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Enzymatic Synthesis of (15*S*)-[15-^{3}H]Prostaglandins and Their Use in the Development of a Simple and Sensitive Assay for 15-Hydroxyprostaglandin Dehydrogenase†

Hsin-Hsiung Tai

ABSTRACT: The stereospecificity of swine renal NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase has been determined. It was found that the enzyme is a B-side specific dehydrogenase. (15*S*)-[15-^{3}H]Prostaglandins were synthesized by stereospecific transfer of the tritium label of D-[1-^{3}H]galactose to prostaglandins by coupling 15-hydroxyprostaglandin dehydrogenase with β-D-galactose dehydrogenase, an enzyme of the same stereospecificity. A simple and sensitive assay for 15-hydroxyprostaglandin dehydrogenase was developed based on the stereospecific transfer of the tritium label of tritiated prostaglandins to glutamate by coupling 15-hydroxyprostaglandin dehydrogenase with glutamate dehydrogenase. The amount of prostaglandin oxidized is determined by the radioactivity of labeled glutamate present in the supernatant after charcoal precipitation of labeled prostaglandin. Concurrent assays with the present tritium release method and the thin-layer chromatography method indicated excellent correlation. The assay was employed to study some of the properties of swine renal 15-hydroxyprostaglandin dehydrogenase in crude extract and the distribution of enzyme activity in various tissues of rat. Enzyme activity was linear for the first 10 min studied and was nonlinear with increasing amounts of crude enzyme, indicating the possible presence of endogenous inhibitor(s). Apparent K_m's for PGE_2, $PGF_{2\alpha}$, and PGA_2 were found to be 2.5, 12.5, and 3.9 μM, respectively. The distribution pattern indicated high levels of enzyme activity in gastrointestinal tract, lung, kidney, and spleen. The assay method may prove to be valuable for studying enzyme turnover and enzyme regulation by hormonal and pharmacological agents.

Conversion of the 15(*S*)-hydroxyl group of prostaglandins to a keto function by NAD^+-dependent[1] 15-hydroxyprostaglandin dehydrogenase (NAD^+-15-hydroxyprostanoate oxidoreductase (EC 1.1.1.141)) is considered to be both the initial and major route for their transformation to inactive metabolites (Anggard and Samuelsson, 1964). This enzyme has been shown to be present in most tissues examined and purification of this enzyme from human placenta (Braithwaite and Jarabak, 1975; Schlegel and Greep, 1975), bovine lung (Nagasawa et al., 1975, Matschinsky et al., 1974), swine lung (Anggard and Samuelsson, 1966), chicken heart (Lee and Levine, 1975), and swine kidney (Tai et al., 1974) has been attempted. The methods employed by these workers for the assay of 15-hydroxyprostaglandin dehydrogenase include development of chromophore at 500 nm (Anggard et al., 1971), measurement of the formation of NADH spectrophotometrically, and application of radioimmunoassay. Development of chromophore at 500 nm induced by alkalinization of the reaction product, 15-oxo-PGE or 15-oxo-PGA, provides a simple assay for this enzyme. However, the assay can not be reliably employed in crude stages of the enzyme preparation because of the interference of hemoproteins at 500 nm. Furthermore, the chromophore has only transient stability. Measurement of the formation of NADH spectrophotometrically is allowed only after a certain degree of enzyme purification simply because the interfering enzymes utilizing NADH are also present in the crude preparation. Although radioimmunoassay provides

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[1] Abbreviations used are: PGE_1, prostaglandin E_1 (11α,15α-dihydroxy-9-oxo-13-*trans*-prostenoic acid); PGE_2, prostaglandin E_2 (11α,15α-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid); PGA_2, prostaglandin A_2 (15(*S*)-hydroxy-9-oxo-5-*cis*,10,13-*trans*-prostatrienoic acid); $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$ (9α,11α,15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid); 15-oxo-PGE_2, 15-oxoprostaglandin E_2 (11α,15α-dihydroxy-9,15-dioxo-5-*cis*,13-*trans*-prostadienoic acid); 15-oxo$PGF_{2\alpha}$, 15-oxoprostaglandin $F_{2\alpha}$ (9α,11α-dihydroxy-15-oxo-5-*cis*,13-*trans*-prostadienoic acid). NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane.

the enzyme assay with greater sensitivity and allows the assessment of enzyme activity in crude extract, it requires the generation of antisera of high specificity and tedious manipulations in assay procedure.

Recently studies on the significance of this enzyme in the physiology and pathophysiology of prostaglandins became more evident. To cite a few examples, high prostaglandin inactivation appears to be an important feature of the early developing tissues (Pace-Asciak and Miller, 1973; Pace-Asciak, 1975), genetic hypertension in rats is accompanied by a defect in renal prostaglandin catabolism (Amstrong et al., 1976), and progesterone and estrogen induce increases in 15-hydroxyprostaglandin dehydrogenase activity during deciduomal growth (Alam et al., 1976). Most of these studies employed the tedious procedure involving incubation with labeled substrate followed by extraction and thin-layer chromatography to assess the enzyme activity. In order to facilitate the studies on the role of this enzyme in the physiology of prostaglandins, development of a simple, sensitive, and reliable assay applicable to crude enzyme extract becomes warranted.

We have recently developed a tritium release assay for 15-hydroxyprostaglandin dehydrogenase based on the stereospecific transfer of tritium label of (15*S*)-[15-^{3}H]prostaglandins to glutamate by coupling 15-hydroxyprostaglandin dehydrogenase with glutamate dehydrogenase. The amount of prostaglandin oxidized is quantitated by the labeled glutamate produced which is separated from labeled prostaglandin by charcoal precipitation. Development of this assay relies on the determination of the stereospecificity of 15-hydroxyprostaglandin dehydrogenase with respect to coenzyme and preparation of stereospecific labeled prostaglandins. This paper describes the studies of these aspects as well as the validation of the assay.

Materials and Methods

PGE_1, PGE_2, PGA_2, $PGF_{2\alpha}$, 15-oxo-PGE_2, and 15-oxo-$PGE_{2\alpha}$ were supplied by Drs. Udo Axen and John Pike of the Upjohn Company. Imipramine hydrochloride, furosemide, and ethacrynic acid were respectively given by CIBA Pharmaceutical Co., Hoechst Pharmaceuticals, Inc., and Merck Sharp & Dohme Research Laboratories. [4-^{3}H]NAD^+ (50 mCi/mmol) was purchased from Amersham/Searle Corporation. [5,6,8,11,12,14,15-^{3}H]PGE_2 (117 Ci/mmol) and D-[1-^{3}H]-galactose (14.2 Ci/mmol) were purchased from New England Nuclear. NAD^+, α-ketoglutarate monosodium salt, triiodothyroacetic acid, indomethacin, DEAE-cellulose, bovine liver glutamic dehydrogenase (51 U/mg), yeast alcohol dehydrogenase (325 U/mg), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (67 U/mg), yeast 3-phosphoglyceric phosphokinase (1600 U/mg), beef heart malic dehydrogenase (4500 U/mg), β-D-galactose dehydrogenase (7.6 U/mg), and pig heart NADH oxidase supplied as diaphorase (3.4 U/mg) were obtained from Sigma Chemical Company. Rabbit muscle lactic dehydrogenase (329 U/mg) was obtained from Calbiochem. Charcoal and dextran (mol wt 86 000) were purchased from Amend Drug and Chemical Company and K & K Laboratories, respectively. Silica gel G plate was obtained from Brinkmann Instrument Inc. Rats weighing 250–300 g were obtained from Camm Research Institute, Inc. Swine kidney was collected from a local slaughterhouse. 15-Hydroxyprostaglandin dehydrogenase was purified from swine kidney by ammonium sulfate precipitation, DEAE-cellulose chromatography, Sephadex G-100 gel filtration, and blue dextran–Sepharose affinity chromatography as previously described (Tai et al., 1974; Tai and Hollander, 1976a). The final preparation was purified 400-fold with a specific activity of 653 mU per mg of protein. This highly purified enzyme was used for the determination of the stereospecificity of the enzyme with respect to coenzyme and the preparation of (15*S*)-[15-^{3}H]prostaglandins.

Preparation of [4-^{3}H]NADH with Swine Renal 15-Hydroxyprostaglandin Hydrogenase. [4-^{3}H]NADH was prepared by incubation of [4-^{3}H]NAD^+ (0.1 μmol, 5 μCi) and PGE_1 (0.1 μmol) with swine renal 15-hydroxyprostaglandin dehydrogenase (420 μg) in 2 ml of 50 mM Tris-HCl, pH 8.0. The incubation was carried out at 37 °C for 1 h. The reaction mixture was diluted with 30 ml of ice-cold water and applied into a DEAE-cellulose column (1 × 4 cm) equilibrated with 50 mM Tris-HCl, pH 8.0. Separation of NAD^+ and NADH was carried out as described by Davies et al. (1972). The column was washed with 20 ml of the same buffer and [4-^{3}H]NADH was then eluted with 100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The eluate was collected in 2-ml fractions. The [4-^{3}H]NADH was usually eluted in fractions 2–4.

Oxidation of [4-^{3}H]NADH with Yeast Alcohol Dehydrogenase and Bovine Liver Glutamic Dehydrogenase. A. With Yeast Alcohol Dehydrogenase. [4-^{3}H]NADH (4.4 nmol) prepared above was incubated with acetaldehyde (100 mM) in the presence of yeast alcohol dehydrogenase (100 μg) in 1 ml of 50 mM Tris-HCl, pH 8.0. The reaction mixture was allowed to proceed until no further decrease in absorption at 340 nm was observed. The reaction mixture after dilution with 9 ml of ice cold water was applied onto a DEAE-cellulose column (1 × 4 cm) equilibrated with 50 mM Tris-HCl, pH 8.0. After washing with 6 ml of 5 mM Tris-HCl, pH 8.0, the NAD^+ was eluted with 10 ml of 50 mM Tris-HCl, pH 8.0, and the NADH was then eluted with 10 ml of 100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The eluate was collected in 2-ml fractions. Both NAD^+ and NADH were eluted in fractions 2–4 by their respective eluent.

B. With Bovine Liver Glutamic Dehydrogenase. [4-^{3}H]NADH (4.4 nmol) prepared above was incubated with α-ketoglutarate (1 μmol) and NH_4Cl (10 μmol) in the presence of bovine liver glutamic dehydrogenase (100 μg) in 1 ml of 50 mM Tris-HCl, pH 8.0. The reaction mixture was diluted and chromatographed on DEAE-cellulose column as described above.

Preparation of (4S)-[4-^{3}H]NADH. (4*S*)-[4-^{3}H]NADH was prepared by incubating yeast alcohol dehydrogenase (100 μg), ethanol (1.73 mmol), and [4-^{3}H]NAD^+ (0.33 μmol, 16.5 μCi) in 1 ml of 100 mM Tris-HCl, pH 8.0. The reaction was carried out at 25 °C and allowed to proceed until no further change in absorption at 340 nm was detected. The reaction mixture was diluted and chromatographed on DEAE-cellulose column equilibrated with 50 mM Tris-HCl, pH 8.0. (4*S*)-[4-^{3}H]NADH was isolated as described previously.

Preparation of (4R)-[4-^{3}H]NADH. (4*R*)-[4-^{3}H]NADH was prepared by incubating glyceraldehyde-3-phosphate dehydrogenase (30 μg), 3-phosphoglyceric phosphokinase (20 μg), K_2HPO_4 (20 μmol), cysteine-HCl (3.3 μmol), ADP (1 μmol), DL-glyceraldehyde 3-phosphate (1.6 μmol), and [4-^{3}H]NAD^+ (0.33 μmol, 16.5 μCi) in 1 ml of 100 mM Tris-HCl, pH 8.0. Addition of 3-phosphoglyceric phosphokinase was to drive the reaction favoring the formation of NADH. (4*R*)-[4-^{3}H]NADH was isolated as described above.

Oxidation of (4R)- and (4S)-[4-^{3}H]NADH with Swine Renal 15-Hydroxyprostaglandin Dehydrogenase. (4*R*)-[4-^{3}H]NADH (3 nmol) and (4*S*)-[4-^{3}H]NADH (5 nmol) prepared as described above were respectively incubated with

TABLE I: Stereospecificity of NAD^+-Dependent Swine Renal 15-Hydroxyprostaglandin Dehydrogenase Studied in the Direction of NAD^+ Reduction.[a]

Enzymes	Radioact. in NADH Fraction (cpm)	Radioact. in NAD^+ Fraction (cpm)	% Recovery of Radioact. in NAD^+
Control	195 000	26 700	
Alcohol dehydrogenase	1 340	24 700	0
Glutamic dehydrogenase	2 180	209 500	95.4

[a] [4-^{3}H]NADH prepared from [4-^{3}H]NAD^+ and PGE_1 by incubation with 15-hydroxyprostaglandin dehydrogenase was allowed to react with the appropriate substrates catalyzed by the dehydrogenases indicated in column 1. The reaction mixture was chromatographed on DEAE-cellulose column. NADH and NAD^+ fractions were respectively collected as detailed in the section on Materials and Methods.

15-oxo-$PGF_{2\alpha}$ (50 nmol) in a final volume of 1 ml of 50 mM Tris-HCl, pH 8.0. The reaction was started by the addition of 15-hydroxyprostaglandin dehydrogenase (110 μg) and allowed to proceed for 1 h at 37 °C. Each reaction mixture was then divided into half. One-half was diluted tenfold with water and chromatographed on a DEAE-cellulose column (1 × 4 cm). The column was eluted with 50 mM Tris-HCl, pH 8.0, followed by 100 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, and fractions of 2 ml were collected as described before. Since both labeled $PGF_{2\alpha}$ and labeled NAD^+ were eluted by 50 mM Tris-HCl, pH 8.0, further separation of these two compounds was achieved by extraction of the eluate with two volumes of ethyl acetate three times after acidification to 3.0. Labeled $PGF_{2\alpha}$ was found in the organic layer. The other half was acidified to pH 3.0 and extracted with three 2-ml portions of ethyl acetate. The combined extracts were evaporated under N_2 at 40 °C. The residue was spotted on silica gel G plate (2 × 20 cm) and developed in the organic layer of the solvent system of ethyl acetate–acetic acid–isooctane–water (11:2:5:10). Strips (1 cm) of the chromatogram were scraped off the plate and the radioactivity was determined by liquid scintillation counting.

Preparation of (15S)-[15-^{3}H]PGE_2 and -$PGF_{2\alpha}$. (15*S*)-[15-^{3}H]PGE_2 and -$PGF_{2\alpha}$ were prepared by incubating D-[1-^{3}H]galactose (3.52 nmol, 50 μCi), NAD^+ (10 nmol), 15-oxo-PGE_2 or 15-oxo-$PGF_{2\alpha}$ (50 nmol), β-D-galactose dehydrogenase (100 μg), and swine renal 15-hydroxyprostaglandin dehydrogenase (179 μg) in a final volume of 1 ml of 20 mM potassium phosphate buffer adjusted to pH 6.0 with 1 N acetic acid. The reaction was allowed to proceed at 37 °C for 3 h for the synthesis of labeled PGE_2 and 6 h for the synthesis of labeled $PGF_{2\alpha}$. Termination and extraction of the reaction mixture as well as thin-layer chromatography of the extract were carried out as described above. Labeled prostaglandins were scraped off the plate and extracted from the gel three times by 1-ml portions of ethanol. The combined extract was kept at −20 °C.

Preparation of (15S)-[15-^{3}H]PGA_2. (15*S*)-[15-^{3}H]PGA_2 was prepared from (15*S*)-[15-^{3}H]PGE_2 by acetic acid–phosphoric acid dehydration as described by Andersen (1969).

Preparation of Soluble Fraction from Swine Kidney. Swine kidney was homogenized in 3 volumes of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM of EDTA. The homogenate was centrifuged at 10 000*g* for 10 min and the resulting supernatant was further centrifuged at 105 000*g* for 60 min. The final supernatant was used as the crude enzyme source for 15-hydroxyprostaglandin dehydrogenase.

15-Hydroxyprostaglandin Dehydrogenase Assay. The incubation mixture contained: NH_4Cl (5 μmol), monosodium α-ketoglutarate (1 μmol), NAD^+ (1 μmol), (15*S*)-[15-^{3}H]PGE_2 (1 nmol, 20 000 cpm), glutamate dehydrogenase (100 μg), and appropriate amount of 15-hydroxyprostaglandin dehydrogenase in a final volume of 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The reaction was started by the addition of 15-hydroxyprostaglandin dehydrogenase and allowed to proceed for 10 min at 37 °C. The reaction was terminated by the addition of 0.3 ml of a charcoal suspension (10% charcoal in 1% dextran solution). The reaction mixture was centrifuged at 1000*g* for 5 min after standing for 5 min at room temperature. The supernatant was decanted and the radioactivity was determined by liquid scintillation counting.

Results and Discussion

Stereospecificity of NAD^+-Dependent 15-Hydroxyprostaglandin Dehydrogenase. The stereospecific oxidation of NADH or reduction of NAD^+ first established by Fisher et al. (1953) has been determined for a number of dehydrogenases. These dehydrogenases can be categorized into two different groups. The A-side specific dehydrogenases catalyze the transfer of *pro-R* hydrogen atom at C-4 of the dihydronicotinamide ring of NADH, whereas the B-side specific dehydrogenases involve the removal of *pro-S* hydrogen atom. Yeast alcohol dehydrogenase (Fisher et al., 1953), rabbit muscle lactic dehydrogenase (Loewus and Stafford, 1960), and pig heart malic dehydrogenase (Graves et al., 1956) belong to the former group, while bovine liver L-glutamic dehydrogenase (Levy and Vennesland, 1957), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Loewus et al., 1959), and NADH oxidase (Weber et al., 1957) are of the latter group.

When 15-hydroxyprostaglandin dehydrogenase is incubated with PGE_1 and [4-^{3}H]NAD^+, the [4-^{3}H]NADH thus produced will be labeled on the 4-*pro-R* position of the dihydronicotinamide ring if 15-hydroxyprostaglandin dehydrogenase is a B-side specific dehydrogenase, and conversely will be on the 4-*pro-S* side if 15-hydroxyprostaglandin dehydrogenase is an A-side specific dehydrogenase. To establish the stereospecificity, [4-^{3}H]NADH prepared by incubation with PGE_1 and 15-hydroxyprostaglandin dehydrogenase was respectively incubated with yeast alcohol dehydrogenase which removed *pro-R* hydrogen atom of the dihydronicotinamide ring and with bovine liver L-glutamic dehydrogenase which eliminated the *pro-S* hydrogen atom of the ring. Table I shows that the tritium of [4-^{3}H]NADH was completely transferred presumably to ethanol which appeared in the flow-through fraction during DEAE-cellulose chromatography when incubated with yeast alcohol dehydrogenase. However, the tritium of [4-^{3}H]NADH was virtually recovered in the NAD^+ fraction (95.4%) when incubated with bovine liver glutamic dehydrogenase. These results indicate that [4-^{3}H]NADH produced by reduction of [4-^{3}H]NAD^+ catalyzed by 15-hydroxyprostaglandin dehydrogenase is labeled at 4-*pro-R* position of the dihydronicotinamide ring. Therefore, NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase is a B-side specific dehydrogenase.

When the reaction was studied in the reversed direction, i.e., reduction of 15-oxoprostaglandins by NADH, stereospecific

TABLE II: Stereospecificity of NAD^+-Dependent Swine Renal 15-Hydroxyprostaglandin Dehydrogenase Studied in the Direction of NADH Oxidation.

Coenzyme	Enzyme	Radioact. in NADH Fraction (cpm)	Radioact. in NAD^+ Fraction (cpm)	% Recovery of Radioact. in NAD^+
(4*R*)-[4-^{3}H]NADH	Control	69 600	15 100	
	15-OH-PGDH	32 700 (36 900)[a]	49 100 (34 000)	92.1
(4*S*)-[4-^{3}H]NADH	Control	115 600	21 700	
	15-OH-PGDH	48 000 (67 000)	34 100 (12 400)	18.3

[a] The parentheses indicate the radioactivity of coenzyme involved in oxidation and reduction. (4*R*)- and (4*S*)-[4-^{3}H]NADH prepared from [4-^{3}H]NAD^+ and the appropriate substrates by incubation with dehydrogenases of known stereospecificity was allowed to react with 15-oxo-$PGF_{2\alpha}$ catalyzed by swine renal 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH). Half of the reaction mixture was chromatographed on DEAE-cellulose column. NADH and NAD^+ fractions were respectively collected as detailed in the section on Materials and Methods.

label of [4-^{3}H]NADH was prepared. (4*R*)-[4-^{3}H]NADH was obtained by reduction of [4-^{3}H]NAD^+ catalyzed by glyceraldehyde-3-phosphate dehydrogenase, a B-side specific dehydrogenase (Loewus et al., 1959), whereas (4*S*)-[4-^{3}H]NADH was prepared by reduction of [4-^{3}H]NAD^+ catalyzed by yeast alcohol dehydrogenase, an A-side specific dehydrogenase (Fisher et al., 1953). When (4*R*)-[4-^{3}H]-NADH was incubated with 15-oxo-$PGE_{2\alpha}$ and 15-hydroxyprostaglandin dehydrogenase, 92.1% of the radioactivity of [4-^{3}H]NADH consumed to reduce 15-oxo-$PGF_{2\alpha}$ was recovered in the NAD^+ fraction as shown in Table II. When (4*S*)-[4-^{3}H]NADH was incubated with 15-oxo-$PGF_{2\alpha}$ and 15-hydroxyprostaglandin dehydrogenase, only 18.3% of the radioactivity of [4-^{3}H]NADH oxidized was found in the NAD^+ fraction as indicated in the same table. That slightly higher radioactivity than expected was recovered in the NAD^+ fraction in this instance was probably due to the incomplete extraction of $PGF_{2\alpha}$ during separation of NAD^+ and $PGF_{2\alpha}$. Extraction of the above two different reaction mixtures by ethyl acetate followed by thin-layer chromatography of the extract on silica gel G plate indicated that the tritium of (4*R*)-[4-^{3}H]NADH was not found in $PGF_{2\alpha}$, while the tritium of (4*S*)-[4-^{3}H]NADH was transferred to $PGF_{2\alpha}$ as shown in Figure 1. These results again indicate that 15-hydroxyprostaglandin dehydrogenase catalyzed stereospecific transfer of 4-*pro-S* hydrogen atom of the dihydronicotinamide ring of NADH.

In order to further determine the fate of 15(*S*) hydrogen of prostaglandins and thus to confirm the stereospecific reduction of NAD^+ catalyzed by 15-hydroxyprostaglandin dehydrogenase, [5,6,8,11,12,14,15-^{3}H]PGE_2 having a 15(*S*) tritium prepared by enzymic cyclization of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid was incubated with NAD^+ and 15-hydroxyprostaglandin dehydrogenase in the presence of an A-side specific dehydrogenase or a B-side specific dehydrogenase. If 15-hydroxyprostaglandin dehydrogenase is a B-side specific dehydrogenase as demonstrated above, the 15(*S*) tritium of

Scheme I

$$(15S)\text{-}[15\text{-}^3\text{H}]PGE_2 + NAD^+ \underset{\text{dehydrogenase}}{\overset{\text{15-hydroxyprostaglandin}}{\rightleftharpoons}} \text{15-oxo-}PGE_2 + (4S)\text{-}[4\text{-}^3\text{H}]NADH$$

$$(4S)\text{-}[4\text{-}^3\text{H}]NADH + H^+ + A \underset{\text{dehydrogenase}}{\overset{\text{A-side specific}}{\rightleftharpoons}} [4\text{-}^3\text{H}]NAD^+ + AH_2$$

$$(4S)\text{-}[4\text{-}^3\text{H}]NADH + H^+ + B \underset{\text{dehydrogenase}}{\overset{\text{B-side specific}}{\rightleftharpoons}} NAD^+ + [^3\text{H}]BH_2$$

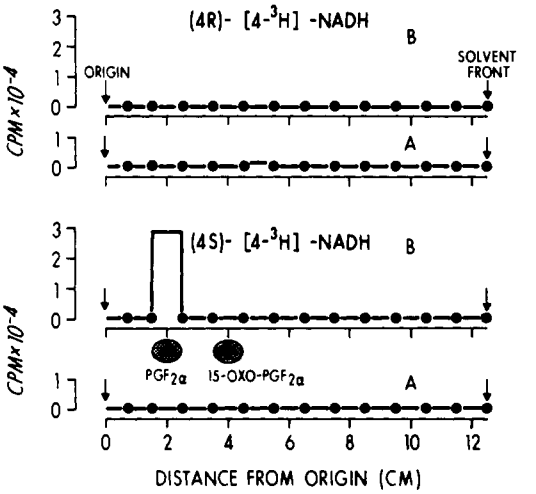


FIGURE 1: Thin-layer radiochromatogram of the ethyl acetate extract of the reaction mixture of 15-oxo-$PGF_{2\alpha}$ and stereospecific labeled NADH catalyzed by swine renal 15-hydroxyprostaglandin dehydrogenase. (A) Control plate. (B) Reaction plate. Half of the reaction mixture described in Table II was extracted and chromatographed as detailed in the Materials and Methods section.

PGE_2 will be transferred to 4-*pro-S* position of NADH which subsequently will be retained at C-4 position of NAD^+ when coupled with an A-side specific dehydrogenase, or the 15(*S*) tritium will be eventually localized to the reduced product of B-side specific dehydrogenase with which 15-hydroxyprostaglandin dehydrogenase is coupled as shown in Scheme I.

Since charcoal binds prostaglandins, NAD^+ and NADH, but not malate, lactate, and glutamate, a simple procedure using charcoal to separate these two different types of compounds was employed to investigate the above scheme. Table III shows that the total releasable tritium of heptatritiated PGE_2 was found virtually in the charcoal precipitate when coupled with A-side dehydrogenases such as lactic dehydrogenase and malic dehydrogenase but was transferred to glutamate or H_2O which stays in the supernatant after charcoal precipitation when coupled with B-side specific dehydrogenases such as NADH oxidase or glutamate dehydrogenase. These data again confirm that wine renal NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase is a B-side specific dehydrogenase.

The above scheme that labeled substrate of the enzyme studied will transfer its tritium to the reduced product of the coupling enzyme if both enzymes are of the same stereospecificity (otherwise, the tritium will retain on NAD^+) serves as a simple principle to determine the stereospecificity of a dehydrogenase provided that labeled substrate can be easily

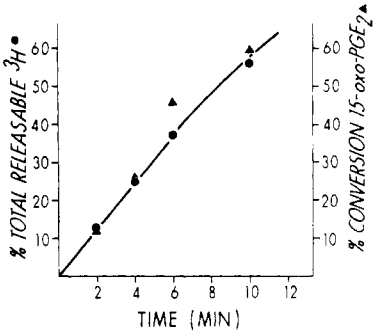


FIGURE 2: Correlation of 15-hydroxyprostaglandin dehydrogenase assays by tritium release method and by thin-layer chromatography method. Duplicated incubations of [5,6,8,11,12,14,15-^{3}H]PGE_2 (20 000 cpm, 1 nmol), NAD^+ (1 μmol), NH_4Cl (5 μmol), sodium α-ketoglutarate (1 μmol), glutamate dehydrogenase (100 μg), and purified 15-hydroxyprostaglandin dehydrogenase (1.1 μg) in a final volume of 1 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA were carried out at 37 °C for the indicated time. One incubation was assayed for tritium release as described in the Materials and Methods section. Percent total releasable tritium was shown in the left abscissa. The other duplicate was acidified and extracted with two 3-ml portions of ethyl acetate. The dried extract was mixed with 20 μg each of PGE_2 and 15-oxo-PGE_2 and chromatographed on silica gel G plate developed in ethyl acetate–acetic acid–isooctane–water (11:2:5:10) system. Both substrate and product were localized by iodine vapor and scraped off the plate and counted. The radioactivity of 15-oxo-PGE_2 zone was multiplied by 7/6 because one tritium was lost during oxidation. Percent conversion to product 15-oxo-PGE_2 was represented in the right abscissa.

TABLE III: Charcoal Unprecipitable Radioactivity after Incubation of [5,6,8,11,12,14,15-^{3}H]PGE_2 with Swine Renal 15-Hydroxyprostaglandin Dehydrogenase and Various Dehydrogenases of Known Stereospecificity.

	cpm
Total releasable radioactivity of [^{3}H]PGE_2	11 469
Control	355
LDH (A)[a]	659
MDH (A)	701
GDH (B)	10 250
NADH oxidase (B)	10 953

[a] The parentheses indicate the stereospecificity of each dehydrogenase. [5,6,8,11,12,14,15-^{3}H]PGE_2 (80 981 cpm, 10 pmol) was incubated with NAD^+ (1 μmol), 15-hydroxyprostaglandin dehydrogenase (44.8 μg), and respective dehydrogenase as indicated in a final volume of 0.1 M potassium phosphate buffer, pH 7.5. The amounts of other dehydrogenases and their substrates used were: rabbit muscle lactate dehydrogenase (LDH, 76 μg), pyruvate (5 μmol); malic dehydrogenase (MDH, 4.4 μg), *cis*-oxaloacetate (5 μmol); bovine liver glutamic dehydrogenase (GDH, 100 μg), NH_4Cl (5 μmol), α-ketoglutarate (1 μmol); pig heat NADH oxidase (200 μg). After incubation of 1 h at 37 °C, the reaction mixture was treated with 0.3 ml of a charcoal suspension (10% charcoal in 1% dextran solution) and allowed to stand for 5 min before centrifugation at 1000*g* for 5 min at room temperature. The supernatant was decanted and the radioactivity was determined by liquid scintillation counting.

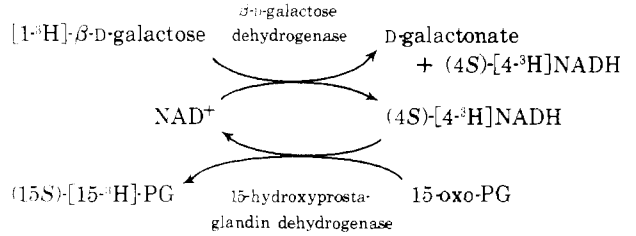
obtained. We have recently employed this principle to determine the stereospecificity of rat kidney NAD^+-dependent 9-hydroxyprostaglandin dehydrogenase in crude extract and found it to be an A-side specific dehydrogenase (Tai and Yuan, 1976).

Development and Validation of 15-Hydroxyprostaglandin Dehydrogenase. The above procedure was further employed to develop a simple and sensitive assay for 15-hydroxyprostaglandin dehydrogenase. According to the scheme, the amount of tritium release should be quantitatively transferred to glutamate or water provided that the coupling reactions were efficient. Since coupling enzymes were added in an excess amount, the interfering enzymes utilizing NADH were overwhelmed by coupling enzyme added. In fact, the oxidation of NADH whereby the transfer of tritium was catalyzed by the amount of coupling enzyme added was achieved in seconds. We have chosen glutamate dehydrogenase as a coupling enzyme because of its inexpensiveness and availability in highly purified preparation. Validation of the assay was then carried out by incubating [5,6,8,11,12,14,15-^{3}H]PGE_2 and purified enzyme in the presence of glutamate dehydrogenase and correlating tritium release by current methods with the formation of 15-oxo-PGE_2 as determined by thin-layer chromatography. Figure 2 shows that there is an excellent correlation between the two methods. The nonlinearity at the later stage of incubation was due to the fact that substantial amounts of substrate had been converted to product whereby substrate became limiting in the reaction.

Enzymatic Synthesis of (15S)-[15-^{3}H]PGE_2 and -$PGF_{2\alpha}$. Although commercially available heptatritiated PGE_2 having a 15(*S*) tritium can be used for substrate, only one-seventh of the tritium labels of the substrate is releasable. Furthermore, the blank of the assay is relatively high as demonstrated in Table III. Ideally (15*S*)-[15-^{3}H]prostaglandins will provide the best substrate for the assay. We first attempted to prepare (15*S*)-[15-^{3}H]$PGF_{2\alpha}$ by tritiated sodium borohydride reduction of 15-oxo-$PGF_{2\alpha}$. Thin-layer chromatography indicated that over 80% of the labeled $PGF_{2\alpha}$ was the undesired epimer. Furthermore, (15*S*)-[15-^{3}H]$PGF_{2\alpha}$ was found to be a relatively poor substrate for 15-hydroxyprostaglandin dehydrogenase.

As indicated earlier, the tritium label of the substrate of one dehydrogenase will be transferred to the product of the coupling enzyme if both enzymes are of the same stereospecificity. This principle provides a simple means to label a substrate stereospecifically. Since β-D-galactose dehydrogenase, a B-side specific dehydrogenase (Eisele and Wallenfels, 1970), and D-[1-^{3}H]galactose are both commercially available, (15*S*)-[15-^{3}H]prostaglandins can be readily prepared according to Scheme II. In order to favor the formation of labeled prostaglandins, NAD^+ was kept at low concentration, while 15-oxo-PG was added at high concentration but not to cause substrate inhibition. Furthermore, the coupling reaction was carried out at pH 6.0 which favors the reversed reaction of 15-hydroxyprostaglandin dehydrogenase (Yamazaki and Sasaki, 1975). Figure 3 shows the time course of such a coupling reaction under the conditions indicated in figure legend. Formation of (15*S*)-[15^{3}H]PGE_2 generally requires 2–3 h to reach equilibrium, while that of (15*S*)-[15-^{3}H]$PGF_{2\alpha}$ requires 6 h or longer to reach equilibrium. This is consistent with the fact that PGE_2 is a more favorable substrate than $PGF_{2\alpha}$ for 15-hydroxyprostaglandin dehydrogenase (Anggard and Samuelsson, 1966). In general, about 30% of the radioactivity of D-[1-^{3}H]galactose can be incorporated into prostaglandins.

Scheme II



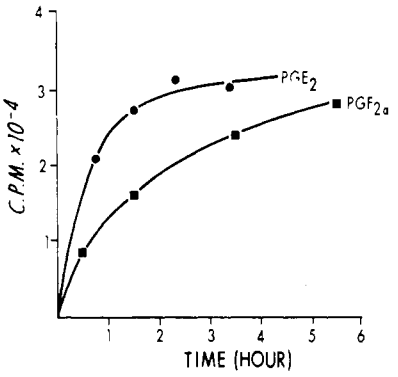


FIGURE 3: The kinetics of the enzymatic synthesis of (15*S*)-[15-^{3}H]PGE_2 and -$PGF_{2\alpha}$. The incubations were carried out as described in the Materials and Methods section. Reaction was terminated at the indicated time by spotting 5 μl of the reaction mixture and 20 μg of PGE_2 or $PGF_{2\alpha}$ on the silica gel G plate which was then developed in the same solvent system as described in Figure 2. PGE_2 or $PGF_{2\alpha}$ was localized by iodine vapor and the respective zone was scraped off the plate and counted.

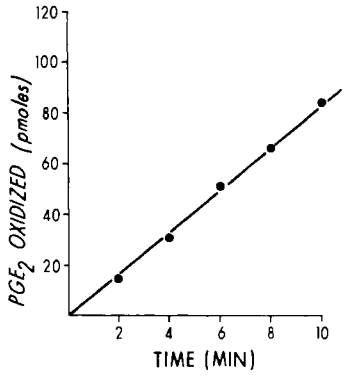


FIGURE 4: Assay of 15-hydroxyprostaglandin dehydrogenase activity as a function of time. Incubation and termination of the reaction mixture were carried out as described in the Materials and Methods section. The soluble fraction of swine kidney (79 μg) was used for assay.

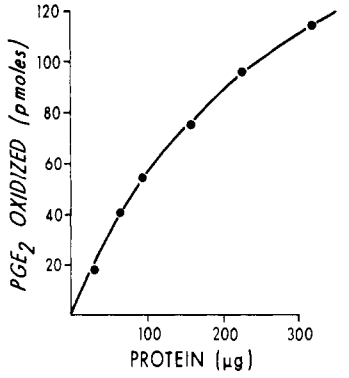


FIGURE 5: Assay of 15-hydroxyprostaglandin dehydrogenase activity as a function of crude enzyme concentration. Incubation and termination of the reaction mixture were carried out as described in the Materials and Methods section, except the soluble fraction of swine kidney was varied as indicated.

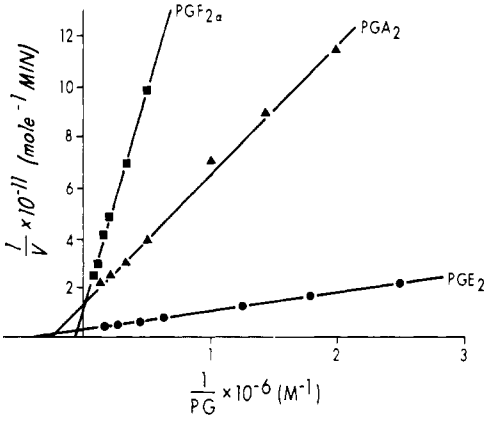


FIGURE 6: Determination of apparent K_m values for PGE_2, $PGF_{2\alpha}$, and PGA_2. The assay systems were the same as described in the Materials and Methods section, except that prostaglandin concentrations were varied. The soluble fraction of swine kidney (158 μg) was used for assay.

The specific radioactivity of labeled prostaglandins thus prepared showed a single radioactive peak as well as the same mobilities on thin-layer plates as those of authentic prostaglandins in two different solvent systems. Furthermore, the blank of the enzyme assay is as low as 50 cpm.

Properties of Swine Renal NAD$^+$-Dependent 15-Hydroxyprostaglandin Dehydrogenase. (15*S*)-[15-^{3}H]PGE_2 was employed to characterize swine renal NAD$^+$-dependent 15-hydroxyprostaglandin dehydrogenase in crude extract. The tritium release induced by the action of crude swine renal enzyme was linear with time provided that substrate was not limiting (Figure 4). The activity shows a nonlinear relationship with the amount of crude enzyme added, indicating the presence of inhibitor(s) in the crude extract (Figure 5). This is consistent with the fact that enzyme activity was increased in the early stage of purification presumably by removing the inhibitor(s) (Tai et al., 1974) and that the linear relationship can be easily demonstrated by using purified enzyme (data not shown). The enzyme–substrate interaction appears to follow Michaelis–Menten kinetics (Figure 6). The apparent K_m for PGE_2, $PGF_{2\alpha}$, and PGA_2 were found to be 2.5, 12.5, and 3.9 μM, respectively. Relative V_{max}'s for PGE_2, $PGF_{2\alpha}$, and PGA_2 were 100, 24.0, and 22.2, respectively. Although introduction of tritium at carbon 15 may show kinetic isotope effects in subsequent enzyme-catalyzed oxidation of 15-hydroxyl group (Richards, 1970), the kinetic constants were in good qualitative agreement with those of partially purified enzyme from swine lung (Nakano et al., 1969).

Application of the current assay to studying the effect of drugs or other agents on 15-hydroxyprostaglandin dehydrogenase requires certain precautions. Although an excessive amount of the coupling enzyme has been added to render the 15-hydroxyprostaglandin dehydrogenase reaction rate limiting, the levels of coupling enzyme should be so saturated that the residual enzyme activity after drug inhibition is still able to maintain maximal tritium transfer. Ethacrynic acid and furosemide which have been reported to be inhibitors of 15-hydroxyprostaglandin dehydrogenase (Paulsrud et al., 1974; Tai and Hollander, 1976b) also inhibited the crude swine renal enzyme in a dose-dependent manner as assayed by the current method (data not shown).

Distribution of NAD$^+$-Dependent 15-Hydroxyprostaglandin Dehydrogenase in Various Tissues of Rat. Table IV shows the level of 15-hydroxyprostaglandin dehydrogenase in a number of rat tissues. Gastrointestinal tract, lung, kidney, and spleen possess high levels of enzyme activity. Lung and kidney from swine (Anggard et al., 1971) and from monkey (Sun et al., 1976) have also been reported to contain high enzyme activity. High level of 15-hydroxyprostaglandin dehydrogenase activity in gastrointestinal tract is of particular interest in view of the fact that prostaglandins are potent stimulants of intestinal water secretion and strong inhibitors of gastric acid secretion (Waller, 1973). The distribution pattern of enzyme activity appeared to differ somewhat in swine,

TABLE IV: NAD^+-Dependent 15-Hydroxyprostaglandin Dehydrogenase Activity in Various Tissues of Rat.[a]

Tissues	Activity (pmol per min per g of tissue)
Brain	11.92 ± 11.20
Adipose	5.44 ± 2.24
Heart	2.08 ± 1.84
Intestine	105.68 ± 76.00
Stomach	527.60 ± 97.90
Kidney	168.00 ± 50.40
Lung	288.80 ± 144.8
Spleen	124.80 ± 27.20
Muscle	9.60 ± 3.20

[a] Three adult rats weighing 250–300 g were killed by decapitation and the tissues were immediately removed and frozen in dry ice. Each tissue was homogenized in 4 volumes of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. The homogenates were centrifuged at 12 000*g* for 15 min. The supernatant was assayed for enzyme activity as described in the Materials and Methods section.

monkey, and rat. Species differences may partially account for these variances. Since the determination of enzyme activity both in swine and in monkey employed the product chromophore method, the presence of different levels of Δ^{13}-15-ketoprostaglandin reductase which catalyzes further the reduction of Δ^{13} double bond in various tissues might underestimate the levels in different degrees in crude homogenates. The unique method of following the tritium release serves as an initial measure of the enzyme activity irrespective of the presence of ensuing enzymes in the catabolic pathway. This method will provide a good estimate of the "true" enzyme activity in the crude extract. Although argument may arise that the substrate concentration (1 μM) in the assay mixture is not saturating because of the need of having a highly sensitive assay, the substrate concentration employed is still much higher than the prostaglandin concentration in the crude extract since the amount of primary prostaglandins present in most tissues is less than 40 ng per g of tissue (Karim et al., 1968). Significant dilution of labeled substrate is considered unlikely when utilizing small aliquots of the crude extract for enzyme assay.

The current method indeed provides a simple and sensitive assay to assess the enzyme activity both in crude extract and in purified preparation. This method will particularly prove valuable in studying the tissue enzyme levels in various diseased states, the enzyme turnover, and in vivo enzyme regulation by various hormonal and pharmacological agents. Furthermore, the enzyme assay can be employed to determine picomole quantities of primary prostaglandins when incubated in small volumes. A standard curve can be constructed based on the decrease of radioactivity released as the result of increasing amount of unlabeled substrate added. This method offers the advantage over fluorometric measurement (Anggard et al., 1969) since relatively crude enzyme can be employed as the enzyme source and the contamination by fluorescent materials is not a factor of concern. The next step necessary for a fully useful enzymatic assay of prostaglandins will be the development of a simple procedure for purifying prostaglandins from biological materials in a form suitable for the enzymatic assay.

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