kidney was purified by a modified and extended method of Tai et al. (1974).

Swine kidneys (198 gm) were cut into small pieces, and homogenized in two volumes of 50 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM DTT, and 1 mM EDTA in a Waring blender for three 1-minute periods separated by cooling. The homogenate was centrifuged at 34,000 xg for 25 minutes in a Sorvall refrigerated centrifuge. The supernatant was referred to as Fraction I.

Ammonium Sulfate Fractionation

To the clear supernatant was added 176 g of ammonium sulfate per liter to reach 30% saturation with constant stirring for one hour. The pH was kept near 7.5 by the addition of 3.0 N NH$_4$OH. The precipitate was removed by centrifuge at 12,000 xg for 20 minutes after stirring for one hour. The clear supernatant was then brought to 55% saturation by adding
162 g ammonium sulfate per liter with stirring for another hour. The pH of this solution was adjusted to pH 7.5. The precipitate collected by centrifugation at 12,000 xg for 20 minutes was dissolved in 116 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 0.2 mM DTT and 1 mM EDTA (Buffer A). This solution was referred to as Fraction II.

**DEAE-Cellulose Chromatography**

The DEAE-cellulose was washed thoroughly according to the procedure of Peterson and Sober (1962). This cellulose was degassed, packed under gravity, and equilibrated with Buffer A. The eluant was tested to insure that the desired pH and ionic strength had been attained before the sample was applied. Fraction II was desalted through a Sephadex G-25 column (5 x 51.0 cm) which had been equilibrated with Buffer A. The desalted fraction (265 ml) was loaded onto a DEAE-cellulose column (5 x 22.5 cm), as shown in Figure 5. The column was washed with 30 ml potassium phosphate buffer, pH 7.5, containing 0.2 mM DTT and 1 mM EDTA, until the OD$_{280}$ of the eluated was near 0.09. Elution was started with a linear gradient containing 700 ml of 30 mM KCl in 10 mM potassium phosphate buffer, pH 7.5, and 700 ml of 600 mM KCl in the same buffer. The enzyme was eluted around 55 mM KCl. The peak activity came off around 0.2 M KCl. Fractions which contained enzyme activity were pooled and concentrated with 472 g ammonium sulfate per liter in order to reach 70% saturation. The mixture was stirred for one hour and centrifuged at 12,000 xg for 20 minutes. The precipitate was dissolved in 42 ml of Buffer A. This solution is referred to as Fraction III. This step removed 77% of the protein. The result is shown in Figure 5.
Figure 5. Chromatography of 15-OH-PGDH on DEAE-cellulose column. Protein concentration was determined by absorption at 280 nm. The enzyme was assayed according to Method A. The conductivity was measured by using Markson's electroMark Analyzer, which had been standardized with conductivity standards. After absorption at 280 nm reached 0.09 through washing with 3 liters of equilibrating buffer, a linear KCl gradient was initiated as indicated. Details of chromatographic conditions are described in the Results section. Fraction volume: 14.1 ml. Flow rate: 130 ml/hr.
**Sephadex G-100 Filtration**

Sephadex G-100 was swollen in water for 2 days, packed under gravity, and equilibrated with Buffer A. Fraction III was applied to a Sephadex G-100 column (5 x 102 cm). Elution was carried out with the same buffer. Active fractions were pooled and concentrated to 47 ml by Amicon Ultrafiltration cells, using 10,000 molecular weight cut-off membrane (PM-10). This fraction was designated Fraction IV. A typical activity profile is shown in Figure 6. Protein and enzyme peaks were well separated. 15-OH-PGDH was eluted from the column following the hemoglobin.

**Affi-Gel Blue Affinity Chromatography**

The Affi-Gel Blue column (1.4 x 38) was packed under gravity and equilibrated with Buffer A. Fraction IV was applied to this column, and the column was then washed with the same buffer until the OD<sub>280</sub> reading was near 0.1. 15-OH-PGDH was eluted from the column by using Buffer A containing 1 mM NADH. The result is shown in Figure 7. Active fractions were pooled and concentrated immediately to 15.3 ml by using Amicon Ultrafiltration cells (with 10,000 M.W. cut-off membrane). The final partially purified enzyme (Fraction V) was stored in small aliquots at -80°C. This single step removed 95% of the protein from the last step and increased purification 14.5-fold. Protein concentration (47 ug/ml) was low in NADH eluate. Inactivation can be minimized by concentration this fraction using Amicon Ultrafiltration cells. All the studies on chemical properties and kinetic mechanism were carried out by this fraction.

Table I summarizes the steps for the purification of swine kidney 15-OH-PGDH. The final preparation showed a 377-fold enrichment with an
Figure 6. Chromatography of 15-OH-PGDH on Sephadex G-100 column. The protein concentrations were determined by absorption at 280 nm. The enzyme was assayed according to Method A. Details of chromatographic conditions are described in the Results section. Fraction volume: 14.5 ml. Flow rate: 100 ml/hr.
Figure 7. Chromatography of 15-OH-PGDH on Affi-Gel Blue affinity column. Fractions of 7.5 ml were collected at a flow rate of 50 ml/hr. Protein concentrations were determined by absorbance at 280 nm before using 1 mM NADH to elute the enzyme. Protein concentrations were measured by Bradford dye reagent after elution with NADH. Enzyme activities were assayed by Method A. Other details of chromatographic conditions were described in the Results section.
overall recovery of activity of 51% under the standard assay of Method A. The specific activity was 1,236 mU per mg.

**Physiochemical Properties**

**Molecular Weight Determination**

Molecular weight determination of 15-OH-PGDH from swine kidneys was performed by Sepahdex G-100 gel filtration (Figure 8). When the elution volume of 15-OH-PGDH was correlated with the elution volumes of proteins of known molecular weights (Andrews, 1964), an apparent molecular weight of 45,000 daltons was estimated for 15-OH-PGDH.

**Substrate Specificity**

15-OH-PGDH was examined for its ability to oxidize a number of prostaglandins. The results are summarized in Table II. Nearly all prostaglandins, with the exception of PGB₂, were metabolized by 15-OH-PGDH. Kᵣ values in increasing order were PGEs, PGAs, PGI₂, 6-keto-PGF₁α, PGFs, TXB₂. Both PGEs and PGAs had Kᵣ values in the range of 10⁻⁶ M, while other prostaglandins had Kᵣ values in the order of 10⁻⁴ M. TXB₂ was a very poor substrate for the enzyme. Since PGE₂ was found to be the best substrate, it was used in all subsequent assays.

**Coenzyme Specificity**

NAD⁺, NADP⁺ and a number of NAD⁺ analogs were examined for their capability to serve as coenzyme for 15-OH-PGDH. Table III shows the activity of 15-OH-PGDH in the presence of each nucleotide at 1 mM. NAD⁺ showed the best activity. NADP⁺ exhibited only one tenth of NAD⁺ activity. Among other NAD⁺ analogs tested, only 3-acetylpyridine adenine dinucleotide and deamino-NAD⁺ showed some coenzyme activity.
TABLE I
PURIFICATION OF 15-HYDROXYPROSTAGLANDIN
DEHYDROGENASE FROM SWINE KIDNEY

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific Activity (units/mg) x 10^6</th>
<th>Purification (fold)</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude Extract</td>
<td>400</td>
<td>9,440</td>
<td>30.9</td>
<td>3.28</td>
<td>----</td>
<td>100</td>
</tr>
<tr>
<td>II. Ammonium Sulfate Precipitate (30 - 55%)</td>
<td>116</td>
<td>4,800</td>
<td>27.0</td>
<td>5.80</td>
<td>1.78</td>
<td>90.3</td>
</tr>
<tr>
<td>III. DEAE-Cellulose</td>
<td>822</td>
<td>1,070</td>
<td>28.0</td>
<td>26.20</td>
<td>8.00</td>
<td>90.6</td>
</tr>
<tr>
<td>IV. G-100 Concentrate</td>
<td>48</td>
<td>310</td>
<td>26.0</td>
<td>85.00</td>
<td>27.20</td>
<td>84.0</td>
</tr>
<tr>
<td>V. Affi-Gel Blue Concentrate</td>
<td>15.3</td>
<td>12.7</td>
<td>15.7</td>
<td>1,236.00</td>
<td>377.00</td>
<td>50.8</td>
</tr>
</tbody>
</table>
Figure 8. Determination of molecular weight of swine kidney 15-OH-PGDH by gel filtration. The logarithm of the molecular weight is plotted against $K_{av}$, the ratio of protein elution volume ($V_e$) to column void volume ($V_o$). A Sephadex G-100 column (2.7 x 38.5 cm) was calibrated with cytochrome C (M.W. 12,384), trypsin inhibitor (M.W. 21,500), bovine serum albumin (M.W. 67,000), alcohol dehydrogenase (M.W. 83,000), lipoxidase (M.W. 102,000), lactic dehydrogenase (M.W. 140,000). Methods of assaying enzyme activities and conditions of chromatography are described in the Methods section.
### TABLE II

**SUBSTRATE SPECIFICITY OF SWINE-KIDNEY 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE**

<table>
<thead>
<tr>
<th>Prostaglandins</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_1$</td>
<td>1.2</td>
</tr>
<tr>
<td>$E_2$</td>
<td>1.2</td>
</tr>
<tr>
<td>$A_1$</td>
<td>1.7</td>
</tr>
<tr>
<td>$A_2$</td>
<td>4.6</td>
</tr>
<tr>
<td>$F_{1\alpha}$</td>
<td>19.2</td>
</tr>
<tr>
<td>$F_{2\alpha}$</td>
<td>12.5</td>
</tr>
<tr>
<td>$I_2$</td>
<td>5.0</td>
</tr>
<tr>
<td>$B_2$</td>
<td>Inactive</td>
</tr>
<tr>
<td>$\text{TXB}_2$</td>
<td>400.0</td>
</tr>
<tr>
<td>6-keto-$\text{PGF}_{1\alpha}$</td>
<td>5.1</td>
</tr>
</tbody>
</table>

All the prostaglandins were respectively dissolved in ethanol at the concentration of 1 mg/ml and diluted in assay buffer before experiment. 

$\text{PGI}_2$ was made fresh. The assay was done by Method C, using 4 μg of enzyme. The concentration of prostaglandin substrate was varied and the concentration of $\text{NAD}^+$ was kept at 0.5 mM. $K_m$ values were determined by the respective double reciprocal plots.
<table>
<thead>
<tr>
<th>Coenzymes</th>
<th>Activity (p mole/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>220</td>
</tr>
<tr>
<td>3-Acetylpyridine Adenine Dinucleotide</td>
<td>38.5</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>20</td>
</tr>
<tr>
<td>Nicotinamide Hypoxanthine Dinucleotide</td>
<td>7</td>
</tr>
<tr>
<td>Thionicotinamide-NAD⁺</td>
<td>0</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-NAD⁺</td>
<td>0</td>
</tr>
<tr>
<td>Deamino NAD⁺</td>
<td>17</td>
</tr>
</tbody>
</table>

The enzyme (6 μg) was assayed in the presence of 1 mM of NAD⁺ or its analogs by Method C.
Effect of Sulfhydryl Inhibitors

Five different sulfhydryl inhibitors, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM), p-hydroxy-mercuribenzoate (PHMB), sodium mersalyl and iodoacetic acid were tested for their effects on 15-OH-PGDH. The results are indicated in Table IV. It is apparent that DTNB is the most potent inhibitor and iodoacetic acid is the least effective one.

Effect of Fatty Acids

A number of saturated and unsaturated fatty acids were investigated for their effects on 15-OH-PGDH since the substrate, prostaglandin is also a fatty acid by itself. Oleic acid (C\textsubscript{18}\textsuperscript{A9}), linoleic acid (C\textsubscript{18}\textsuperscript{A9,12}), arachidonic acid (C\textsubscript{20}\textsuperscript{A5,8,11,14}), palmitic acid (C\textsubscript{16}) and stearic acid (C\textsubscript{18}) were found to be inhibitory to 15-OH-PGDH at low \textmu M range, as shown in Table V. Oleic acid, the most potent inhibitor, has a $K_I$ of 1.25 \textmu M. Inhibition as examined by Dixon plot was of non-competitive type.

Effect of Diuretic Drugs

Since PGE\textsubscript{2} and PGA\textsubscript{2} have been shown to have some diuretic activity, and 15-OH-PGDH greatly reduces that activity, a series of known diuretic compounds was tested for their ability to inhibit 15-OH-PGDH, as shown in Table VI. The organomercurial diuretics, meralluride and sodium mersalyl, were found to be potent inhibitors of 15-OH-PGDH with $I_{50}$ of 3.3 \textmu M and 1.5 \textmu M respectively. The 'loop' diuretics - furosemide, a sulfonamide derivative, and ethacrynic acid, an aryloxyacetic acid derivative, are also good inhibitors of 15-OH-PGDH, with $I_{50}$ of 91 \textmu M and 109 \textmu M. Thiazide diuretics such as chlorothiazide and hydrochlorothiazide,
<table>
<thead>
<tr>
<th>Additions</th>
<th>((I_{50}) (\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Mersalyl</td>
<td>1.5</td>
</tr>
<tr>
<td>P-Hydroxymercuribenzoate</td>
<td>2.5</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>36.0</td>
</tr>
<tr>
<td>Dithiobis Nitrobenzoic Acid</td>
<td>0.91</td>
</tr>
<tr>
<td>Iodoacetic Acid</td>
<td>650.0</td>
</tr>
</tbody>
</table>

\(I_{50}\) is defined as the concentration of inhibitor required to produce 50% inhibition of enzyme activity under the standard assay conditions of Method C. The concentration of the compounds listed varied from 0 to 1 mM. Enzyme activity at zero concentration was taken as 100%. Five \(\mu g\) of enzyme was used.
### TABLE V

**EFFECT OF FATTY ACIDS ON SWINE KIDNEY 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE**

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>$K_I$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oleic Acid</td>
<td>1.25</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>4.10</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>6.00</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>4.00</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>40.00</td>
</tr>
</tbody>
</table>

Fatty acids were dissolved in ethanol and diluted in assay buffer before experiment. The assay was done by Method C, using 5 $\mu$g of enzyme. Fatty acid concentrations were varied at two fixed concentrations of $\text{PGE}_1$, $5.6 \mu$M and $28.3 \mu$M. The Dixon plot (1953) was used to estimate $K_I$.  


### TABLE VI
**EFFECT OF DIURETIC DRUGS ON SWINE KIDNEY 15-HYDROXYPROSTAGLANDIN DEHYDROGENASE**

<table>
<thead>
<tr>
<th>Additions</th>
<th>( I_{50} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Mersalyl</td>
<td>1.5</td>
</tr>
<tr>
<td>Meralluride</td>
<td>3.3</td>
</tr>
<tr>
<td>Ethacyrnic Acid</td>
<td>109.0</td>
</tr>
<tr>
<td>Furosemide</td>
<td>91.0</td>
</tr>
<tr>
<td>Chlorothiazide</td>
<td>240.0</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>1,030.0</td>
</tr>
<tr>
<td>Acetazolamide</td>
<td>&gt; 1,000.0</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>277.0</td>
</tr>
</tbody>
</table>
and the aldosterone antagonist, spironolactone, are relatively weak inhibitors. Acetazolamide, a carbonic anhydrase inhibitor, was not inhibitory at the concentrations studied.

**Kinetic Studies**

In order to elucidate the kinetic mechanism of the swine kidney 15-OH-PGDH in terms of its possible relationship to the kidney prostaglandin metabolism, a relatively extensive kinetic study was performed.

**Initial Velocity**

The initial velocity patterns for the forward reaction are shown in Figures 9 and 10. When NAD$^+$ was plotted as the variable substrate, with different concentrations of PGE$_2$ as the changing fixed substrate (Figure 9), an intersecting pattern in the double reciprocal plot was obtained. When PGE$_2$ was plotted as the variable substrate, with different concentrations of NAD$^+$ as the changing fixed substrate, an intersecting pattern was again observed (Figure 10). When the data were fitted to Equation 1, the Michaelis constants for NAD$^+$ and PGE$_2$ were found to be $38.4 \pm 6.4 \mu$M and $1.58 \pm 0.19 \mu$M, respectively. The dissociation constant for NAD$^+$ was $81.6 \pm 8.37 \mu$M. That the dissociation constant is higher than the Michaelis constant for NAD$^+$ is indicated by the fact that the intersecting point is above the horizontal axis.

**Product Inhibition**

With NAD$^+$ as the variable substrate, 15-keto-PGE$_2$ gave linear noncompetitive inhibition (Figures 11 and 12). $K_{is}$ and $K_{ii}$ were calculated to be $32 \pm 2.4 \mu$M and $31 \pm 2.37 \mu$M respectively. With PGE$_2$ as the variable substrate, 15-keto-PGE$_2$ gave also linear noncompetitive inhibition (Figures
13 and 14). $K_{iS}$ and $K_{ii}$ were determined to be $21 \pm 1.6 \mu M$ and $19.6 \pm 1.5 \mu M$, respectively. With NAD$^+$ as the varied substrate, NADH gave linear competitive inhibition (Figures 15 and 16). $K_{iS}$ was determined to be $13.5 \mu M$. Since the available data from product inhibition studies posed at least two possibilities with respect to the order of addition of substrates and the order of release of products, dead-end inhibition and alternate substrate studies were performed.

**Dead-End Inhibition**

Adenosine diphosphate ribose (ADPR), a moiety of NAD$^+$ and an inhibitor of a number of dehydrogenase, was selected as the dead end inhibitor. With NAD$^+$ as the variable substrate, ADPR gave linear competitive inhibition (Figures 17 and 18). With PGE$_2$ as the variable substrate, ADPR gave non-competitive inhibition (Figures 19 and 20). A summary of the kinetic constants for product and dead-end inhibitions and their kinetic patterns is shown in Table VII.

**Table VII**

**PRODUCT AND DEAD-END INHIBITION STUDIES**

<table>
<thead>
<tr>
<th>Varied Substrates</th>
<th>15-keto-PGE$_2$</th>
<th>Inhibitors</th>
<th>NADH</th>
<th>ADPR</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD$^+$</td>
<td><strong>Type</strong></td>
<td><strong>Constants</strong></td>
<td><strong>Type</strong></td>
<td><strong>Constants</strong></td>
</tr>
<tr>
<td>NC</td>
<td>$K_{iS} = 31 \pm 2.3$</td>
<td>C</td>
<td>$K_{iS} = 13.5 \pm 1.1$</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>$K_{ii} = 32 \pm 2.4$</td>
<td></td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>NC</td>
<td>$K_{iS} = 19.6 \pm 1.5$</td>
<td>-----</td>
<td>NC</td>
</tr>
<tr>
<td></td>
<td>$K_{ii} = 21 \pm 1.60$</td>
<td>-----</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Type: "NC"—non-competitive, "C"—competitive. **Constants are in $\mu M$.}
Figure 9. Double reciprocal plot of initial velocity study with NAD\(^+\) as the varied substrate at different fixed concentrations of PGE\(_2\). The concentrations of NAD\(^+\) was varied between 0.02 mM and 0.25 mM. The concentrations of PGE\(_2\) were 0.75 \(\mu\)M (□), 1.0 \(\mu\)M (△), 1.5 \(\mu\)M (○), 2.0 \(\mu\)M (×), and 4.0 \(\mu\)M (●).
Figure 10. Double reciprocal plot of initial velocity study with PGE$_2$ as the varied substrate at different fixed concentrations of NAD$^+$. The concentration of PGE$_2$ was varied between 0.5 $\mu$M and 4.0 $\mu$M. The concentrations of NAD$^+$ were: 0.020 mM (□), 0.033 mM (△), 0.045 mM (×), 0.065 mM (○), and 0.25 mM (●).
\[
\frac{1}{PGE_2} \times 10^{-6} (M^{-1})
\]
Figure 11. Double reciprocal plot of product inhibition by 15-keto-PGE$_2$ with NAD$^+$ as the varied substrate. The concentration of NAD$^+$ was varied from 17.5 µM to 67.5 µM. The concentration of PGE$_2$ was kept constant at 2 µM. The concentrations of 15-keto-PGE$_2$ were none (X), 10.4 µM (O), 17.6 µM (△), 24.0 µM (□).
\frac{1}{V} \times 10^{-10} \text{(Mole}^{-1}\text{min)}

\frac{1}{\text{NAD}^+} \times 10^{-4} \text{(M}^{-1})

2 \quad 4 \quad 6
Figure 12. Slope and intercept replots of product inhibition by 15-keto-PGE₂ with NAD⁺ as the varied substrate. Conditions of the experiment are described in the legend to Figure 7. The slope replot is indicated by ( ○ ) and the intercept replot by ( ● ).
Figure 13. Double reciprocal plot of product inhibition by 15-keto-PGE$_2$ with PGE$_2$ as the varied substrate. The concentration of PGE$_2$ was varied from 0.65 μM to 3.0 μM. The concentration of NAD$^+$ was kept constant at 1 mM. The concentrations of 15-keto-PGE$_2$ were: none (○), 8.8 μM (△), 15.0 μM (×), 22.4 μM (□).
\[
\frac{1}{PGE_2} \times 10^{-6} (\text{M}^{-1})
\]

\[
(W_{\text{Mole/min}} - 0.10) \times \frac{A}{l}
\]
Figure 14. Slope and intercept replots of product inhibition by 15-keto-PGE$_2$ with PGE$_2$ as the varied substrate. Conditions of the experiment are described in the legend to Figure 9. The slope replot is indicated by (○) and the intercept replot by (●).
Figure 15. Double reciprocal plot of product inhibition by NADH with NAD$^+$ as the varied substrate. The concentration of NAD$^+$ was varied from 25 μM to 100 μM. The concentration of PGE$_2$ was kept constant at 28.3 μM. The concentrations of NADH were none (△), 15.6 μM (○), 22.2 μM (●). The amount of enzyme used per assay was 8 μg. The enzyme was assayed under the conditions of Method D.
\[
\frac{1}{NAD^+} \times 10^{-4} (M^{-1})
\]

\[
(\text{Mole}_{\text{min}}) \times 10^{-g} \frac{\Lambda}{l}
\]
Figure 16. Slope replot of product inhibition by NADH with NAD$^+$ as the varied substrate. Conditions of the experiment are described in the legend to Figure 11.
Figure 17. Double reciprocal plot of dead-end inhibition by ADPR with NAD\(^+\) as the varied substrate. The concentration of NAD\(^+\) was varied between 25 \(\mu\)M and 80 \(\mu\)M. The concentration of PGE\(_2\) was kept constant at 2 \(\mu\)M. The concentrations of inhibitor used were none (X), 0.2 mM ( ), 0.4 mM ( ), 0.6 mM ( ).
Figure 18. Slope replot of dead-end inhibition by ADPR with NAD$^+$ as the varied substrate. Conditions of the experiment are described in the legend to Figure 11.
Figure 19. Double reciprocal plot of inhibition by ADPR with PGE$_2$ as the varied substrate. The concentration of PGE$_2$ was varied between 0.2 μM and 1.35 μM. The concentration of NAD$^+$ was kept constant at 1.0 mM. The concentrations of inhibitor used were none (X), 0.5 mM (○), 1.2 mM (△), 1.8 mM (□).
Figure 20. Slope and intercept replots of dead-end inhibition by ADPR with PGE$_2$ as the varied substrate. Conditions of the experiment are described in the legend to Figure 13. The slope replot is indicated by (O) and the intercept replot by (●).
Alternate Substrate Studies

3-Acetyl-NAD$^+$ exhibited some coenzyme activity, as indicated in coenzyme specificity studies. When 3-acetyl-NAD$^+$ was mixed with NAD$^+$ at the same concentration (1 mM) and PGE$_2$ was varied in a wide range of concentrations, a concave upward curve with two different slopes was observed in a double reciprocal plot (Figure 21). Extrapolation of two lines with different slopes intersected with the horizontal axis at two points which gave apparent $K_m$ values of 4.0 \mu M and 1.1 \mu M for PGE$_2$, respectively.

When PGE$_2$ and PGF$_2$ were mixed at the same concentration (1 \mu M) and NAD$^+$ was varied in different concentrations, a linear plot was observed. The apparent $K_m$ for NAD$^+$ determined with mixed substrates was identical with the apparent $K_m$ for NAD$^+$ obtained by either using PGE$_2$ or PGF$_2\alpha$ as substrate. This evidence by three lines intersecting at the same point on the horizontal axis (Figure 22).
Figure 21. Double reciprocal plot of mixed coenzyme studies. The concentrations of NAD$^+$ and 3-acetyl-NAD$^+$ used were both 1.0 mM (○○○). The concentration of PGE$_2$ was varied between 0.6 μM and 10 μM. [15-3H]-PGE$_2$ was 20,000 cpm/ml. When 1 mM NAD$^+$ was used as substrate, PGE$_2$ concentration was varied between 0.8 μM and 2 μM (□□□). When 1 mM 3-acetyl-NAD$^+$ was used as substrate, the concentration of PGE$_2$ was varied between 0.6 μM and 4 μM (▵▵▵). The amount of enzyme used per assay was 1.7 μg.
\[ \frac{1}{V} \times 10^{-10} \text{(Mole}^{-1}\text{min)} \]

\[ \frac{1}{V} \times 10^{-11} \text{(Mole}^{-1}\text{min)} \]

\[ \frac{1}{V} \times 10^{-12} \text{(Mole}^{-1}\text{min)} \]

Plot with data points and lines.
Figure 22. Double reciprocal plot of mixed prostaglandin substrates study. The PGE\textsubscript{2} and PGF\textsubscript{2α} concentrations were both at 1 μM. The specific activity of [15-\textsuperscript{3}H]-PGE\textsubscript{2} and [15-\textsuperscript{3}H]-PGF\textsubscript{2α} were both at 20,000 cpm/nmole. When PGE\textsubscript{2} was used as substrate, NAD\textsuperscript{+} concentration was varied between 15 μM and 100 μM (■■■■). When PGF\textsubscript{2α} was used as substrate, NAD\textsuperscript{+} concentration was varied between 80 μM and 500 μM (●●●●). When PGE\textsubscript{2} and PGF\textsubscript{2α} were mixed as substrates, NAD\textsuperscript{+} concentration was varied between 40 μM and 200 μM (△△△). The amount of enzyme used was 3.5 μg.
DISCUSSION

15-OH-PGDH was assayed by several methods, and each method was validated by checking the linearity of 15-OH-PGDH activity as a function of time and enzyme concentrations.

Spectrophotometric Assay at 340 nm

The reduction of NAD\(^+\) is followed by the increase in absorbance at 340 nm. Interference occurs with this assay if crude extracts or early fractions of the purification process contain NADH oxidase or other enzymes utilizing pyridine nucleotides. This method is not as sensitive as the fluorometric or tritium transfer methods. The sensitivity of detecting 15-OH-PGDH activity is up to nmole range. Relatively large amounts of enzyme have to be used.

Spectrophotometric Assay at 500 nm

This assay was based on the development of a strong and transient chromophore at 500 nm following alkaline treatment of the reaction product of 15-keto-PG (Anggard and Samuelsson, 1966). This method is not suitable for kinetic studies, since the development of the chromophore is transient and unstable. However, it is convenient, fast, and economical for assaying a large number of chromatographic fractions.

Tritium-transfer Assay

Enzyme activity was determined by measuring the transfer of tritium from 15(S)-[15-\(^3\)H]-PGE\(_2\) to glutamate by coupling 15-OH-PGDH with excess glutamate dehydrogenase. This method provides a sensitive assay for the
enzyme activity in both crude extract and purified preparation.

The tritium transfer assay serves as an initial measurement of the enzyme activity irrespective of the presence of $\Delta^{13}$-15-keto-prostaglandin reductase in the catabolic pathway. This has a major advantage over other assays. The sensitivity of detecting 15-OH-PGDH activity is up to picomole range of substrate conversion. This method is fast and reproducible for kinetic studies. But this method has its limitations; for example, product inhibition by NADH cannot be performed, since an NAD$^+$ regenerating system was included. In addition, certain precautions are required for studying the effect of drugs or other agents on 15-OH-PGDH activity, since those substances may also affect the activity of the coupling enzyme.

Fluorometric Assay

The 15-OH-PGDH was determined by following the formation of NADH fluorometrically. This assay can detect NADH in the picomole range. However, studies on product inhibition by NADH are difficult to perform since the fluorescence of NADH at the concentrations used is beyond the range of the instrument.

Swine kidney was previously shown to be a rich source of 15-oG-PGDH (Ånggärd et al., 1971). Attempts to purify this enzyme by classical chromatographic techniques alone failed to obtain a preparation of sufficiently high specific activity with good recovery. Efforts to purify this enzyme were directed to the use of affinity chromatographic techniques. Several types of NAD$^+$-agarose having agarose derivatized to attach to different regions of the nucleotide were tested for possible affinity purification of swine renal 15-OH-PGDH. None of these affinity columns
was able to specifically retain swine renal 15-OH-PGDH. This is in contrast to human placenta 15-OH-PGDH, which could be specifically purified by NAD$^+$-hexane-agarose (Braithwaite and Jarabak, 1975). 5'-AMP-Sepahrose, which has been shown to be an effective affinity gel for the purification of a number of dehydrogenases (Guilford et al., 1972), also failed to bind swine renal 15-OH-PGDH. PGF$_2\alpha$-aminohexyl-sepharose was also proven to be of little value although it was reported to be useful for purifying bovine lung and swine kidney 15-OH-PGDH (Nagasawa et al., 1974; Oliw et al., 1976). The binding of 15-OH-PGDH to prostaglandin-sepharose column reported in the latter two cases was probably due to non-specific binding, since the enzyme could only be eluted with acetate buffer at pH 4.0. This is supported by the finding that the kinetic order of addition of substrates for 15-OH-PGDH favors NAD$^+$, being the first substrate added, followed by prostaglandin. The only affinity gel which was found to specifically bind swine renal 15-OH-PGDH was Affi-Gel Blue, or Blue Sepharose. This type of affinity gel has been increasingly used to purify enzyme having dinucleotide fold (Thompson et al., 1975). Swine renal 15-OH-PGDH was found to bind tightly to the Cibachron blue dye. Elution of the enzyme could not be effected even with 10 mM NAD$^+$ unless 1 mM of NADH was added in the buffer. This procedure resulted in efficient purification in one step. Although the specific activity of the purified enzyme was found to be lower than that of homogeneous enzyme from human placenta (Braithwaite and Jarabak, 1975) (1.2 U per mg vs. 1.7 U per mg), it was higher than those reported from swine lung (Marrazzi and Matschinsky, 1972), bovine lung (Nagasawa et al., 1974), and swine kidney (Tai and Hollander, 1976). The highly purified enzyme was devoid of any
other enzymes involved in prostaglandin metabolism, namely, 9-keto-prosta-
glandin reductase (Leslie and Levine, 1973), Type II 15-OH-PGDH (Lee and
Levine, 1975), 9-OH-PGDH (Pace-Asciak, 1975), and Δ13-15-keto-prostaglandin
reductase (Ånggård et al., 1971). It was of sufficient purity to carry
out some aspects of characterization and kinetic studies. A summary of
the partial purification of 15-OH-PGDH from swine kidney is shown in Table
I. Near-quantitative recovery of enzyme activity in early steps may in-
dicate that some endogenous inhibitors were present in the crude prepara-
tion which were gradually removed during purification, but it may as well
reflect a contamination with Δ13-15-keto-porstaglandin reductase, which
catalyzes further the reduction of Δ13-double bond, resulting in the
underestimation of the enzyme activity in early fraction.

The molecular weight of 15-OH-PGDH from swine kidney was estimated
to be 45,000. It is comparable to those from bovine lung, 40,000 (Hansen,
1974), and human placenta, 51,000 (Braithwaite and Jarabak, 1975). All
these values were estimated by Sephadex gel filtration.

The substrate specificity of 15-OH-PGDH from swine kidney was
compared to that from other tissues and species, as shown in Table VIII.

15-OH-PGDH from swine kidney appeared to have lower $K_m$ values for the
same prostaglandins than those from other sources. The difference may be
cased by differences in the incubation conditions, the purity, and the
source of the enzyme. It is worth noting that PGI$_2$ and its stable hydro-
lyzed product, 6-keto-PGF$_{1\alpha}$, are good substrates for swine renal 15-OH-
PGDH. This finding is consistent with the recent reports that PGI$_2$ and
6-keto-PGF$_{1\alpha}$ are rapidly metabolized to 6,15-diketo-13,14-dihydro-PGF$_{1\alpha}$
by rabbit and rat kidney (Wong et al., 1979 and Pace-Asciak et al., 1977).
### TABLE VIII

**APPARENT **$K_m^*$\_\text{values of 15-hydroxyprostaglandin dehydrogenase**}

<table>
<thead>
<tr>
<th>Tissues</th>
<th>PGE$_1$</th>
<th>PGE$_2$</th>
<th>PGA$_1$</th>
<th>PGA$_2$</th>
<th>PGF$_1\alpha$</th>
<th>PGF$_2\alpha$</th>
<th>PGI$_2$</th>
<th>PGB$_2$</th>
<th>TXB$_2$</th>
<th>Bibliograph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swine lung</td>
<td>7.7</td>
<td>12.0</td>
<td>14.0</td>
<td>25.0</td>
<td>25.0</td>
<td>31.0</td>
<td>---</td>
<td>---</td>
<td>not subs.</td>
<td>Nakano \textit{et al.}, 1969</td>
</tr>
<tr>
<td>Bovine lung</td>
<td>4.0</td>
<td>3.0</td>
<td>4.0</td>
<td>6.0</td>
<td>14.0</td>
<td>6.0</td>
<td>---</td>
<td>---</td>
<td>50.0</td>
<td>Matschinsky \textit{et al.}, 1974</td>
</tr>
<tr>
<td>Rhesus monkey lung</td>
<td>14.5</td>
<td>11.9</td>
<td>---</td>
<td>95.0</td>
<td>56.0</td>
<td>7.4</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>McGuire and Sun, 1978</td>
</tr>
<tr>
<td>Human placenta</td>
<td>7.7</td>
<td>5.3</td>
<td>8.7</td>
<td>---</td>
<td>---</td>
<td>30.0</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Jarabak, 1972</td>
</tr>
<tr>
<td>Human placenta</td>
<td>1.33</td>
<td>2.5</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Jarabak and Braithwaite, 1976</td>
</tr>
<tr>
<td>Human placenta</td>
<td>18.0</td>
<td>10.0</td>
<td>33.0</td>
<td>32.0</td>
<td>---</td>
<td>59.0</td>
<td>7.4</td>
<td>---</td>
<td>---</td>
<td>Schlegel and Greep, 1976</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>1.0</td>
<td>0.36</td>
<td>8.8</td>
<td>5.13</td>
<td>---</td>
<td>1.53</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>Wright \textit{et al.}, 1976</td>
</tr>
<tr>
<td>Swine kidney</td>
<td>1.2</td>
<td>1.2</td>
<td>1.7</td>
<td>4.6</td>
<td>19.2</td>
<td>12.5</td>
<td>5.0</td>
<td>not subs.</td>
<td>400</td>
<td>This thesis.</td>
</tr>
</tbody>
</table>

*$K_m^*$ values are in $\mu$M.
The fact that TXB$_2$ is a poor substrate for 15-OH-PGDH suggests that the enzyme may require a cyclopentane ring in addition to the presence of a 15(S)-hydroxyl group. However, it should be emphasized that these results are from in vitro data. Other factors may influence the metabolic fate of these compounds when administered to the intact animal. A good example is that PGAs circulate through the lung without appreciable loss of biological activity (Piper, et al., 1970) despite the fact that they are rapidly oxidized by purified lung 15-OH-PGDH (Anggard and Samuelsson, 1966).

Studies of the structural requirements for binding to NAD$^+$ site indicated that any modification of the NAD$^+$ molecule results in substantial loss of coenzyme activity. Alteration of the carboxamide group of the nicotinamide moiety appears to affect more of the coenzyme capacity than changing in the adenosine portion. For instance, NAD$^+$ analogs modified in the carboxamide group, such as thionicotinamide-NAD$^+$ and 3-pyridinealdehyde, could not serve as coenzyme. On the other hand, analogs modified in the adenosine portion, such as NADP$^+$ and deamino-NAD$^+$, showed some coenzyme activity. This finding is in contrast with the observation reported for swine lung 15-OH-PGDH (Marrazzi and Matschinsky, 1972). The difference in the source and the purity of the enzyme may account for the discrepancy. Selective control of 15-OH-PGDH activity through the NAD$^+$ site had limited pharmacological potential because of the presence of a similar site on numerous other dehydrogenases. Nevertheless, a study of the NAD$^+$ site, which is an essential feature of 15-OH-PGDH, contributes to a better understanding of how 15-OH-PGDH interacts with the coenzyme.

Virtually all sulfhydryl inhibitors except iodoacetic acid were found to be potent inhibitors of swine renal 15-OH-PGDH. This suggests that
certain sulfhydryl groups of the enzyme are crucial to the catalytic function. The fact that the enzyme is very liable in the absence of reducing agents in the buffer is consistent with this observation.

The effects of saturated and unsaturated fatty acids on swine renal 15-OH-PGDH were very similar to those reported for swine lung 15-OH-PGDH (Marrazzi and Matschinsky, 1972). Fatty acids, saturated or unsaturated, are apparently potent inhibitors of 15-OH-PGDH. The significance of this inhibition is not clear. However, it is known that free fatty acids are present at low concentrations intracellularly. They are released in high concentrations upon humoral or other stimulations. The released arachidonic acid is rapidly converted to prostaglandins and the related biologically active compounds. It is possible that the free fatty acids released along with arachidonic acid serve as endogenous modulators to regulate the half life of prostaglandins.

The effects of diuretic drugs on swine renal 15-OH-PGDH activity are particularly interesting. The results seem to suggest a slight correlation between the inhibitory potency and diuretic efficacy. There are four groups of diuretics, divided largely on the basis of their efficacy as saluretic agents. The first group includes diuretics such as ethacrynic acid, furosemide, and organomercurials. The second group consists of widely used diuretics of moderate potency, the chlorothiazide, hydrochlorothiazide, benzothiadiazine, and related compounds. The third group comprises the potassium-sparing agents, which are particularly valuable in antagonising the action of aldosterone. The fourth group is made up of various miscellaneous substances which do not act primarily by inhibiting the transport of sodium by renal tubular cells, but function as vasodilator
or osmotic agents. Mercurial diuretics are among the most potent diuretics. Mercury is presumed to inhibit enzymes involved in the reabsorption of sodium in the proximal tubules by combination with sulfhydryl groups of the enzyme. According to the efficacy of diuresis, mercurials produce a 20-25% loss in filtered sodium in the human system. Ethacrynic acid and furosemide produce a 25-35% loss in filtered sodium. These drugs exert their diuretic effects by blocking active chloride transport in the ascending limb of Henle's, probably in the form of a complex (Burg and Green, 1973). The thiazides produce a 10-15% loss in filtered sodium, and act primarily on the early distal tubule (cortical diluting segment) and on the sodium transporting mechanism. Spironolactone is structurally related to the natural hormone, aldosterone, and acts by competing with aldosterone for its target site in the distal tubule. It will produce a 3% loss of filtered sodium as a diuretics; however, it is primarily used for its potassium-sparing abilities. The present results suggest a correlation between inhibition of 15-OH-PGDH and diuretic efficacy. The first group of diuretics is indeed the most potent inhibitors of swine renal 15-OH-PGDH among all other groups studied. The second and third groups of diuretics are relatively weak inhibitors of the enzymes, as shown by higher $I_{50}$ values of the thiazide diuretics and spironolactone. Similar findings were previously reported for 15-OH-PGDH from human placenta (Paulsrud et al., 1974).

The results of kinetic studies on the initial velocity, product inhibition, dead-end inhibition, and alternate substrates are consistent with the following Ordered Bi-Bi mechanism.
where NAD$^+$ (A) is added first, followed by PGE$_2$ (B) and 15-keto-PGE$_2$ (P) is then released, followed by NADH (Q).

The study of initial velocity patterns in which one substrate is varied at different fixed levels of the second substrate provides a means of distinguishing between sequential and non-sequential kinetic mechanisms. In sequential mechanisms, addition of substrates may be ordered or random, but all must be enzyme-bound before product release can occur. Mechanisms such as the "ping-pong" type are non-sequential, and the first product is released before the second substrate is added. Double reciprocal plots which yield intersecting lines in the second quadrant are indicative of sequential mechanisms while plots which give parallel lines are consistent with the non-sequential mechanism (Cleland, 1963c).

Most sequential reactions conform to Equation (1), as formulated in the Methods section. Taking the reciprocal form of Equation (1), after rearranging, one obtains Equations (6) and (7). B is the changing fixed substrate and A is varied.

$$\frac{1}{v} = \frac{K_a}{V} \left(1 + \frac{K_{iA}K_B}{K_{AB}} \right) \left(\frac{1}{A} \right) + \left(1 + \frac{K_B}{B} \right) \frac{1}{V}$$  \hspace{1cm} (6)

A is the changing fixed substrate when B is varied.

$$\frac{1}{v} = \frac{K_B}{V} \left(1 + \frac{K_{iA}}{A} \right) \frac{1}{B} + \frac{1}{V} \left(1 + \frac{K_A}{A} \right)$$  \hspace{1cm} (7)

The slope of the two equations is
\[
\frac{K_a}{V} (1 + \frac{K_{ia}K_b}{K_{ia}B}) \text{ and } \frac{K_b}{V} (1 + \frac{K_{ia}}{A}) \text{ respectively for } A \text{ or } B \text{ as the variable substrate.}
\]

These slopes are a function of the nonvaried substrate. Therefore, intersecting reciprocal plots will be observed. Figures 5 and 6 show intersecting patterns for varying either NAD\(^+\) or PGE\(_2\), and thus they are compatible with a sequential mechanism for this enzyme.

Equation (1) is given by any sequential mechanism which include Ordered, Theorell-Chance and Rapid Equilibrium Random. To elucidate the precise kinetic mechanism, it is necessary to conduct further kinetic investigations. Product inhibition, dead-end inhibition, and alternate substrate studies have been the basic tools to achieve the objective. For any sequential Bi-Bi mechanism, the rate equation can be derived from the basic rate equation for the ordered mechanism, which is shown in Equation 8.

\[
\begin{align*}
V = & \frac{V_i \left(AB - \frac{PQ}{K_{eq}}\right)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_{iQ}}{K_{iq}} + \frac{K_{ia}K_p}{K_{ip}}} + \\
& \frac{K_{ia}K_{BPQ}}{K_{ip}K_{iq}} + \frac{K_{bK_{AP}}}{K_{iq}} + \frac{K_{aBQ}}{K_{iq}} + \frac{K_{ABP}}{K_{ip}} + \frac{K_{ia}K_bBPQ}{K_{ip}K_{iq}ib}
\end{align*}
\]

The rate equation for the Theorell-Chance mechanism (where there is no kinetically significant ternary complex) is the same except that it lacks the denominator terms in ABP and BPQ, while that for Rapid Equilibrium Random (where the order of addition of A and B is not obligatory, but the rate limiting step is solely the conversion of EAB to EPQ) lacks both of the AP and BQ terms as well (Cleland, 1963c).
For product inhibition studies, the rate equations can be derived from Equation (8) by setting either P or Q equal to zero. After rearranging, we obtain the following three equations for the product inhibition studies reported in this thesis.

Varying A, inhibit with P:
\[
\frac{1}{V} = \frac{K_a}{V} \left[ 1 + \frac{K_a K_b}{K_a} \left( 1 + \frac{p}{K_p K_{iq}} \right) \frac{1}{B} \right] + \frac{1}{V} (1 + \frac{K_b}{B}) + \frac{K_b K_a}{K_{iq} K_p} \frac{1}{K_{ip}} P
\]

Varying B, inhibit with P:
\[
\frac{1}{V} = \frac{K_b}{V} \left( 1 + \frac{K_a}{A} \right) \left( 1 + \frac{p}{K_p K_{iq}} \right) \frac{1}{B} + \frac{1}{V} \left( 1 + \frac{K_a}{A} \right) \left( 1 + \frac{p}{K_{ip}} \right) \frac{1}{1 + \frac{K_a}{A}}
\]

Varying A, inhibit with Q:
\[
\frac{1}{V} = \frac{K_a}{V} \left( 1 + \frac{K_a K_b}{K_a B} \right) \left( 1 + \frac{Q}{K_{iq}} \right) \frac{1}{A} + \frac{1}{V} \left( 1 + \frac{K_b}{B} \right)
\]

Equations (9) and (10) predict a linear noncompetitive inhibition, while Equation (11) predicts a linear competitive inhibition. The linear noncompetitive inhibition patterns as seen by 15-keto-PGE$_2$ with respect to NAD$^+$ (Figures 7 and 8) and PGE$_2$ (Figures 9 and 10) conform to Equations (9) and (10). These data clearly rule out Rapid Equilibrium Random mechanism since no competitive inhibition pattern as observed. The linear competitive inhibition pattern as observed by NADH with respect to NAD$^+$ (Figures 11 and 12) conforms to Equation (11). The results of these
product inhibition studies, however, do not distinguish between an Ordered Bi-Bi mechanism (12) and an Iso-Theorell-Chance mechanism (13).

\[
\begin{align*}
\text{NAD}^+ & \quad \text{PGE}_2 & \quad \text{15-keto-PGE}_2 & \quad \text{NADH} \\
\downarrow & \quad \downarrow & \quad \uparrow & \quad \uparrow \\
E & \quad E.\text{NAD} & \quad (E.\text{NAD}.\text{PGE}_2) & \quad E.\text{NADH} & \quad E.\text{NADH}.\text{15-keto-PGE}_2
\end{align*}
\]

\[
\begin{align*}
\text{PGE}_2 & \quad \text{NAD}^+ & \quad \text{NADH} & \quad \text{15-keto-PGE}_2 \\
\downarrow & \quad \downarrow & \quad \uparrow & \quad \uparrow \\
E & \quad E.\text{PGE}_2 & \quad F.\text{15-keto-PGE}_2 & \quad F & \quad E
\end{align*}
\]

Both mechanisms, (12) and (13), will be expected to give linear noncompetitive inhibition with respect to either substrate when 15-keto-PGE\textsubscript{2} is used as a product, and linear competitive inhibition with respect to NAD\textsuperscript{+} when NADH is used as a product.

To differentiate between these two possible mechanisms, dead-end inhibition and alternate substrate studies were carried out. The use of dead-end inhibitor has been known to be particularly useful for determining the order of addition of substrates in cases where product inhibition studies cannot or do not give an unequivocal answer. When ADPR was used as a dead-end inhibitor, it demonstrated a linear competitive inhibition with respect to NAD\textsuperscript{+} (Figures 13 and 14) and a linear noncompetitive inhibition with respect to PGE\textsubscript{2} (Figures 15 and 16). These patterns are consistent only with ordered addition of NAD\textsuperscript{+} followed by PGE\textsubscript{2}, as shown in the mechanism described by (12), since reverse order of addition of substrates will predict an uncompetitive inhibition with respect to PGE\textsubscript{2}. The kinetic mechanism actually can be further supported by the use of dead-end inhibitors, competitive to PGE\textsubscript{2}. This kind of inhibitor should yield
uncompetitive pattern with respect to NAD$^+$. Unfortunately, no such kind of inhibitor has been found.

Further substantiation of the kinetic mechanism of swine renal 15-OH-PGDH being an Ordered Bi-Bi mechanism was provided by alternate substrate studies. The use of alternate substrate to elucidate the kinetic order of addition of substrates was first suggested by Wong and Hanes (1962). If a mixture of two $A$ substrates is used, namely $A_1$ and $A_2$, and $A$ is the first substrate on the enzyme, $B$ will now have two enzymic forms ($A_1E$) and ($A_2E$) with which to react. The result will be a mechanism now second degree in $B$, and the standard plots will be curved unless $A_1$ and $A_2$ coincidently have identical effect of $K_m^B$. If, however, $A$ is the second substrate, $B$ reacts only with free enzyme and no increase in degree results. This behavior permits the experimental distinction of the first the second substrates. When a mixture of 3-acetyl-NAD$^+$ and NAD$^+$ was used, the double reciprocal plot of $1/V$ vs. $1/PGE_2$ appeared to be concave upward. $K_m$ values for $PGE_2$ determined from two apparent slopes were significantly different from that obtained by the presence of either coenzyme alone. Apparently the affinity for $PGE_2$ is significantly altered in the presence of both nucleotides. In contrast, when a mixture of $PGE_2$ and $PGF_{2\alpha}$ was used, the double reciprocal plot of $1/V$ vs. $1/NAD^+$ did not depart from linearity. $K_m$ values for NAD$^+$ were the same irrespective of the presence of $PGE_2$ and $PGF_{2\alpha}$, either alone or in a mixture. Obviously, the affinity for NAD$^+$ is not affected by the presence of different substrates. These results clearly suggest that NAD$^+$ is the first substrate added, followed by prostaglandins.

Schlegel and Greep (1975 and 1976) and Rückrich et al. (1975 and
1976) have separately employed product inhibition studies to deduce the kinetic mechanism of human placental 15-OH-PGDH. They found that NADH exerted a linear competitive inhibition with respect to NAD$^+$, and that 15-keto-prostaglandins $E_1$ and $F_2\alpha$ showed a linear noncompetitive inhibition with respect to the corresponding prostaglandins. Based on these studies, they suggested an Ordered Bi-Bi mechanism in which NAD$^+$ was added first, followed by prostaglandins for human placental 15-OH-PGDH. Critically, these product inhibition studies do not distinguish between an Ordered Bi-Bi mechanism, as described by (12), and an Iso-Theorell-Chance mechanism as described by (13), as pointed out by Hansen (1976).

Jarabak and Braithwaite (1976) conducted a more detailed kinetic analysis of the mechanism of human placental 15-OH-PGDH. They carried out product inhibition studies at two different pH values, and also employed alternate substrate studies. They have concluded that human placental 15-OH-PGDH proceeds by a single displacement mechanism. Addition of the substrates is ordered, with NAD$^+$ binding first. The lifetime of the ternary complex is affected by the pH of the reaction mixture. At pH 7.0 a kinetically significant ternary complex is formed, while at pH 9.0 the ternary complex is not kinetically significant (Theorell-Chance mechanism). The results of the present study on swine renal 15-OH-PGDH (conducted at pH 7.5) appeared to agree well with the mechanism proposed for human placental 15-OH-PGDH at pH 7.0.

The significance of these kinetic studies can be manifold. The $K_m$ values for prostaglandins were found to be much higher than the prostaglandin concentrations present in animal kidneys (Karim et al., 1968; van Dorp, 1971). It is anticipated that renal 15-OH-PGDH should operate
at a reaction rate far below the maximum velocity with respect to prosta-
glandins. The $K_m$ values for NAD$^+$ were much lower than the actual
concentration of NAD$^+$ (Villee, 1962). The potential reaction rate would
be at near-maximal rate with respect to NAD$^+$. Regulation of renal 15-OH-
PGDH reaction rate by the renal levels of prostaglandins is much more
likely than a regulation by the NAD$^+$/NADH relation. Furthermore, because
of the high $K_i$ of 15-keto-PGE$_2$, neither inhibition of the enzymatic
reaction by 15-keto-prostaglandins nor a possible backward reaction seems
to be of physiological significance.
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