ISOLATION AND CHARACTERIZATION OF TWO ENZYME PROTEINS CATALYZING OXIDO-REDUCTION AT C-9 AND C-15 OF PROSTAGLANDINS FROM SWINE KIDNEY

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Two swine kidney proteins (PI 4.8 and 5.8) both possessing 9-prostaglandin ketoreductase (9-PGKR) and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) activities were purified to homogeneity. Purification increased specific activities in parallel.

Molecular weight, subunit size, amino acid composition, coenzyme and substrate specificity and antigenicity of both proteins were similar. Gel filtration and SDS-polyacrylamide gel electrophoresis molecular weights of 29,500 and 29,000, respectively, suggested a single subunit.

Although a variety of prostaglandins served as substrates, the best for 15-PGDH was PGB, while PGA₁₁-GSH showed the lowest Km for 9-PGKR. Rabbit antibody against the PI 5.8 protein crossreacted with both purified renal enzymes and with extracts from rat spleen, lung, heart, aorta, and liver.
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INTRODUCTION

In 1930 Kurzrok and Lieb showed that human semen could induce either a strong contraction or relaxation when applied to a human uterus. A few years later, Goldblatt (1933) and Von Euler (1934) independently demonstrated the presence of a vasodepressor agent and a stimulator of smooth muscle in human seminal plasma and in the sheep vesicular gland. The biologically active material was named prostaglandin (PG) by Von Euler (1936), who also indicated that it was lipid-soluble and had acidic properties. The elucidation of the chemical structure of prostaglandins was achieved by Bergström and Sjövall (1957). They isolated and identified crystalline prostaglandin E₁ (PGE₁) and prostaglandin F₁α (PGF₁α) from sheep seminal vesicular extracts. Within a short time, a number of other prostaglandins were isolated. The naturally occurring prostaglandins may be regarded as derivatives of prostanoic acid, an organic acid with a substituted cyclopentane unit. Subsequent studies by some investigators led to the important discovery that prostaglandins were derived from certain polyunsaturated essential fatty acids via the formation of short-lived prostaglandin endoperoxides (PGG and PGH) (Bergström et al., 1964; Van Dorp et al., 1964; Hamberg et al., 1975). Three fatty acids: dihomo-ß-linolenic acid (C₂₀:8,11,14),
arachidonic acid \((C_{20:5,8,11,14})\) and eicosopentaenoic acid \((C_{20:5,8,11,14,17})\) have been identified as precursors of prostaglandins of the 1,2 and 3 family with 1,2 and 3 double bonds respectively in the side chains. Generally speaking, prostaglandins of the 3 family are biologically less active than those of the other two families. The biosynthesis of prostaglandins of the 2 family chosen as an example is shown in Scheme I.

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 finally to prostaglandins of the B type as shown in Scheme II.

![Scheme II: Biosynthesis of PGA, PGB and PGC](image)

Recently two potent and short-lived compounds, thromboxane \(A_2(TXA_2)\) \((\text{Hamberg et al., 1975})\) and prostacyclin \((\text{PGI}_2)\) \((\text{Moncada et al., 1976})\) were discovered. Both of which are derived from arachidonic acid via prostaglandin
Scheme I  Biosynthesis of Prostaglandin 2 family
endoperoxides (PGH₂) and are hydrolyzed readily to much less active thromboxane B₂ (TXB₂) and 6-ketoprostaglandin F₁α (6-keto-PGF₁α) respectively as shown in Scheme III.

Prostaglandins are versatile and powerful substances found in almost all mammalian tissues. Although the physiological role of prostaglandins has yet to be clearly defined, the pharmacological actions of these compounds are numerous: PGEs relax smooth muscle, lower blood pressure, inhibit lipolysis and platelet aggregation. The PGFs raise the blood pressure as vasopressors, relax or contract smooth muscle depending on the species examined and initiate luteolysis. The PGDs are more potent inhibitors of platelet aggregation than the PGEs. The PGAs and the PGCs cause vasodilatation with a subsequent lowering of venous return which contributes to the hypotensive effect. The PGBs do not appear to have any prominent biological activity. TXA₂ contracts smooth muscle and is the most powerful agent causing irreversible platelet aggregation, while PGI₂ relaxes smooth muscle and is the most potent inhibitor of platelet aggregation. The opposing actions of TXA₂ and PGI₂ as well as PGEs and PGFs have led to the speculation that the regulation of the synthesis of these prostaglandins may be of vital importance in physiological function and disease.

The catabolism of the biologically active prostaglandins in most tissues is initiated by the oxidation of the
Scheme III  Biosynthesis of Thromboxane A$_2$ and Prostacyclin
15(S)-hydroxyl group, followed by the reduction of the Δ^{13}-double bond forming 15-keto-13,14-dihydroprostaglandins (Änggärd et al., 1965). Further catabolism includes β-oxidation from the carboxyl end and ω-oxidation of the terminal carbon resulting in the formation of C_{16}-dicarboxylic acids which are predominant prostaglandin metabolites observed in urine (Hamberg and Samuelsson, 1971). A representative catabolic pathway of PGE\textsubscript{2} and PGF\textsubscript{2α} is illustrated in Scheme IV.

The enzyme involved in the first step of catabolic degradation of these naturally occurring prostaglandins is 15-hydroxyprostaglandin dehydrogenase (15-PGDH). This enzyme catalyzes the oxidation of 15(S)-hydroxyl group resulting in a significant loss of biological activity of prostaglandins and has been considered the major enzyme responsible for biological inactivation of prostaglandins (Änggärd, 1966). Two types of 15-PGDH have been described. Type I needs NAD\textsuperscript{+} as coenzyme, while type II requires NADP\textsuperscript{+} as coenzyme (Lee and Levine, 1975). In addition to coenzyme requirements, these two types of enzymes can be distinguished by their chromatographic properties, relative affinities for prostaglandins, and sensitivities to inhibition by reduced pyridine nucleotides (Lee et al., 1975). Because of its lower Km values for the the prostaglandins and the relative abundance of cytoplasmic NAD\textsuperscript{+}, type I 15-PGDH
Scheme IV  Interconversion and catabolism of PGE$_2$ and PGF$_{2\alpha}$
may be primarily responsible for the inactivation of prosta-
taglandins. The physiological significance of type II 15-
PGDH is not clear.

Type I 15-PGDH catalyzes NAD$^+$-dependent oxidation of
prostaglandins with the exception of PGB and TXB (Nakano
and Samuelsson, 1969; Braithwaite and Jarabak, 1975;
Kung-Chao and Tai, 1980). It is ubiquitously present in
the cytosolic fraction of almost all mammalian tissues
(Ånggärd et al., 1971; Sun et al., 1976; Tai, 1976; Moore
and Houlet, 1977). Isolation and characterization of this
enzyme from various tissues have been attempted by several
investigators. In one instance the enzyme from human
placenta has been purified to homogeneity (Braithwaite and
Jarabak, 1975). The enzyme in general has M.W. of 45,000-
51,000 and is sulfhydryl sensitive, inhibited by indome-
thacin, thyroid hormones and diuretic drugs (Tai and Hollander,
1974; Braithwaite and Jarabak, 1975; Hansen, 1976; Kung-Chao
and Tai, 1980). A different type I 15-PGDH which is speci-
fic for PGA has been found in rabbit kidney papilla (Olien
et al., 1976).

Type II 15-PGDH catalyzes NADP$^+$-dependent oxidation of
virtually all prostaglandins although PGB$_1$ being the best
substrate (Jarabak and Fried, 1979). The enzyme has been
demonstrated to be present in various animal tissues and
has been isolated and characterized from monkey brain (Lee
and Levine, 1975), human erythrocytes (Kaplan et al., 1975),
swine kidney (Lee et al., 1975), chicken heart (Lee and Levine, 1975), chicken kidney (Hassid et al., 1977) and human placenta (Westbrook et al., 1977; Line and Jarabak, 1978). The enzyme from human placenta has been purified to homogeneity (Lin and Jarabak, 1978). The enzyme in general is sulfhydryl sensitive, inhibited by indomathacin and diuretic drugs, but not by thyroid hormones (Lee and Levine, 1975, Sun and Tai, 1977; Lin and Jarabak, 1978). The enzyme isolated from human placenta appeared to exist in two forms having M.W. of 31,000 and 33,000 respectively (Westbrook et al., 1977). A heat stable inhibitory protein having M.W. of 6,000-7,000 has also been demonstrated in human placenta (Westbrook et al., 1977). Recently, a different type II 15-PGDH specific for PGD has been isolated from swine brain. The M.W. as estimated by Sephadex G-100 gel filtration was 62,000 (Watanabe et al., 1980).

Earlier studies by Hamberg and Samuelsson (1971) showed that metabolism of PGE₂ in guinea pig liver led to the formation of PGF₂α and 13,14-dihydro-PGE₂ in addition to 15-keto-PGE₂ and 15-keto-13,14-dihydro-PGE₂ indicating the presence of prostaglandin 9-ketoreductase (9-PGKR) and prostaglandin 15-ketoreductase (15-PGKR). Leslie and Levine (1973) first provided evidence for the presence of a prostaglandin E₂-9-ketoreductase in various rat organs using radioimmunological assay of PGF₂α generated from PGE₂. Subsequently, Lee and Levine (1974) demonstrated that
interconversion of PGF$_{2\alpha}$ and PGE$_2$ in the presence of NADP or NADPH could be also effected by the cytosolic fraction although it was not certain that both directions of reaction were catalyzed by the same enzyme protein. The same investigators also showed that a second type of 9-PGKR was present in the microsomal fraction and used NADH more efficiently than NADPH. Nevertheless, demonstration of the presence of enzyme(s) which catalyzes the interconversion of functionally opposite prostaglandins in various tissues has generated a great deal of interest since this enzyme might play a major role in regulating the ratio of PGE$_2$ to PGF$_{2\alpha}$ (Lee and Levine, 1974). Attempts to purify and to study the regulatory properties of NADPH-dependent 9-PGKR were made in several systems, including monkey brain (Lee and Levine, 1975 a), human erythrocytes (Kaplan et al., 1975), chicken heart (Lee and Levine, 1975 b), rabbit kidney (Stone and Hart, 1975), swine kidney (Lee et al., 1975; Sun and Tai, 1977), chicken kidney (Hassid and Levine, 1977) and human placenta (Westbrook et al., 1977). The enzyme was able to catalyze the reduction of 9-keto group of PGE and PGA and their metabolites. Although the 15-keto function of prostaglandins could be reduced by the partially purified preparations, it was not clear whether the reduction was catalyzed by the same enzyme protein. Km values of these 9- and 15-keto prostaglandins were relatively high (100 µM). Interestingly, Cagen et al., (1979) discovered that the
glutathione conjugate of PGA's was a much better substrate (20 μM) showing a specific activity that was at least three orders of magnitude greater than that when PGA₂ or PGE₂ was used as a substrate. The enzyme was found to be sensitive to sulfhydryl inhibitors, indomethacin and certain diuretics such as furosemide and ethacrynic acid. A heat-stable inhibitory factor has been demonstrated in chick breast muscle (Lee et al., 1976) and in human placenta (Westbrook et al., 1977). A heat-stable stimulatory factor has also been shown to be present in chicken heart (Lee and Levine, 1975 b). The enzyme appeared to exist in multiple forms from chicken heart (Lee and Levine, 1975 b), chicken kidney (Hassid and Levine, 1977), swine kidney (Sun and Tai, 1977) and human placenta (Westbrook et al., 1977; Lin and Jarabak, 1978). In some instances where type II 15-PGDH activity was also determined, 9-PGKR copurified with 15-PGDH. It has been suspected that 9-PGKR activity and type II 15-PGDH activity might be associated with the same enzyme protein. Lin and Jarabak (1978) provided strong evidence that it was the case by purifying the enzyme from human placenta to homogeneity and showing both activities resided in the single protein. Whether this observation holds true in other tissues remains to be determined.

Mammalian kidneys are known to play a central role in the regulation of fluid and electrolyte homeostasis, the regulation of systemic arterial blood pressure, and the
maintenance of plasma volume. The discovery that the kidney medulla possesses prostaglandins (PGE$_2$ and PGA$_2$) with potent antihypertensive and natriuretic activities has stimulated speculation that these compounds may act intra-renal to regulate renal blood flow, arterial blood pressure, and sodium and water secretion (Lee et al., 1967, 1968). Not only do mammalian kidneys have high activity of biosynthesis of prostaglandins (Tai and Hollander, 1976; Pong and Levine, 1976), but also active catabolism of these biologically potent substances (Larsson and Änggärd, 1973; Tai et al., 1974). Type I 15-PGDH and prostaglandin-$\Delta^1$ reductase were shown to be richest in kidney among all swine tissues examined (Änggärd et al., 1971). Type II 15-PGDH and 9-PGKR were also found to be abundant in mammalian kidneys (Leslie and Levine, 1973). Studies on the distribution of these catabolic enzymes in swine kidney have indicated that 11 times more type I 15-PGDH activity was found in the cortex than in the medulla. However, twice as much type II 15-PGDH activity was found in the medulla as in the cortex, and 9-PGKR activity was equally distributed between cortex and medulla (Lee et al., 1975). Partial purifications of type II 15-PGDH and 9-PGKR from swine kidney have indicated non-parallel increment of specific activities although two different enzyme activities failed to separate from each other during various chromatography (Lee et al., 1975). These observations
argue against the idea, type II 15-PGDH and 9-PGKR activities are associated with the same enzyme protein in swine kidney. It was postulated that these two enzyme activities are coupled to regulate the relative levels of PGE$_2$ and PGF$_{2\alpha}$ in kidney (Lee and Levine, 1974; Lee et al., 1975).

The possible roles of type II 15-PGDH and 9-PGKR in renal function and blood pressure regulation have been explored in at least two different systems. Limas and Limas (1977) found that type I as well as type II 15-PGDH activities were lower in the Japanese spontaneously hypertensive rat kidney than in the age-paired normotensive control at all ages, suggesting an impaired catabolism of renal prostaglandins in hypertensive rats. Weber et al. (1977) observed an increase of renal cortical and medullary 9-PGKR activity during high salt intake in rabbit. The increase of renal 9-PGKR activity shifts intrarenal prostaglandin formation from PGE$_2$ to PGF$_{2\alpha}$ which results in a decrease in renin activity. They proposed that 9-PGKR might serve as a mediator of salt intake-related prostaglandin-renin interaction. The significance of type II 15-PGDH and 9-PGKR in renal function and blood pressure regulation becomes more apparent.

This study was initiated by isolating type II 15-PGDH and 9-PGKR from swine kidney and purifying it to apparent homogeneity. It was found that both enzyme activities were
associated with each of the two isozymic proteins. In addition, 15-PGKR and 9-PGDH activities were also found to be present in either isozymes. Apparently both isozymes are capable of catalyzing the oxido-reduction at C-9 and C-15 of prostaglandins. Partial characterization of both isozymes with regard to molecular weight, subunit structure, coenzyme and substrate specificities, amino acid composition, kinetics of oxido-reduction at C-9 and C-15 and immunological properties was conducted. The results provide valuable information for future investigation of mechanism and function of type II 15-PGDH/9-PGKR.
MATERIALS AND METHODS

Materials

TEAE-cellulose, coomassie brilliant blue R-250, NADP⁺, NAD⁺, NADPH, NADH, Trizma base, indomethacin, EDTA, albumin (egg), trypsinogen, lysozyme, glutathione, β-mercaptoethanol, nitro blue tetrazolium, phenazine methosulfate, horse liver alcohol dehydrogenase (0.5 unit/mg), bovine serum albumin, soybean lipoxidase, cytochrome-C, linoleic acid were obtained from Sigma Chemical Co. Dye-reagent concentrate (protein assay), ammonium persulfate, sodium dodecyl sulfate (SDS), acrylamide, Affi-Gel Blue (100-200 mesh), N,N'-methylene-bis-acrylamide were obtained from Bio-Rad Laboratories. Glacial acetic acid, 1-butanol, glycerol, phosphoric acid, acetone, methanol, gelatin, trichloroacetic acid were purchased from Fisher Scientific Co. Sephadex G-100, G-50, G-25, ampholine carrier ampholytes were obtained from Pharmacia Fine Chemical Co. Sucrose (density gradient grade) was obtained from Schwarz/Mann Co. Potassium phosphates (dibasic & monobasic) were obtained from Matheson Coleman & Bell Manufactory Chemists Co. Ethacrynic acid was obtained from Merck Sharp & Dohme Research Lab. Complete Freund's adjuvant was obtained from Calbiochem Co. Immuno-diffusion disc was obtained from Miles Laboratories, Inc.
Ethanolamine was obtained from Eastman Chemical Co.
Prostaglandin A_1 (PGA_1), prostaglandin A_2 (PGA_2), prostaglandin B_2 (PGB_2), prostaglandin D_2 (PGD_2), prostaglandin E_1 (PGE_1), prostaglandin E_2 (PGE_2), prostaglandin F_2(1alpha) (PGF_2(1alpha)), prostacyclin (PGI_2), 6-keto-prostaglandin F_1(1alpha) (6-keto-PGF_1(1alpha)), 15-keto-prostaglandin E_2 (15-keto-PGE_2), 15-keto-prostaglandin F_2(1alpha) (15-keto-PGF_2(1alpha)) and thromboxane B_2 (TxB_2) were kind gifts of Dr. John Pike of the Upjohn Co.

Methods

Enzyme Assays

Enzyme activities catalyzing oxidoreduction of prostaglandins at C-9 and C-15 were determined by a variety of methods. The enzymatic reactions catalyzed by these enzymes are shown in Scheme IV.

Method A. (for 15-PGDH) -- The 15-PGDH activity was assayed by quantitating the production of 15-keto-PGE_2 (or 15-keto-PGE_1) from PGE_2 (or PGE_1) as described by Anggard and Samuelsson (1966). The assay is based on the development of a strong and transient chromophore at 500 nm following alkali treatment of the reaction product 15-keto-PGE_2. The assay mixture contained: PGE_2, 56.8 uM; NADP^+, 0.2 mM; and enzyme in a final volume of 1 ml of 0.1 M potassium phosphate buffer, pH 7.5. The reaction mixture
was incubated at 37°C for 40 minutes, then terminated by the addition of 0.1 ml of 2N NaOH. The absorbance of the chromophore was determined spectrophotometrically at 500 nm and the concentration of 15-keto-PGE$_2$, was estimated using a molar extinction coefficient of 27,000 M$^{-1}$cm$^{-1}$ (Oliw et al., 1976). This method was used for the rapid analysis of the chromatographic fractions.

Method B. (for 9-PGDH and 15-PGDH) -- 15-PGDH activity was also determined by measuring the rate of formation of NADPH spectrofluorometrically at 25°C in a reaction mixture containing 0.2 mM NADP$^+$ and proper amount of substrate and enzyme in a final volume of 0.75 ml of 0.1 M potassium phosphate buffer, pH 7.5. The reaction was initiated by the addition of enzyme and the NADPH formed was recorded by the increase in fluorescence at 468 nm with excitation at 347 nm, using an Aminco SPF-500 spectrofluorometer coupled to an Aminco X-Y recorder. The instrument was standardized by different concentrations of NADPH, which was determined by direct measurement of the absorbance at 340 nm with a Gilford 250 spectrophotometer using a molar extinction coefficient of 6.22 x 10$^3$ M$^{-1}$cm$^{-1}$ (Horecker and Kornberg, 1949). This method was used to determine the substrate specificity of 15-PGDH.

Method C. (for 9-PGKR and 15-PGKR) -- Enzyme activity of 9-PGKR and 15-PGKR was measured by following the decrease
of NADPH spectrophotometrically at 25°C in a reaction mixture containing 0.2 mM NADPH and proper amount of substrate and enzyme in a final volume of 0.75 ml of 0.1 M potassium phosphate buffer, pH 7.5. The decrease in absorbance at 340 nm was recorded using Gilford 250 spectrophotometer attached to a recorder. This method was used to determine the substrate specificity of 9-PGKR and 15-PGKR.

**Method D.** (for 9-PGKR, 15-PGKR, 9-PGDH and 15-PGDH) --
The products of these enzymatic reactions, as shown in Scheme IV, were measured by radioimmunoassay (RIA). The reaction mixture (0.2 ml) contained: 0.5 mM NADP⁺ (or NADPH), 1 μg of prostaglandin, enzyme and 0.1 M potassium phosphate buffer, pH 7.5. It was incubated at 37°C for 10 minutes and then terminated by boiling for 2 minutes. After centrifugation to remove denatured proteins, reaction products in the clear solution were analyzed by RIA.

Antisera against PGE₂, PGF₂α and 15-keto-PGF₂α from New Zealand rabbits were prepared following immunization with the corresponding prostaglandin-BSA conjugate according to the procedure of Tai and Yuan (1978). Antisera at 1 to 25,000, 150,000 and 10,000 final dilution were used for the RIA of PGE₂, PGF₂α and 15-keto-PGF₂α, respectively. [¹²⁵I] labeled prostaglandin tyrosine methyl ester conjugate were prepared according to the procedure of Tai and Yuan (1978). The RIA incubation mixture (0.4 ml) contained: 0.2 ml of
sample (or standard), 0.1 ml of diluted antiserum and 0.1 ml of labeled hapten (ca. 10,000 cpm) in RIA buffer, 50 mM Tris-HCl, pH 7.5 containing 0.1% gelatin. After one hour of incubation at room temperature, the separation of free from bound hapten was achieved by the addition of 0.2 ml of a 3% charcoal suspension containing 1% bovine γ-globulin to the reaction mixture and incubation for 10 minutes before precipitating the charcoal-bound fraction by centrifugation at 1000 xg for 10 minutes. Antibody-bound hapten remained in the supernatant, while the free hapten was absorbed by the added charcoal. The amount of prostaglandins formed during the enzymatic reaction was estimated from a standard displacement curve after logit transformation, as described by Tai and Chey (1976). This method provided a more sensitive quantitation of enzyme activity than the other methods. It was used in enzyme purification, heat inactivation studies, inhibition and kinetic studies.

Alkaline pH Discontinuous Polyacrylamide Gel Electrophoresis

Resolving gel (7.5%) and stacking gel (3%) were prepared in gel tubes (0.5 x 10 cm) according to the method of Maizel (1969). Sample (20 μg of protein) with volume up to 50 μl was mixed with an equal volume of sample buffer, containing 10% glycerol, 0.01 M 2-mercaptoethanol, 0.03 M H₃PO₄, 0.06 M Tris base and 0.0025% bromophenol blue as tracking dye in H₂O. The sample solution was transferred
to the top of the gel, then carefully overlaid with electrode buffer (1.2 g Tris base and 5.76 g glycine in one liter H₂O, pH 8.3). Gel electrophoresis was run in cold room with voltage set at 300-400 volts and current set at 3 mA per tube. When the run was completed, the gel was removed from the tube and fixed in 12% trichloroacetic acid (TCA) for 30 minutes. The gel was then transferred into staining solution, 0.05% coomassie brilliant blue R-250 in 12% TCA solution, for at least 6 hours. Finally the gel was destained with destaining solution (5% methanol and 7% glacial acetic acid in distilled water) till the background of the gel was clear. This method was used for determining the homogeneity of the native enzyme and providing some information on the electrophoretic properties of the enzyme.

Molecular Weight Determination

A. Determination of the molecular weight of native enzyme by gel filtration.

Sephadex G-100 gel was swollen and equilibrated in 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA and 0.25% sucrose for two days. The Sephadex G-100 was then packed into a column (2.7 x 55 cm) and washed with buffer for 24 hours at 4°C until the bed height and flow rate (30 ml per hour) were stabilized. The void volume was determined with blue dextran (M.W. 2,000,000). Protein of known molecular weights, namely cytochrome C, trypsin
inhibitor, horse liver alcohol dehydrogenase and lipoxidase, were used to calibrate the column (Andrews, 1964).

Cytochrome C and trypsin inhibitor were estimated spectrophotometrically at 412 nm and 215 nm respectively. Alcohol dehydrogenase was assayed by following the production of NADH. The assay mixture contained 2 M ethanol, 0.5 mM NAD\(^+\) in 0.1 M Tris-HCl buffer, pH 8.0 containing 1 mM EDTA, and proper amount of alcohol dehydrogenase from the column eluate to effect an absorbance change at 340 nm. The assay for lipoxidase activity involved mixing 0.5 mM of linoleic acid and column eluate in 0.2 M Borate buffer at pH 9.0. The oxidation of linoleate was detected by an increase in absorbance at 234 nm.

**E. Determination of subunit molecular weight of the enzyme by SDS gel electrophoresis.**

The subunit molecular weight was determined by comparing the electrophoretic mobility of the enzyme with known protein markers. A linear relationship is obtained if the electrophoretic mobilities (Rf) are plotted against the logarithm of known polypeptide chain molecular weights (Weber et al., 1969).

SDS polyacrylamide gels (7.5%) were prepared in gel tubes (0.5 x 10 cm). The sample (5 to 25 \(\mu\)g of protein) was mixed with an equal amount of sample buffer (0.1% SDS, 10% glycerol, 0.01 M 2-mercaptoethanol, 0.01 M sodium
phosphate buffer, pH 7.2 and 0.002% bromophenol blue as tracking dye in H₂O) in a small test tube. The test tube was placed into a boiling water bath for 5 minutes. The sample mixture was then transferred to gel tube and carefully overlaid with electrode buffer: 0.1 M sodium phosphate buffer, pH 7.2 containing 0.1% SDS. The SDS gel electrophoresis was run at room temperature. The running current was set at 8 mA per tube. When the electrophoresis was completed, the gel was removed from the tube and was fixed with 12% TCA for 30 minutes. The gel was then stained overnight with 0.05% coomassie brilliant blue R-250 stain solution. The gel was finally destained with destaining solution, same as that for alkaline pH discontinuous gel electrophoresis. This method was used to determine the homogeneity and the number of subunits of the enzyme.

**Production Of Antiserum**

Antiserum was generated against one form of the purified enzyme (PI 5.8) in an adult rabbit by intramuscular injections of 250 µg each of the enzyme in 1 ml of saline emulsified with an equal volume of Freund's complete adjuvant at five different occasions. With the exception that the second injection was done two weeks after the first one, the next three injections were made at monthly intervals. The antiserum was collected two weeks after each injection, beginning with the third injection. Control
rabbit serum was collected before the first injection.

**Immunodiffusion Studies**

Double immunodiffusion analyses were performed with Ouchterlony discs, containing 0.9% agarose, 0.01% merthiolate and borate saline buffer, pH 8.5. The antiserum was placed in the central well, while the different enzyme preparations were placed in surrounding wells. After 48 hours incubation, the gels were soaked in 1% NaCl for six hours, upon which fresh NaCl was added and the gel allowed to soak overnight. The gels were rinsed with distilled water for one hour, dried and stained in an Amido black stain solution (0.5 g Amido black in one liter of the solution, containing 40% ethanol and 10% acetic acid in distilled water) for 20 minutes. The gels then were destained with destaining solution, containing 45% (v/v) of methanol, 10% acetic acid in distilled water, until the background of the gels were clear.

**Analysis Of Amino Acid Composition**

Purified enzyme with PI 5.8 and PI 4.8 were dialyzed against distilled water for two days. The samples (200 µg) were then lyophilized and hydrolyzed with 0.3 ml 6 N HCl in an evacuated sealed tube at 110°C for 22, 48 and 72 hours. The hydrolysates were dried thoroughly in vacuum over P_2O_5. Aliquots of the hydrolysates were applied to a
Durrum Amino Acid Peptide Analyzer using o-phthaldialdehyde/2-mercaptoethanol for amino acid detection. The data were analyzed using a Perkin Elmer Sigma 10 data station. Values for serine and threonine were extrapolated to "zero time" hydrolysis. Cystine and tryptophan were not determined due to destruction by acid hydrolysis.

**Preparation Of Prostaglandin-Glutathione Conjugates**

Glutathione conjugates of PGA₁, PGA₂ and 15-keto-PGF₂α were prepared by the method of Cagen et al., (1976). Prostaglandin (1.2 mg) and trace amount of [³⁵S]-labeled one were dissolved in 1 ml of 0.2 M Tris-HCl, pH 7.4, and mixed with 6 mg of glutathione. The reaction mixture was incubated with stirring at 37°C for 70 minutes. The reaction mixture was acidified with 45 μl of 1 M HCl and the unreacted prostaglandins were removed by extraction with ethyl acetate. The prostaglandin-glutathione conjugate was quantitated by determining the amount of radioactivity remained in the aqueous phase.

**Protein Determination**

Protein concentrations were determined by Bio-Rad Protein Assay method using bovine γ-globulin as a standard (Bradford, 1976). Protein concentrations of column eluates were determined by absorbance at 280 nm.
RESULTS

Purification Of 9-PGKR, 15-PGKR, 9-PGDH And 15-PGDH From Swine Kidney

Purification of the enzyme was carried out at 4°C. A typical purification requires one week and yields the enzyme as a homogeneous protein.

Preparation of crude extract (Fraction I) -- Swine kidneys (500 g) were homogenized in two volumes of 10 mM Tris·HCl buffer, pH 7.5, containing 1 mM EDTA (Buffer A) in a Waring blender for two minutes. The homogenate was centrifuged at 40,000 x g for 20 minutes in a Sorval refrigerated centrifuge. The supernatant solution (940 ml) was passed through a glasswool plugged funnel to remove excess fatty materials. This fraction was designated as "crude extract".

Acetone fractionation (Fraction II) -- Dry ice chilled acetone (-68°C) was slowly added with stirring to the crude extract to 33% (v/v). Precipitate was removed by centrifugation at 40,000 x g for 15 minutes. Acetone was further added to the supernatant to 43%, and the precipitate was removed by centrifugation. Acetone was then added to the final supernatant to 67% and the precipitate was dissolved in 50 ml of Buffer A. This fraction was designated as
Fraction II. A small portion of this fraction (1 ml) was immediately passed through a sephadex G-25 column (1 x 25 cm) equilibrated and eluted with Buffer A to remove acetone. The active fractions were pooled, concentrated and stored at -80°C for further estimation of specific activity.

**Sephadex G-100 chromatography (Fraction III)** -- Sephadex G-100 was swollen in water for two days, packed in a column (6 x 100 cm) under gravity and equilibrated with Buffer A. Fraction II was applied immediately to the column and elution was carried out with the same buffer. Enzyme activity profiles of 15-PGDH, 9-PGKR and 15-PGKR overlapped as shown in Figure 1. The active fractions were pooled and concentrated to 20 ml with an Amicon ultrafiltration PM 10 membrane.

**TEAE-cellulose chromatography (Fraction IV)** -- TEAE-cellulose was washed thoroughly according to Peterson and Sober (1962). A column of TEAE (1.5 x 25 cm) was packed under gravity and equilibrated with Buffer A. Fraction III was applied to the column and the column was eluted with 10 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA. Again, three different enzyme activity profiles overlapped, as shown in Figure 2. The active fractions were pooled and concentrated to a volume of 20 ml with an Amicon ultrafiltration.
Figure 1. Column chromatography of 15-PGDH, 9-PGKR and 15-PGKR on Sephadex G-100. Fraction II (50 ml containing 862 mg of protein) was applied to a Sephadex G-100 column (6 cm x 100 cm) which was equilibrated with Buffer A. The column was eluted with the same buffer at a flow rate of 90 ml per hour. Fractions of 8.8 ml were collected and assayed for enzyme activities of 9-PGKR (●), 15-PGDH (■), and 15-PGKR (▲), and for protein concentration (×). The enzyme activity in each fraction was indicated by its percent activity of that in the most active fraction.
Figure 2. Column chromatography of 15-PGDH, 9-PGKR and 15-PGKR on TEAE-cellulose. Fraction III (20 ml containing 177 mg of protein) was applied to a TEAE-cellulose column (1.5 cm x 25 cm) which was equilibrated with Buffer A. The column was eluted with 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA at a flow rate of 40 ml per hour. Fractions of 8.2 ml were collected and assayed for the enzyme activities of 9-PGKR (●), 15-PGDH (■), and 15-PGKR (▲), and for protein concentration (×). The enzyme activity in each fraction was indicated by its percent activity of that in the most active fraction.
Preparative isoelectric focusing (Fraction V and Fraction VI) -- Isoelectric focusing was carried out in a sucrose density gradient using 2.5% ampholine, pH range 4-6, in a 110 ml LKB column as described by Vesterberg et al., (1967). Fraction IV was applied as part of the less dense gradient solution. The cathode solution (2% (v/v) ethanolamine in distilled water) was at the bottom of the column, while the anode solution (1% phosphoric acid in distilled water) was at the top. Electrofocusing was performed at 3°C at 1600 volts for 16 hours. When focusing was completed, 1-ml fractions were collected and assayed for their pH value, protein concentration and enzyme activities. The results as shown in Figure 3 indicated three different enzyme activity profiles overlapped and appeared in two peaks. Active fractions from two peaks were respectively pooled, concentrated and passed through a Sephadex G-50 (1 x 15 cm) column equilibrated with 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The enzyme-containing fractions were concentrated and dialysed against 10 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA for 24 hours. The enzyme was stored at -80°C. The first active peak (PI 5.8) was designated as Fraction V, while the second one (PI 4.8) was designated as Fraction VI. A summary of the purification of 9-PGDH, 15-PGDH, 9-PGKR and 15-PGKR from swine kidney is shown in Table I. A nearly equal increase
Figure 3. Isoelectric focusing of 15-PGDH, 9-PGKR and 15-PGKR. Fraction IV (20 mg of protein) was applied to an isoelectric focusing column containing 2.5\% ampholines (pH 4.0 - 6.0) as described in "Methods". It took 16 hours to complete the isoelectric focusing under the running voltage of 1600 V. Fractions of 1 ml were collected and assayed for the enzyme activities of 9-KPR (●), 15-PGDH (■), and 15-PGKR (▲), for protein concentration (×), and for pH (...). The enzyme activity in each fraction was indicated by its percent activity of that in the most active fraction.
### TABLE I

Purification of 9-PGKR (I), 9-PGDH (II), 15-PGKR (III) and 15-PGDH (IV) from Swine Kidney

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>PROTEIN (MG)</th>
<th>ENZYME ACTIVITY (UNITS)</th>
<th>SPECIFIC ACTIVITY (UNITS/MG)</th>
<th>PURIFICATION (FOLD)</th>
<th>RECOVERY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I  II  III  IV</td>
<td>I  II  III  IV</td>
<td>I  II  III  IV</td>
<td>I  II  III  IV</td>
</tr>
<tr>
<td>I. CRUDE EXTRACT</td>
<td>9,360</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II. ACETONE FRACTIONATION (43-67%)</td>
<td>862.5</td>
<td>70  104  24  49</td>
<td>0.08  0.12  0.03  0.06</td>
<td>1  1  1  1</td>
<td>100 100 100 100</td>
</tr>
<tr>
<td>III. SEPHADEX G-100</td>
<td>176.8</td>
<td>83  120  25  55</td>
<td>0.47  0.68  0.14  0.31</td>
<td>5.8  5.7  5  5.5</td>
<td>118 115 104 112</td>
</tr>
<tr>
<td>IV. TEAE-CELLULOSE</td>
<td>71.4</td>
<td>58  82  22  41</td>
<td>0.81  1.15  0.31  0.57</td>
<td>10  9.6  11  10.1</td>
<td>83  79  91  84</td>
</tr>
<tr>
<td>ISOELECTRIC FOCUSING</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V. pH 5.8</td>
<td>2.9</td>
<td>22  33.1  7.7  19.8</td>
<td>7.5  11.3  2.6  6.8</td>
<td>93  94  93  119</td>
<td>31  32  32  40</td>
</tr>
<tr>
<td>VI. pH 4.8</td>
<td>2.0</td>
<td>18.6  27.4  6.3  15</td>
<td>9.3  13.6  3.1  7.5</td>
<td>115  113  111  132</td>
<td>27  26  26  30</td>
</tr>
</tbody>
</table>
in the specific activities of four different enzyme activities in every purification step was observed.

Criteria Of Purity

Fraction V (20 μg) and Fraction VI (20 μg) were respectively applied to 7.5% alkaline pH discontinuous polyacrylamide gels and subjected to electrophoresis and stained as described in "Methods". The results are shown in Figure 4. Only one major protein band was observed in the gel of Fraction V and Fraction VI respectively. The two purified enzymes were also separated by electrophoresis with $R_f$ value of 0.28 for Fraction V and $R_f$ value of 0.34 for Fraction VI. Furthermore, both Fraction V (20 μg) and Fraction VI (20 μg) were respectively subjected to 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS. Only one major protein band was again observed in either Fraction as shown in Figure 5.

Determination Of Native Molecular Weight

The native enzyme (Fraction IV) mixed with protein standards was subjected to Sephadex G-100 gel filtration as described in "Methods". When the elution volume of the enzyme was correlated with the elution volume of proteins of known molecular weight, a molecular weight of 29,000 was obtained as shown in Figure 6.
Figure 4. Disc alkaline gel electrophoresis of Fraction V and Fraction VI. Electrophoresis in polyacrylamide gel (7.5%) was run as described in "Method". From left to right, Gel A, Fraction VI (PI 4.8, 20 µg); Gel B, mixture of Fraction V (PI 5.8, 20 µg) and Fraction VI (PI 4.8, 20 µg); Gel C, Fraction V (PI 5.8, 20 µg).
Figure 5. SDS-polyacrylamide gel electrophoresis of Fraction V and Fraction VI. Electrophoresis of polyacrylamide gels (7.5%) in the presence of 0.1% SDS were run as described in "Method". From left to right, Gel 1, Fraction V; Gel 2, Fraction VI.
Figure 6. Determination of molecular weight of swine renal 15-PGDH, 9-PGKR and 15-PGKR. The logarithm of the molecular weight is plotted against $K_a v$, defined as $(Ve - Vo)/Vo$, where $Ve$ is the protein elution volume and $Vo$ is the column void volume. A Sephadex G-100 column (2.7 x 55 cm) was calibrated with cytochrome C (M.W. 12,384), trypsin inhibitor (M.W. 21,500), liver alcohol dehydrogenase (M.W. 83,000) and lipoxidase (102,000).
**Determination Of Subunit Molecular Weight**

Sample (Fraction V or Fraction VI) mixed with protein standards, lysozyme (M.W. 14,300); trypsinogen (M.W. 24,000); egg albumin (M.W. 45,000), bovine albumin (M.W. 66,000) were subjected to 7.5% polyacrylamide gel electrophoresis in the presence of 0.1% SDS as described in "Methods". As shown in Figure 7, comparison of the mobility of standard proteins with Fraction V and Fraction VI yielded enzyme subunit molecular weight of 29,500 for both Fraction V and Fraction VI. Judged from the native and subunit molecular weights, the enzyme is a monomer.

**Coenzyme Specificity**

The coenzyme specificities of 15-PGDH and 9-PGKR were investigated. The results are summarized in Table II. For oxidation reaction, NADP+ showed almost one thousandth Km value of that of NAD+. For reduction reaction, NADPH showed 30 times lower Km value than that of NADH.

**Substrate Specificity**

The enzyme was examined for its ability to oxidize and to reduce a number of prostaglandins. The results are summarized in Tables III and IV.

For 15-PGDH, Km values in increasing order were $\text{PGA}_2 > \text{PGE}_2 > \text{PGE}_1 > \text{PGA}_1 > \text{PGB}_2 > 6$-keto-PGF$_{1\alpha}$ > PGB$_1$ > PGD$_2$ > TXB$_2$. PGB$_1$ gave unusually high Vmax as compared to other
Figure 7. Determination of subunit molecular weight by SDS-gel electrophoresis. Proteins were run on 7.5% polyacrylamide gels in the presence of 0.1% SDS. Protein standards used were lysozyme (M.W. 14,300); trypsinogen (M.W. 24,000); egg albumin (M.W. 45,000); bovine albumin (M.W. 66,000). The logarithm of the molecular weight is plotted against the relative mobility, the ratio of the distance of protein migration to that of the tracking dye migration.
TABLE II

Coenzyme Specificity Of Swine Kidney 15-PGDH And 9-PGKR

<table>
<thead>
<tr>
<th>Coenzyme</th>
<th>Enzyme Preparations</th>
<th>Km (µM)</th>
<th>Vmax (nmole/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td><strong>15-PGDH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP⁺</td>
<td>PI 5.8</td>
<td>1.4</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>1.4</td>
<td>6.50</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>PI 5.8</td>
<td>1,500.0</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>1,100.0</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>9-PGKR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>PI 5.8</td>
<td>80.0</td>
<td>63.00</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>100.0</td>
<td>43.00</td>
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<tr>
<td>NADH</td>
<td>PI 5.8</td>
<td>3,300.0</td>
<td>24.00</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>2,600.0</td>
<td>21.00</td>
</tr>
</tbody>
</table>

The coenzyme specificity of swine kidney 15-PGDH were assayed spectrofluorometrically as described in enzyme assay method B using 40 to 100 µg of enzyme. The concentration of NADP⁺ (or NAD⁺) was varied and the concentration of substrate, PGE₂, was kept at 0.2 mM.

The coenzyme specificity of swine kidney 9-PGKR was assayed spectrophotometrically using 40 to 100 µg of enzyme. The concentration of NADPH (or NADH) was varied and the concentration of substrate, PGAl-GSH, was kept at 0.15 mM. Km and Vmax values were determined by the respective double reciprocal plots.
<table>
<thead>
<tr>
<th>Prostaglandins</th>
<th>Enzyme Preparations</th>
<th>Km (µM)</th>
<th>Vmax (nmole/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA₁</td>
<td>PI 5.8</td>
<td>180</td>
<td>12.00</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>250</td>
<td>18.00</td>
</tr>
<tr>
<td>PGA₂</td>
<td>PI 5.8</td>
<td>80</td>
<td>4.10</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>76</td>
<td>5.40</td>
</tr>
<tr>
<td>PGE₁</td>
<td>PI 5.8</td>
<td>153</td>
<td>6.20</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>200</td>
<td>6.10</td>
</tr>
<tr>
<td>PGE₂</td>
<td>PI 5.8</td>
<td>140</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>100</td>
<td>0.21</td>
</tr>
<tr>
<td>PGB₁</td>
<td>PI 5.8</td>
<td>560</td>
<td>150.00</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>670</td>
<td>220.00</td>
</tr>
<tr>
<td>PGB₂</td>
<td>PI 5.8</td>
<td>200</td>
<td>17.00</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>227</td>
<td>24.00</td>
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<tr>
<td>PGD₂</td>
<td>PI 5.8</td>
<td>670</td>
<td>9.60</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>500</td>
<td>8.70</td>
</tr>
<tr>
<td>6-keto-PGF₁α</td>
<td>PI 5.8</td>
<td>250</td>
<td>2.90</td>
</tr>
<tr>
<td>TXB₂</td>
<td>PI 5.8</td>
<td>270</td>
<td>2.30</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>20,000</td>
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<tr>
<td></td>
<td>PI 4.8</td>
<td>16,000</td>
<td>15.00</td>
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<tr>
<td>15-keto-PGF₂α</td>
<td>PI 5.8</td>
<td>83</td>
<td>0.50</td>
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<td></td>
<td>PI 4.8</td>
<td>90</td>
<td>0.42</td>
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<tr>
<td>15-keto-PGE₂</td>
<td>PI 5.8</td>
<td>Inactive</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>Inactive</td>
<td>----</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. The assay was done by spectrofluorometric method as described in enzyme assay method B using 40 to 100 µg of enzyme. The concentration of substrate was varied and the concentration of NADP⁺ was kept at 0.27 mM. Km and Vmax values were determined by the respective double reciprocal plots.
TABLE IV

Substrate Specificity Of Swine Kidney 9-PGKR And 15-PGKR

<table>
<thead>
<tr>
<th>Prostaglandins</th>
<th>Enzyme Preparations</th>
<th>Km (μM)</th>
<th>Vmax (nmole/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>9-PGKR</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PGA$_2$</td>
<td>PI 5.8</td>
<td>670</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>400</td>
<td>1.8</td>
</tr>
<tr>
<td>PGE$_2$</td>
<td>PI 5.8</td>
<td>200</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>180</td>
<td>6.9</td>
</tr>
<tr>
<td>PGA$_2$-GSH</td>
<td>PI 5.8</td>
<td>43</td>
<td>22.0</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>18</td>
<td>18.0</td>
</tr>
<tr>
<td>PGA$_1$-GSH</td>
<td>PI 5.8</td>
<td>11</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>13</td>
<td>14.0</td>
</tr>
<tr>
<td><strong>15-PGKR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15-keto-PGF$_{2\alpha}$</td>
<td>PI 5.8</td>
<td>200</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>160</td>
<td>6.9</td>
</tr>
<tr>
<td>15-keto-PGF$_{2\alpha}$-GSH</td>
<td>PI 5.8</td>
<td>20,000</td>
<td>110.0</td>
</tr>
<tr>
<td></td>
<td>PI 4.8</td>
<td>12,500</td>
<td>140.0</td>
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</tbody>
</table>

All the prostaglandins were respectively dissolved in ethanol at the concentration of 1 mg/ml and diluted in assay buffer before experiment. The assays of 9-PGKR with PGE$_2$ as substrate and of 15-PGKR with 15-keto-PGF$_{2\alpha}$ as substrate were done by RIA method (Method D), while the other assays were done by spectrophotometric method (Method C). The concentration of substrate was varied and the concentration of NADPH was kept at 0.3 mM. Km and Vmax values determined by the respective double reciprocal plots.
prostaglandins. In order to determine whether or not 15-PGDH catalyzes the oxidation of 9α or 11α hydroxyl group of prostaglandins, 15-keto-PGE₂ and 15-keto-PGF₂α, which possess a single 11α hydroxyl group and both 9α and 11α hydroxyl groups, respectively, were examined. 15-keto-PGE₂ was found not to be a substrate, while 15-keto-PGF₂α was shown to be a substrate with Km values of 83 and 90 μM for Fraction V (PI 5.8) and Fraction VI (PI 4.8), respectively. These results indicate that the enzyme catalyzes the oxidation of 9α hydroxyl group but not the 11α hydroxyl group of prostaglandins.

As for 9-PGKR and 15-PGKR, PGA₁-GSH and PGA₂-GSH conjugates were better substrates than PGA₁ and PGA₂ for 9-PGKR and 15-keto-PGF₂α-GSH is reduced greater than 15-keto-PGF₂α for 15-PGKR. Although the Km for the conjugate is unusually high, the Km values of enzyme with PI 5.8 and PI 4.8 for the same substrate did not show great difference. The enzyme did not catalyze the reduction of 11α hydroxyl group since no conversion of PGD₂ to PGF₂α was observed.

**Double Immunodiffusion Analysis**

Antiserum against purified Fraction V (PI 5.8) was placed in the central well of Ouchterlony disc and the different enzyme preparations were placed in the surrounding wells. The double immunodiffusion was performed as described in "Methods". The result is shown in Figure 8 which
Figure 8. Ouchterlony diffusion analysis of the enzyme prepared from various purification steps. Antibody prepared against the pure enzyme of Fraction V (PI 5.8) was placed in the center well. Well 1, Fraction III; well 2, Fraction IV; well 3, Fraction V (PI 5.8); well 4, Fraction VI (PI 4.8); well 5, mixture of Fraction V and VI; well 6, blank. The amount of protein used for each well is around 50 μg.
indicated that antibody against Fraction V showed cross-
reaction with Fraction VI and that the two precipitation
line appeared to merge into a single arc. The immunodi-
ffusion analysis also showed that the antibody crossreacted
with two different proteins in Sephadex G-100 fraction.
One of the precipitation lines merged into the line resulted
from Fraction V.

**Amino Acid Analysis**

Amino acid composition analyses for purified Fraction
V (PI 5.8) and Fraction VI (PI 4.8) were performed as des-
cribed in "Methods". The results were summarized in Table
V. The amino acid compositions of both Fractions show some
difference in certain amino acid residues.

**Enzyme Inactivation Studies**

Enzyme was incubated at 60°C in a shaking water bath.
The rate of denaturation of 9-PGKR and 15-PGDH were deter-
mined. The result is shown in Figure 9. The relative decay
rates of both enzyme activities of either from are very
similar. The time required to inactivate 50% of the enzyme
activities is slightly less for Fraction VI (PI 4.8) than
for Fraction V (PI 5.8).

**Inhibition Studies**

The inhibitory effect of indomethacin on the enzyme
activities of 9-PGKR and 15-PGDH were determined. The
TABLE V

Amino Acid Composition Analysis Of Fraction V (PI 5.8) And VI (PI 4.8) Collected From Isoelectric Focusing

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues Per Mole</th>
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<tbody>
<tr>
<td></td>
<td>PI 5.8</td>
</tr>
<tr>
<td>ASX</td>
<td>30</td>
</tr>
<tr>
<td>THR</td>
<td>15</td>
</tr>
<tr>
<td>SER</td>
<td>13</td>
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<tr>
<td>GLX</td>
<td>36</td>
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<tr>
<td>GLY</td>
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<td>ALA</td>
<td>21</td>
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<td>MET</td>
<td>3</td>
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<td>VAL</td>
<td>25</td>
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<td>ILE</td>
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<td>PHE</td>
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<td>ARG</td>
<td>14</td>
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<tr>
<td>HIS</td>
<td>7</td>
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</tbody>
</table>

Protein samples were hydrolyzed in vacuo at 110°C for 22, 48 and 72 hours in 6 N HCl containing 0.02% (v/v) 2-mercaptoethanol. Amino acid analyses were carried out using o-phthaldialdehyde fluorescence detection system. Values for serine and threonine were extrapolated to "zero time" hydrolysis. All other values were the average of the data of the hydrolyses at different time intervals. Cysteine, tryptophan and proline were not determined by this method.
Figure 9. Effect of heat upon enzyme activities of 15-PGDH and 9-PGKR of Fraction V (PI 5.8) and VI (PI 4.8). Enzyme was incubated at 60°C in 0.1 M potassium phosphate buffer, pH 7.5. At the indicated time, aliquots were assayed for the enzyme activities of 9-PGKR (●) and 15-PGDH (■) using assay Method D. A, Fraction V (PI 5.8); B, Fraction IV (PI 4.8).
results as shown in Figure 10 indicate that both enzyme activities of either form were inhibited to a similar degree at several concentrations of indomethacin (Figure 10). However, the enzyme activities of Fraction V (PI 5.8) appeared to be more sensitive to indomethacin inhibition than those of Fraction VI (PI 4.8).

**Kinetics Of Oxido-Reduction Of Prostaglandins At C-9 And C-15**

In order to compare the oxido-reduction rates of prostaglandins at C-9 and C-15, PGF$_{2\alpha}$ or 15-keto-PGE$_2$ which has hydroxyl groups or keto groups at both C-9 and C-15 was chosen as substrate for oxidation or reduction reaction. The oxidation rate of PGF$_{2\alpha}$ at C-15 was slightly faster than that at C-9 as evidenced by the greater formation of 15-keto-PGF$_{2\alpha}$ than of PGE$_2$ shown in Figure 11. The reduction rate of 15-keto-PGE$_2$ at C-15 was much faster than that at C-9 as shown by the far greater formation of PGE$_2$ than of 15-keto-PGF$_{2\alpha}$ indicated in Figure 12.
Figure 10. Inhibition of the enzyme activities of 15-PGDH and 9-PGKR of Fraction V (PI 5.8) and VI (PI 4.8) by indomethacin. The enzyme was preincubated with cofactor and inhibitor for 5 minutes before initiating the enzymatic reactions by adding substrates and incubating for another ten minutes. Enzyme was assayed for activities of 9-PGKR (●) and 15-PGDH (■) using assay method D. A, Fraction V (PI 5.8); B, Fraction VI (PI 4.8).
Figure 11. Comparison of oxidation of PGF$_2\alpha$ at C-9 and C-15 positions by Fraction V (PI 5.8) and Fraction VI (PI 4.8). Enzyme was incubated at 37°C. Aliquots of the enzyme solution were removed and assayed at various time intervals using assay Method D. A, Fraction V (PI 5.8); B, Fraction IV (PI 4.8).
Figure 12. Comparison of reduction of 15-keto-PGE$_2$ at C-9 and C-15 positions by Fraction V (PI 5.8) and Fraction VI (PI 4.8). Aliquots of the enzyme solution were removed and assayed at various time intervals using assay Method D. A, Fraction V (PI 5.8); B, Fraction IV (PI 4.8).
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[Diagram A]

- **PGE2**
- **15-KETO-PGF2α**

[Diagram B]

- **PGE2**
- **15-KETO-PGF2α**

**PRODUCT (10^-10 mole)**

**TIME (MINUTE)**
DISCUSSION

Mammalian kidneys are a rich source of a variety of prostaglandin catabolizing enzymes. Both type I 15-PGDH and 15-keto-prostaglandin-\(\Delta^{13}\)-reductase are abundant in swine kidney (Ånggärd et al., 1971) and have been partially purified and characterized (Tai et al., 1974; Oliw et al., 1976; Tai and Yuan, 1977; Kung-chao and Tai, 1980). NAD\(^+\)-dependent 9-hydroxyprostaglandin dehydrogenase is uniquely present in rat kidney (Pace-Asciak, 1976) and has been purified to homogeneity and characterized (Yuan et al., 1980). Type II 15-PGDH and 9-PGKR have been shown to be present in swine kidney (Lee et al., 1975) and in rabbit kidney (Stone and Hart, 1976). This study describes the purification and characterization of type II 15-PGDH and 9-PGKR from swine kidney to homogeneity. The results show that both enzyme activities are associated with each of the two isoenzymic proteins and that each isozyme also possesses 9-hydroxyprostaglandin dehydrogenase (9-PGDH), as well as 15-ketoprostaglandin reductase (15-PGKR) activities.

Purification of 15-PGDH and 9-PGKR employed several assay methods. Each method has its own advantages and limitations. The spectrophotometric method of enzyme assay is simple, but relatively insensitive (nmole range).
Spectrofluorometric method is much more sensitive (pmole range) when assaying oxidative direction of the reaction than the reverse reaction, since blank-out of the NADPH fluorescence at high sensitivity setting is impossible. Radioimmunological method provides extreme sensitivity for either directions of the reaction. Although crossreactions of each antibody with other prostaglandins are generally low (1%), the substrate concentration needs to be optimized to avoid crossreaction interference by the excessive amount of unreacted substrate. Furthermore, for each reaction a zero time sample needs to be run in order to subtract the concentration estimated from nonspecific inhibition of labeled hapten binding. This situation is more evident when crude extract is used for enzyme assay.

Throughout the purification procedure, from crude homogenate to homogeneous protein, 9-PGKR activity was found concomitantly with 15-PGDH activity. Acetone fractionation proved to be a very efficient step in that it separated the enzyme from most of the other proteins with good activity recovery. As mentioned previously, enzyme assays for the crude extract were difficult due to endogenous inhibitors of the enzyme or competing factors in the crude extract. Acetone fractionation appeared to remove most of these interfering factors. Acetone denatured the enzyme rapidly, so that the operation temperature and
time are very critical factors for this purification step. Sephadex G-100 gel filtration gave about a 5-fold purification for these enzymes. Subsequent TEAE column chromatography showed another 2-fold purification for all enzymes. TEAE chromatography separated type II 15-PGDH (associated with enzyme activities of 9-PGKR and 15-PGKR) from type I 15-PGDH and other proteins. Type II 15-PGDH was eluted with 10 mM potassium phosphate buffer, pH 7.5, while type I 15-PGDH was retained on the column. Blue-dextran Sepharose affinity chromatography greatly enhanced the specific activity of the enzymes but yielded a poor recovery of the enzyme activities as well as little separation of two different forms of the enzyme and hence was not routinely used for preparative purposes. A 10-fold purification of the enzyme was achieved using preparative isoelectric focusing following TEAE-cellulose chromatography. This step gave two electrophoretically pure proteins. The enzyme activities of 9-PGKR, 15-PGKR, 9-PGDH and 15-PGDH appeared to be associated with each of the two enzyme proteins. Studies on the sensitivities of different enzyme activities of either enzyme proteins to heat and indomethacin inhibition further support the contention that various enzyme activities are associated with the same enzyme protein.

The existence of multiple forms of 9-PGKR or 15-PGDH was first noted by Lee and Levine (1975). They purified
9-PGKR from chicken heart and found that two peaks of activity could be resolved by phosphocellulose chromatography. Either peak could catalyze the reduction of 9-keto group as well as 15-keto group of prostaglandins although it was not certain both reductions were effected by the same enzyme protein. Subsequently, Hassid and Levine (1977) demonstrated three different 9-PGKR activities (PI 5.7, 7.8 and 8.2) from chicken kidney by isoelectric focusing. All three different reductase preparations catalyze the reversible oxido-reduction at C-9 and C-15 of prostaglandins. However, the impure preparation did not exclude the possibility that the multiple functions were catalyzed by separated enzyme proteins. A more conclusive evidence that the multiple functions of oxido-reduction at C-9 and C-15 are catalyzed by the same enzyme protein was provided by Lin and Jarabak (1978). They isolated two proteins from human placenta to homogeneity and showed that both proteins act as the catalyst of oxido-reduction at the C-9 and C-15 of prostaglandins. Whether both proteins from human placenta are identical or similar to the two isozymic forms isolated from swin kidney remains to be determined. The use of antibody against Fraction V (PI 5.8) should help determine at least whether they have similar antigenic determinants.

The properties of two different forms of enzyme proteins are similar in many aspects. These include molecular
weight, amino acid composition, coenzyme specificity, substrate specificity and kinetic properties. Sensitivities to increased temperature and indomethacin inhibition are slightly different. However, the properties of swine renal enzyme appeared to be at more variance with the enzyme isolated from other sources in many aspects.

The molecular weight of swine renal enzyme was estimated to be 29,000 daltons for both forms by gel filtration. The subunit molecular weight, determined by SDS gel electrophoresis was 29,500 daltons for both forms of the enzyme. Therefore, it is clear that the enzyme is a monomer. The native molecular weight of human placental enzyme determined by Westbrook et al. (1977) was 31,000 daltons. The molecular weight of 9-PGKR from rabbit kidney and monkey brain were estimated to be 12,800 daltons and 33,500 daltons, respectively (Stone and Hart, 1975). The molecular weight of both forms of 9-PGKR from chicken heart was estimated to be about 45,000 to 55,000 (Lee and Levine, 1975). Difference in these values suggests a tissue and species specificity of 9-PGKR and type II 15-PGDH.

The amino acid compositions of the two forms of swine renal enzyme are similar but not identical. In fact there are significant differences in several amino acid residues, notably glycine and valine. Whether one form is derived from the other by exopeptidase action or they are two
separate gene product is not known since both carboxyl and amino terminal residues were not determined.

The double immunodiffusion analysis showed that these two forms of the enzyme had identical or very similar antigenic determinants. This suggests that the antibody binding sites of both forms are comprised of amino acid residues of very similar sequence. Regions of significant difference in two separate forms particularly those containing glycyl and valinyl residues are not likely to be the sites of interaction with the antibody. Double precipitin lines exhibited in Ouchterlony double diffusion analysis by partially purified preparation indicated that there are other proteins in the tissues that possess the cross-reaction antigenic determinants. These proteins may represent inactive proenzyme or altered enzyme form that contains immunoreactive region of the enzyme protein. The antibody appeared to cross-react also with certain proteins in the soluble fraction of a variety of rat tissues, including spleen, lung, kidney, heart, aorta and liver, as revealed by diffusion analysis. Whether these protein are catalytic enzyme or inactive enzyme or other protein remain to be determined.

Studies on coenzyme specificity indicate type II 15-PGDH utilizes NADP⁺ far better than NAD⁺, and 9-PGKR employs NADPH much better than NADH. These results attest to a
previous observation using partially purified enzyme (Lee et al., 1975). Since both enzyme activities are associated with the same enzyme protein, the expression of the enzyme as an oxidative or reductive enzyme will depend on the NADP⁺/NADPH ratio and will control the interconversion of PGE and PGF and their further metabolism.

The 15-PGDH activity of the enzyme catalyzes the oxidation of 15(S)-hydroxyl group of virtually every type of prostaglandins. However, the 1 series of prostaglandins appears to be better substrate than the 2 series. PGB₁ was found to oxidize far better than any other prostaglandins including PGB₂. Similar findings were reported for human placental enzyme (Jarabak and Fried, 1979). The unusual specificity for PGB₁ of swine renal enzyme is not clear since the physiological importance of PGB₁ remains unknown. The Km values for different prostaglandins are also unusually high as compared to those of type I 15-PGDH at low μM range (Kung-Chao and Tai, 1980). The kinetic parameters of type II 15-PGDH of swine kidney are compared to the enzyme from other sources as summarized in Table VI. Regardless of the sources of the enzyme, Km values are much higher than those for type I 15-PGDH.

In addition to catalyzing the oxidation at C-15, the enzyme also effected the oxidation at C-9 as evidenced by the formation of PGE₂ from PGF₂α. However, the enzyme did
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<th>PGB</th>
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not catalyze the oxidation at C-11 since 15-keto-PGE$_2$ was found not to be a substrate. Studies on the relative rate of oxidation at C-9 and C-15 using PGF$_2$$\alpha$ as a substrate indicated that oxidation appeared to occur slightly faster at C-15.

The 9-PGKR activity of the enzyme catalyzes the reduction of 9-keto group of PGE$_2$, PGA$_2$ and PGA-GSH conjugates. Km values for PGE$_2$ and PGA$_2$ are also unusually high. However, Km values are significantly decreased and Vmax values are greatly increased if PGA-GSH conjugates are used as substrates. Similar observations were reported previously for the enzyme partially purified from chicken heart and kidney although the Vmax's were more dramatically increased for avian enzyme (Cagen and Pisano, 1979). The Km values for PGA-GSH conjugates were found to be at low $\mu$M range. In view of the unusually high Km values, the primary prostaglandins may not be the natural substrate for the 9-PGKR activity of the enzyme protein. PGA-GSH conjugates are more likely to be candidate for the natural substrate. Although the natural occurrence of PGA-GSH conjugates has not been established, several lines of evidences support its presence in the biological systems. Cagen et al. (1976) showed that PGA$_1$ was converted into PGA$_1$-GSH and its 9-hydroxy metabolite when incubated with human red cell suspension. Gross and Gillis (1976) found that PGA$_1$ was conjugated with GSH during passage through the pulmonary
vasculature of rabbits. Considering the large amounts of reduced glutathione normally circulating in the blood (ca. 300 μg/ml), it is likely that the conjugate forms of PGA is readily formed in the circulation. If PGA-GSH conjugates are the natural substrate, the role of 9-PGKR activity of the enzyme protein awaits to be defined, since the biological activities of PGA-GSH conjugates and their 9-hydroxy catabolites remain unknown. For the reduction of 15-keto group of prostaglandins, both 15-keto-PGF$_{2α}$ and 15-keto-PGE$_2$ are reduced by the 15-PGKR activity of the enzyme protein. Conjugation of GSH with 15-keto-PGF$_{2α}$ increased the Km values of 15-keto-PGF$_{2α}$ as well as the Vmax. This is in contrast to conjugation of GSH with PGA's. The explanation for this difference is not apparent. The enzyme did not appear to catalyze the reduction of 11-keto group since PGD$_2$ was found not to be a substrate. When comparing the rate of reduction at C-9 and C-15 using 15-keto-PGE$_2$ as a substrate, it was found that reduction at C-15 was very much favored. These results indicate that reversal of an biologically inactive metabolite back to an active substance is a preferred process to conversion to another biologically inactive metabolite. Whether such a reversal actually occurs in vivo may depend on the ratio of NADPH to NADP$.^+$.

The sensitivities of two different forms of the enzyme to increased temperatures and indomethocin were
found to be slightly different. Fraction VI (PI 4.8) was more labile to heat but was less sensitive to indomethacin inhibition as compared to Fraction V (PI 5.8). Differential sensitivities of various forms of an enzyme to heat and to inhibitors are noted in several instances. The five rabbit lactate dehydrogenase isozymes are known to have different heat stabilities (Plagemann et al., 1961). They also differ in the degree of inhibition by sulfite (Wieland and Pfleider, 1957) and a peptide inhibitor isolated from human urine (Wacker and Schoenenberger, 1966). Recent work on two human triosephosphate isomerase isozymes also showed that one isozyme was more labile than the other (Yuan et al., 1979). The molecular basis of these differential sensitivities to heat and to inhibitors by isoenzymes is not clear. Their physiological relevance is also far from being understood. Obviously our understanding on the structure and function of 9-PGKR/15-PGDH isozymes requires further investigation.

The significance of this study is several fold. Previously known activities of type II 15-PGDH and 9-PGKR in swine kidney are actually alternate activities of the same enzyme proteins. In addition, 9-PGDH and 15-PGKR activities are also found to be associated with the same enzyme proteins. The enzyme exists in two isozymic forms and each form is capable of catalyzing oxido-reduction at C-9 and C-15 of prostaglandins. This finding may extend to the same enzyme existing in kidneys of different species and in
other organs as well. The multiple functions of the enzyme may represent the versatility of the enzyme and the expression of each enzyme activity may depend on the availability of each particular prostaglandin substrate and the ratio of NADPH/NADP⁺. The significance of this enzyme in renal function as well as in other functions should be interpreted in light of this multiple roles of the enzyme. Furthermore, the molecular, kinetic and immunological properties revealed in this study should be of great value to future investigations on the mechanism and function of the enzyme.
BIBLIOGRAPHY


