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ENZYMATIC SYNTHESIS OF TRITIUM - LABELLED PROSTAGLANDIN D_2 AND ITS CONVERSION TO OTHER PROSTAGLANDINS

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SUMMARY

The one-stage enzymatic synthesis of tritium-labelled prostaglandin D_2 from labelled arachidonic acid was performed by using the enzyme system PGH-synthetase/PGH-PGD-isomerase. By enzymatic and chemical transformation of [^{3}H]PGD_2 the following compounds were obtained: 15-keto-13,14-dihydro-[^{3}H]PGD_2, 9α,11β-[^{3}H]PGF_2, 9-deoxy-Δ^9-[^{3}H]-PGD_2 ([^{3}H]PGJ_2) and Δ^{12}-13,14-dihydro-[^{3}H]PGJ_2. It was found that L-selectride is a more effective reducing agent than sodium borohydride in the synthesis of 9α,11β-[^{3}H]PGF_2.

Key words: *Tritium Labelled Prostaglandin D_2, Enzymatic Synthesis, [^{3}H]PGD_2 Derivatives, L-Selectride*

INTRODUCTION

Tritium incorporation into eicosapolyenoic acids by hydrogenation of their acetylene precursors and further conversion of labelled acids by using different enzyme systems provides a universal scheme for synthesing high molar radioactivity eicosanoids. A number of such studies have been carried out in our laboratory [1,2,3]. E-type prostaglandins (PG's) provided a single precursor for the synthesis of A-, B- and F-type prostaglandins, prostacycline and 6-keto-$PGF_{1\alpha}$.

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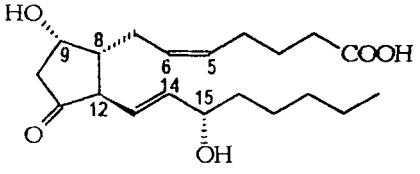
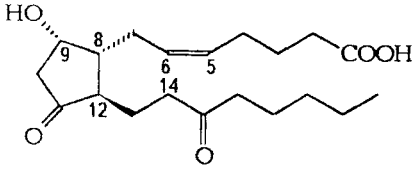
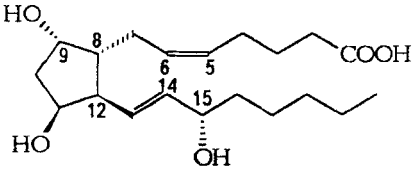
This study was devoted to the elaboration of a one stage synthesis method for another key prostaglandin [^{3}H]PGD_2, 1, obtainable from tritium-labelled arachidonic acid (AA), and its conversion to other prostaglandins. Different ways of PGD_2 metabolism as well as its biological role and mechanisms of action are currently of great interest [4].



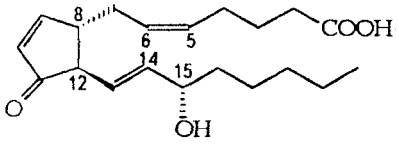
[5,6,8,9,12,14,15(n)-^{3}H] PGD_2 1



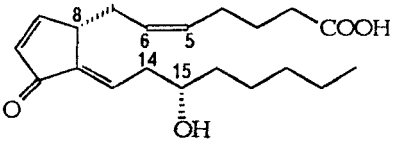
15-keto-13,14-dihydro-[^{3}H]PGD_2 2



9α,11β-[^{3}H]PGF_2 3



9-deoxy-Δ^9-[^{3}H]PGD_2 ([^{3}H]PGJ_2) 4



Δ^{12}-13,14-dihydro-[^{3}H]PGJ_2 5

RESULTS AND DISCUSSION

1 was prepared from both [^{3}H]AA and its cyclooxygenase product [^{3}H]PGH_2 (Fig. 1). The conversion of [^{3}H]PGH_2 by using a purified preparation of PGH-PGD-isomerase allowed a higher yield of 1 than in the case of crude enzyme [5]. 1 formed also during a long incubation of [^{3}H]AA in the presence of PGH-synthetase or as a result of non enzymatic transformation of [^{3}H]PGH_2.

In the case of 1 synthesis from [^{3}H]AA the yield of 1 was diminished due to the PGH-PGD isomerase inhibition by AA and the sorption of AA on ballast proteins (Fig. 1). The influence of these factors were reduced by successive addition of enzymes to the reaction mixture (Fig. 2). A 1.5-fold increase in the yield of 1 was observed at 1.5-2min intervals between successive additions of enzymes.

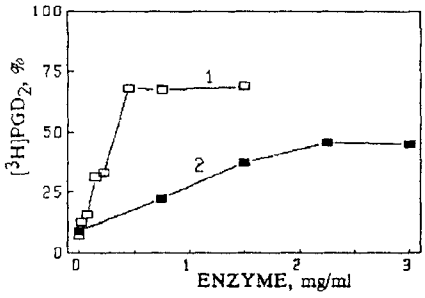


Fig. 1

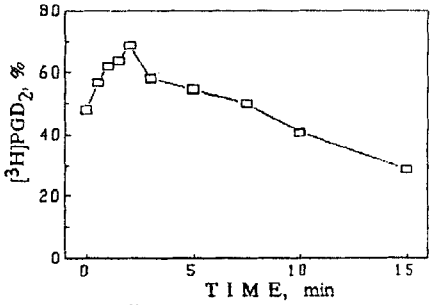


Fig. 2

Fig. 1 [^{3}H]PGD_2 synthesis by the incubation of PGH/PGD-isomerase with [^{3}H]AA - 50μM (curve 1) or [^{3}H]PGH_2 - 10μM (curve 2).

Fig. 2 [^{3}H]PGD_2 yield dependence on time intervals between successive addition of PGH-synthetase and PGH-PGD-isomerase into the reaction mixture with [^{3}H]AA (50μM).

The decrease in the yield of 1 at higher time intervals was due to the degradation of [^{3}H]PGH_2.

The isomeriation of PGH_2 in the presence of defatted serum albumin shifts towards PGD_2 formation [6]. However, the use of albumin appeared to be ineffective in the synthesis of 1 directly from [^{3}H]AA (data not shown).

Various labelled prostaglandins of natural configuration were synthesized through the intermediate 1 by using a number of enzymatic and chemical reactions.

The conversion of 1 to 15-keto-13,14-dihydro-[^{3}H]PGD_2, 2, was carried out by using enzyme preparation isolated from human platelets. This preparation possessed the NADP-dependent prostaglandin dehydrogenase activity at C15 position and the NADPH-dependent Δ^{13} -reductase activity towards 15-keto-PGD_2.

The 15-hydroxy-PG-dehydrogenase reaction was initiated by adding oxidized NADP to the reaction mixture containing 1 and the enzyme from platelets. The Δ^{13}-reductase reaction was stimulated by NADPH formed in the course of 1 dehydrogenation. Addition of the glucose-6-phosphate into the reaction mixture 1.5-2h after the start of the reaction, when the concentration of

Table 1. Synthesis of 9α,11β-[^{3}H]PGF_2 by chemical and enzymatic reduction of [^{3}H]PGD_2

Composition of reaction mixture	9α,11β-[^{3}H]PGF_2,%	Ratio of 11α/11β-isomers of [^{3}H]PGF_2
[^{3}H]PGD_2/$NaBH_4$	16	83:17
[^{3}H]PGD_2/L-Selectride	32	51:49
[^{3}H]PGD_2/Ketoreductase	88	0:100

15-keto-[^{3}H]PGD_2 was maximal, led to a quicker reduction of NADP and acceleration of the Δ^{13}-reductase reaction. No other product except 2 was found in the extract. Its yield was about 50% at high concentrations of the enzyme.

Thus, the presence of several enzymatic activities in the preparation isolated from human platelets made it possible to conduct one-stage synthesis of 2 and to prevent the accumulation of 1 degradation products. 9α,11β-[^{3}H]PGF_2, 3, was produced by enzymatic and chemical reduction of 1 (Table 1). The bulky reducer L-selectride appeared to be a more effective reagent than sodium borohydride ($NaBH_4$), in the production of 3. However, unlike [^{3}H]PGE_2 [1], 1 converted into a mixture of two hydroxy isomers of [^{3}H]PGF_2 in the presence of L-selectride. The use of liver enzyme preparation containing 11-PG-ketoreductase activity led to a practically complete conversion of 1 to 3. Changing the reaction mixture pH from 5 to 10 did not affect the yield of 3.

A.W.Neff et al. [7] described a method for producting 9α,11β-[11-^{3}H]PGF_2 by [^{3}H]PGD_2 reduction with sodium [^{3}H]-borohydride. However, the applications of 9α,11β-[11-^{3}H]PGF_2 were limited due to the low specific activity obtained.

9-Deoxy-Δ^9-PGD_2 (PGJ_2) was the main product of the long PGD_2 incubation in aqueous solutions [8]. Its formation is connected with the spontaneous dehydration reaction of PGD_2. It has been shown [9] that PGJ_2 is able to convert into other prostaglandins in the presence of albumins or blood plasma.

We studied the effect of temperature, pH, 1 concentration and incubation time on the yield of 9-deoxy-Δ^9-[^{3}H]PGD_2 ([^{3}H]PGJ_2) 4, during reaction in 0.1M potassium-phosphate buffer (Table 2). It was found that apart from the main reaction product, 4, the extract contained a minor component to be identified as Δ^{12}-13,14-dihydro-[^{3}H]PGJ_2 5. The ratio was independent on pH or 1 concentration and was about 9:1. The addition of human serum albumin into the reaction mixture led to an increase in the conversion degree of 1 and to a change of 4 and 5 ratio up to the 1:6.

As a result of chromatographic purification of the compounds produced from 1 their content in the final preparations was no less than 95%. The chromatographic characteristics of the labelled preparations obtained and corresponding non-labelled standards were identical.

Table 2. Synthesis of $[^3H]PGJ_2$ by dehydration of $[^3H]PGD_2$ in 0.1 M potassium-phosphate buffer (pH 7) during 12h

Incubation conditions			$[^3H]PGD_2$,%	$[^3H]PGJ_2$,%
$[^3H]PGD_2$ concentration, mg/ml	pH	t°C		
0.01	7	37	49	36
0.1	7	37	47.5(32)*	36(45)*
1	7	37	49	33
0.1	5	37	55.5	31.5
0.1	9	37	42	38
0.1	7	22	72	18
0.1	7	32	57	29

* Results obtained for a 36h incubation are given in brackets.

EXPERIMENTAL

Materials

L-Adrenaline and $NaBH_4$ were purchased from Serva Feinbiochemica GmbH & Co. Human serum albumin (essentially fatty acid-free), hemin, glucose-6-phosphate and PGD_2 were from Sigma Chemical Co. Arachidonic acid and glucose-6-phosphate dehydrogenase from yeast (250U/mg protein) were obtained from Fluka Chemie AG, NADP from E.Merk Darmstadt, L-Selectride (lithium tri-sec-butyl-borohydride) from Aldrich Chemical Co. and SEP PAC C_{18} cartridges from Millipore Associates. PGD_2 from the Institute of Chemistry, Tallinn, Estonia, was also used in this study. Ion exchange resins, DEAE-cellulose and P-cellulose were from Whatman Ltd. [5,6,8,9,11,12,14,15(n)-^{3}H]arachidonic acid (180Ci/mmol) was produced in our laboratory as previously described [1]. PGH_2 and [5,6,8,9,11,12,14,15(n)-^{3}H]PGH_2 (150Ci/mmol) were produced from AA and $[^3H]$AA by using PGF-synthetase according to Graff's method [10]. 15-keto-13,14-dihydro-PGD_2 and 9α,11β–PGF_2 were obtained by enzymatic synthesis from PGD_2 according to procedures described for corresponding labelled compounds. 9-Deoxy-Δ^9–PGD_2 (PGJ_2) and 9-deoxy-$\Delta^{9,12}$–13,14-dihydro-PGD_2 (Δ^{12}–13,14-dihydro-PGJ_2) were produced from PGD_2 by using well-known methods [9,11].

The purification of tritium-labelled prostaglandins was conducted by HPLC on a 3.3x150mm column with Separon C_{18}, 5μM on a Gilson chromatograph equipped with a flow through

radioactivity detector or on a 2x60mm column with Silasorb C_{18}, 5μM on a Milichrom chromatograph (Russia) with a multy-wave UV-detector. TLC analysis and purification of prostaglandins were carried out using precoated Kieselgel plates with F254 (Fluka Chemie AG) and Silica gel plates Silufol (Kavalier, Czechoslovakia). Radioactivity distribution along the plates was measured by a Berthold 2027 Scanner. Spectrophotometric measurements were taken on a UV/VIS Specord M40 spectrophotometer (Germany).

Enzyme preparations

Microsomes of sheep vesicular glands obtained according to the method of Van der Ouderaa et al. [12] were used as PGH-synthetase preparation (EC 1.14.99.1). PGH-PGD-isomerase (EC 5.3.99.2) was isolated from rat brain according to Urade et al. [13]. The production of enzymatic preparation included several procedures: preparation of the cytozole fraction (105000g of supernatant), fractionation by ammonium sulfate (0.3-0.7 saturation), dialysis, P-cellulose bathwise chromatography, DEAE-cellulose column chromatography. The preparation possessing 15-hydroxy-PG-dehydrogenase and 15-keto-PG-reductase activities was isolated from human platelets according to Watanabe et al. [14]. PGD-11-ketoreductase was isolated from rabbit liver by the method of Wong [15].

[5,6,8,9,12,14,15(n)-^{3}H] PGD_2, 1

55mCi of [5,6,8,9,11,12,14,15(n)-^{3}H] AA (180Ci/mmol) in 11ml of ethanol was placed in a 100ml cone flask. After removal of the solvent 1ml of 0.2M Tris-HCl (pH 8), L-adrenalin (8nmol), hemin (1.5pmol) were added as well as water to a volume of 3.4ml. After 5min of shaking at 25^{O}C, the reaction was initiated by adding a suspension of sheep vesicular gland microsomes (0.6ml, 1.25mg, 3units of PGH-synthetase activity). After 1.5 min. of incubation 13ml of PGH-PGD-isomerase solution (20mg, 4U) in 0.13M Tris-HCl (pH 9) containing dithiotreithol (1.3mM) was rapidly introduced into the vessel. The incubation was carried out during 90min at 20^{O}C with continuous stirring. The reaction mixture was acidified with HCl to pH 3, and extraction of prostaglandins was conducted with the help of SEP PAK C_{16} cartridges as described by Powell [16]. According to TLC data for system A, chloroform/methanol/acetic acid, 90:9:1 (v/v/v), the content of 1 in the extract was 52%. After HPLC purification (acetonitrile/water/acetic acid, 30:70:0.1 (v/v/v)) 13.4mCi of 1 of 96% purity and molar radioactivity of 120Ci/mmol was obtained.

15-Keto-13,14-dihydro-[^{3}H]PGD_2, 2

Upon removal of solvent, 1.5ml of 0.2M Tris-HCl (pH9) containing dithiotreitol (1mM), aqueous solution of NADP (0.6mmol) and enzyme preparation from platelets containing 15-hydroxy-PG-dehydrogenase (3mU) and 15-keto-PG-Δ^{13}-reductase (4.3mU) activities was added to 3mCi of

1 (120Ci/mmol). The volume of reaction mixture after addition of enzyme was 3ml. After 1.5h incubation at 32°C with continuous stirring, an aqueous solution of glucose-6-phosphate (15 mol) was added to the reaction mixture. After 30min. of incubation, the reaction mixture was acidified with citric acid to pH3, and then the prostaglandin was extracted with ethylacetate (3x10ml). According to TLC data for system B, benzene/ethylacetate/acetic acid, 50:50:2 (v/v/v), about 45% of the total radioactivity in the extract belonged to 2. Purification was carried out by using TLC in system B. R_f values of 2 and prostaglandins B_2 and D_2 (standards) in system B were 0.48, 0.38 and 0.19, respectively.

9α,11β-[^{3}H]PGF$_2$, 3

2mCi of 2 (120Ci/mmol) in 2ml of ethanol were placed in a cone flask. After removal of the solvent, the reaction was initiated by adding 1ml of PGD-11-ketoreductase solution (6.7mg, 3.7mU) in 0.1M potassium-phosphate buffer (pH 7) containing NADP (0.5μmol), glucose-6-phosphate (5μmol) and glucose-6-phosphate dehydrogenase (0.25U). Incubation was carried out during 2h at 32°C with constant stirring of the reaction mixture. The prostaglandin was extracted as described previously. The yield of 3 was no less than 85% according to TLC results in system C, chloroform/methanol/acetic acid, 85:14:1 (v/v/v). Purification was conducted by using HPLC in the solvent system acetonitrile/water/acetic acid, 33:67:0.1 (v/v/v). Rf values of 3 and prostaglandins $F_{2\alpha}$, E_2 and D_2 (standards) in system C were 0.46, 0.5, 0.63 and 0.68 respectively.

9-Deoxy-Δ^9-[^{3}H]PGD$_2$ ([^{3}H]PGJ$_2$), 4

0.5ml of 0.1M potassium-phosphate buffer (pH7) was added to 2.5mCi of 1 (120Ci/mmol) upon removal of solvent, and incubated with continuous stirring during 24h at 37°C. Prostaglandin extraction was performed with ethylacetate (3x5ml) at pH 3. According to TLC data for system B, the content of 4 in the extract was 40%. Purification was conducted by using TLC in system B.

Δ^{12}-13,14-Dihydro-[^{3}H]-PGJ$_2$, 5

The reaction conditions with 1 (2.5mCi) were the same as for the 4 preparation, except that the reaction mixture contained human serum albumin (5mg/ml). Extraction from reaction mixture, analysis and purification of 5 was conducted in the same way as in the case of 4. The 5 yield according to radioactivity was about 50%. R_f values of 4, 5 and prostaglandins B_2 and D_2 (standards) in system B were 0.37, 0.43, 0.39 and 0.18 respectively.

Reduction of 1 by Na borohydride and L-selectride

Treatment of 1 with sodium borohydride and L-selectride was conducted as previously described for reactions with [^{3}H]PGE$_2$ [1,17].

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