A QUANTITATIVE STEREOSELECTIVE SYNTHESIS OF RADIOLABELLED $PGF_{2\alpha}$ FROM ARACHIDONATE OR PGE_2

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SUMMARY

A simple, efficient and relatively cheap procedure for synthesising radiolabelled $PGF_{2\alpha}$ from arachidonate or PGE_2 is described. The method exploits the stereospecific enzymatic reduction of PGE_2 by PGE_2-9-ketoreductase from rabbit renal cortex.

INTRODUCTION

Studies on the biology of prostaglandins have increased logarithmically following the implication of these acidic lipids in control mechanisms for the cardiovascular system, the respiratory system, gastro-intestinal function, mechanisms of inflammation, fever, thrombosis and reproduction. The demand for radiolabelled prostaglandins has increased accordingly. Recently, we described a cytoplasmic NADPH-dependent PGE_2-9-ketoreductase which occurs in rabbit renal cortex and which has the capacity to quantitatively convert PGE_2 to $PGF_{2\alpha}$ (1). In this paper we report the utilisation of this reductase in a simple, efficient and relatively cheap procedure for synthesising radiolabelled $PGF_{2\alpha}$ from either arachidonate or PGE_2.

MATERIALS AND METHODS

Chloroform was redistilled before use, all other solvents were of Analar grade. Chromatography on 0·25 mm thin-layers of Kieselgel 60 (Merck) utilised the following solvent systems: 1% (v/v) formic acid in ethyl acetate, System 1; ethyl acetate-acetone-acetic acid (90 : 10 : 1, v/v/v), System 2; ethyl acetate-methanol-acetic acid (100 : 10 : 1, v/v/v), System 3.

Preparation of Enzymes

Prostaglandin synthetase was prepared from homogenates of ovine seminal vesicles by precipitation with citric acid followed by extraction with acetone and pentane as previously described (2). Prostaglandin-E_2-9-ketoreductase was prepared from female 6 - 8 week old Himalayan rabbits. Animals were killed by cervical dislocation, the kidneys were removed and the renal cortex obtained by dissection. This material was cut into small pieces and homogenised in 3 volumes of 0·1M potassium phosphate buffer pH 7·3, containing 4 mM $MgCl_2$ and 0·1 mM dithiothreitol. The homogenate was centrifuged at 10,000 x g for 20 min and the supernatant re-centrifuged at 100,000 x g for 60 min. The precipitate was discarded and the supernatant, which contained 20 mg protein/ml, was stored at - 17° until required. All operations concerning the preparation of this enzyme were carried out at 0 - 4°.

Preparation of [1-^{14}C]-$PGF_{2\alpha}$ from [1-^{14}C]-arachidonic acid

The enzyme preparations utilised in this synthesis were added to incubation mixtures in excess in order to ensure rapid and efficient conversions of substrate to product. Similar percentage yields may be obtained with larger amounts of high specific activity substrates without using proportionally greater amounts of enzymes. Silanised or siliconised glassware was used throughout.

15 mg of acetone-pentane powder were homogenised in a Potter-Elvjhem homogeniser with 0·2 ml 0·1M Tris-HCl buffer, pH 8·2 and 0·1 ml of 25 mM L-epinephrine (Sigma (London) Chemical Co. Ltd.). The mixture was incubated at 30° for 5 min before the addition of 0·2 ml of arachidonic acid solution containing 5 µCi [1-^{14}C]-arachidonic acid (54 mCi/mmol, The Radiochemical Centre, Amersham, Bucks.) dissolved in an anti-oxidant solution consisting of 0·01 ml benzyl-alcohol, 4 mg disodiumethylenediaminetetra-acetic acid, 2 mg propylgallate, 2 mg butylatedhydroxytoluene and 40 mg sodium metabisulphite per ml of solution adjusted to pH 9·8 with NaOH. Tris-HCl buffer (4 ml, of a 0·1 M solution pH 8·2), and a solution in buffer of reduced glutathione (0·2 ml, 2 mg) were added before the reaction was incubated at 30° for 30 min.

Half of the reaction mixture (2·25 ml) was transferred to a 15 ml centrifuge tube containing 1 ml of a 2 M citric acid, 3·3 M NaCl solution, and thoroughly mixed. The mixture was extracted four times each with 2 ml of ethyl acetate. The organic phase which separated on centrifugation was combined (Sample A).

To the remaining 2·25 ml of reaction mixture the following were added: glucose-6-phosphate (Boehringer, 9·1 mg), glucose-6-phosphate dehydrogenase (Sigma, 6·5 Units), NADPH (Boehringer 0·25 mg), $MgCl_2$ (2·46 mg), dithiothreitol (BDH, 46·7 μg), 0·5 ml of the 100,000 x g supernatant from rabbit renal cortex, and the volume was adjusted to 3·0 ml with Tris-HCl buffer 0·1 M pH 8·2. The reaction was incubated for a further 90 min at 37° after which it was acidified with citric acid, NaCl solution and extracted with ethyl acetate as before to give Sample B.

Approximately 10 μl of Sample A and Sample B were each subjected to chromatography using system 1.

Purification of radioactive products

Sample A and Sample B were each purified as follows.

The organic extract was evaporated to dryness under a stream of oxygen-free nitrogen and applied, redissolved in 2 x 0·2 ml 1% (v/v) methanol/chloroform to a 5 cm x 0·4 cm column of silicic acid (Sil-A-200, Sigma) which had been previously washed with 40% (v/v) methanol/chloroform followed by chloroform. The column was packed in a pasteur pipette with chloroform and eluted with increasing concentrations of methanol. Fractions were collected as follows:

Sample A: 6 ml $CHCl_3$, 6 ml 1% (v/v) methanol in chloroform, six 1·5 ml fractions of 2·5%, 1·5 ml of 8% and 4·5 ml of 8%.

Sample B: 6 ml $CHCl_3$, 6 ml 1%, 4·5 ml 2·5%, 1·5 ml 2·5%, and four 1·5 ml fractions of 8%.

RESULTS

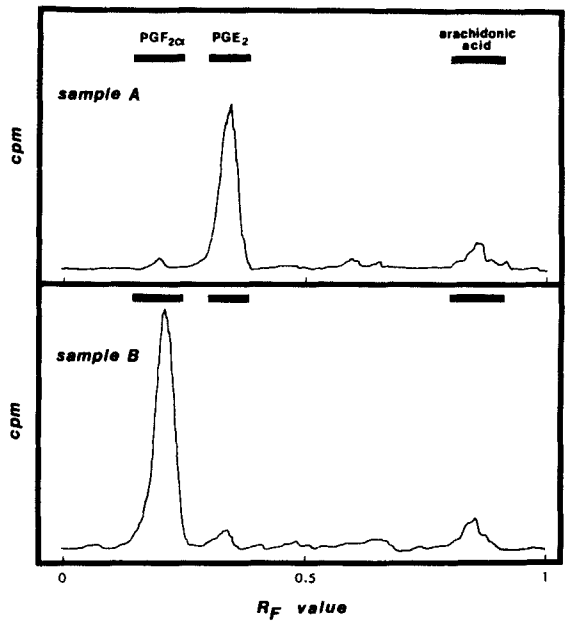
Products of prostaglandin synthetase

Incubation of prostaglandin synthetase from ovine seminal vesicles with arachidonate in the presence of reduced glutathione produces PGE_2 as the major product (3). Thin-layer chromatography with three different solvent systems of a portion of Sample A confirmed that the major peak of radioactivity (75% of the total radioactivity) co-chromatographed with authentic PGE_2, lesser amounts of radioactivity chromatographed with $PGF_{2\alpha}$ (4%) and arachidonate (12%). Products less polar than PGE_2 accounted for 9% of the radioactivity (Fig. 1A).

Products of PGE_2-9-ketoreductase

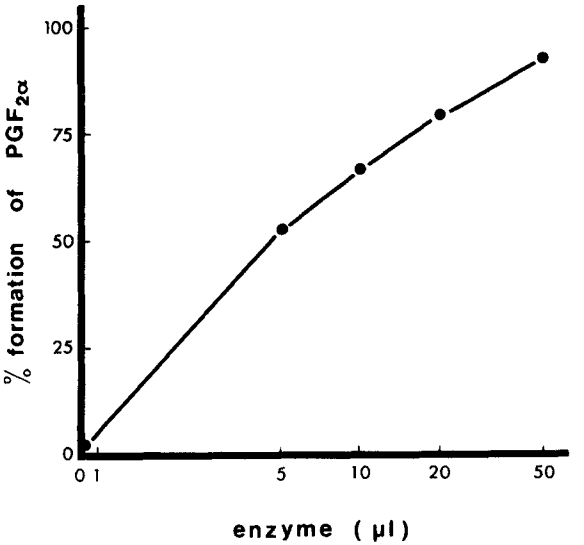
Chromatography of a portion of Sample B in system 1 identified the major radioactive component (77% of the total radioactivity) of the reaction mixture as PGF_2. Arachidonic acid accounted for 11%, PGE_2 for 5% and other products for 7% of the recovered radioactivity (Fig. 1B). From these data it may be concluded that 93 - 95% of the PGE_2 synthesised by the seminal vesicle enzyme was converted to PGF_2 by the renal

FIGURE 1 Enzymatic synthesis of (1-^{14}C)-PGE_2 and (1-^{14}C)-$PGF_{2\alpha}$ from (1-^{14}C)-Arachidonic acid



Samples were chromatographed on Kieselgel 60 with 1%(v/v) formic acid in ethyl acetate as solvent.

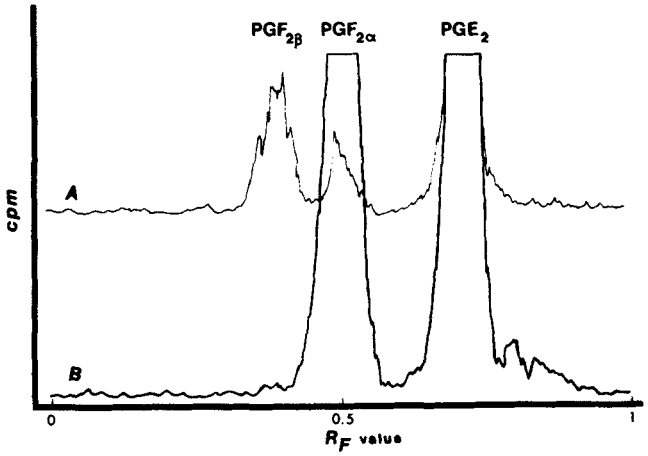
FIGURE 2 Formation of (1-^{14}C)-$PGF_{2\alpha}$ from (1-^{14}C)-PGE_2



PGE_2 (4µg) was reacted with enzyme and NADPH (10^{-4}M) for 40min in a volume of 0.25ml.

enzyme. This result agrees with incubations on a smaller scale in which purified [1-^{14}C]-PGE_2 was converted quantitatively and exclusively to $PGF_{2\alpha}$ by PGE_2-9-ketoreductase (Fig. 2). Chromatography of this radiolabelled product in system 2 followed by redevelopment of the chromatogram in system 3 (4) (with $PGF_{2\alpha}$ and $PGF_{2\beta}$, prepared by the action of $NaBH_4$ on [1-^{14}C]-PGE_2 (5), as reference compounds) resulted in a single radioactive peak which corresponded to the $F_{2\alpha}$ isomer (Fig. 3). When the rate of enzymatic production of $PGF_{2\alpha}$ was monitored by radioimmunoassay using antibodies specific for $PGF_{2\alpha}$ and by measurement of radioactivity following chromatography, the data coincided (1). These results support the chromatographic evidence for the identity of the product as $PGF_{2\alpha}$.

FIGURE 3 **Stereospecific reduction of PGE_2**



A Partial reduction of PGE_2 with $NaBH_4$

B Partial reduction of PGE_2 with renal PGE_2-9-ketoreductase

Column chromatography

Preliminary experiments showed that arachidonic acid, PGE_2 and $PGF_{2\alpha}$ may be efficiently separated on short columns of silicic acid with mixtures of $CHCl_3$ and MeOH. The particular type of silicic acid used in the procedure described above resulted in no apparent dehydration of PGE_2 to PGA_2. Recovery of radioactivity from the column was routinely 90 - 100%. Elution profiles for Samples A and B are shown in Table 1. Thin-layer chromatography of the column fractions confirmed the product separation. Fraction E1 plus E2 (Table 1) contained PGE_2 with a radiopurity of > 93% and represented an overall yield of 62%. Fraction F1 plus F2 (Table 1) contained $PGF_{2\alpha}$ with a radiopurity of > 98% and represented an overall yield of 58%.

TABLE 1. Purification of Radioactive PGE_2 and $PGF_{2\alpha}$ by Chromatography on Silicic Acid

Eluent	Sample A* (4.97×10^6 dpm)		Sample B* (4.96×10^6 dpm)	
	dpm $\times 10^3$	%	dpm $\times 10^3$	%
6 ml $CHCl_3$	574	11.7	594	12.9
6 ml 1% MeOH/$CHCl_3$	170	3.5	171	3.7
1.5 ml 2.5% MeOH/$CHCl_3$	206	82.9	418	12.6
"	2169 (E1)			
"	1353 (E2)			
"	197		163	
"	55			
"	70			
1.5 ml 8% MeOH/$CHCl_3$	80	1.9	1601 (F1)	70.7
"	14		1555 (F2)	
"			63	
"			51	
Recovery	4.89×10^6 dpm (98%)		4.62×10^6 dpm (93%)	

* Organic extracts obtained after incubating [1-^{14}C]-arachidonic acid with either prostaglandin synthetase (Sample A) or prostaglandin synthetase followed by PGE_2-9-ketoreductase (Sample B).

TABLE 2

Yields and Recoveries of [1-^{14}C]-PGE_2 and [1-^{14}C]-$PGF_{2\alpha}$ Synthesised from [1-^{14}C]-arachidonate

Analysis of Sample A*

% arachidonate converted to PGE_2 (measured by TLC)	75%
% of total radioactivity extracted from incubation A	90%
% recovery of radioactivity from column chromatograph	98%
Calculated yield of [1-^{14}C]-PGE_2	67%
Overall yield of radioactivity in fractions E1 + E2	66%
Overall yield of [1-^{14}C]-PGE_2 in fractions E1 + E2 = 66·4 x 0·93	= 62% (1·55 μCi)

Analysis of Sample B*

% of arachdionate converted to $PGF_{2\alpha}$ (measured by TLC)	77%
% of total radioactivity extracted from incubation B	90%
% recovery of radioactivity from column chromatograph	93%
Calculated yield of [1-^{14}C]-$PGF_{2\alpha}$	64%
Overall yield of radioactivity in fractions F1 + F2	59%
Overall yield of [1-^{14}C]-$PGF_{2\alpha}$ in fractions F1 + F2 = 59·4 x 0·98	= 58% (1·45 μCi)

Efficiency of the enzymatic reduction of [1-^{14}C]-PGE_2 to [1-^{14}C]-$PGF_{2\alpha}$	93 - 95%

* Organic extracts obtained after incubating [1-^{14}C]-arachidonic acid with either prostaglandin synthetase (Sample A) or prostaglandin synthetase followed by PGE_2-9-ketoreductase (Sample B).

Table 3. Potential Products Using Commercially Available Substrates

Substrate	Supplier	Specific Activity	Label	Product
Eicosatrienoic Acid	(1)	40 - 60 mCi/mmol	1-^{14}C	$PGF_{1\alpha}$
Eicosatrienoic Acid	(2)	5 - 20 mCi/mmol	1-^{14}C	$PGF_{1\alpha}$
PGE_1	(3)	40 - 60 mCi/mmol	2-^{14}C	$PGF_{1\alpha}$
PGE_1	(3)	> 60 Ci/mmol	5,6-^{3}H(N)	$PGF_{1\alpha}$
PGE_1	(1)	40 - 60 Ci/mmol	5,6-^{3}H(N)	$PGF_{1\alpha}$
Arachidonic Acid	(1)	> 50 mCi/mmol	1-^{14}C	$PGF_{2\alpha}$
Arachidonic Acid	(2)	5 - 20 mCi/mmol	1-^{14}C	$PGF_{2\alpha}$
Arachidonic Acid	(3)	60 - 100 Ci/mmol	5,6,8,9,11,12,14,15-^{3}H(N)	$PGF_{2\alpha}$
PGE_2	(3)	100 - 150 Ci/mmol	5,6,8,9,11,12,14,15-^{3}H(N)	$PGF_{2\alpha}$
PGE_2	(1)	> 100 Ci/mmol	5,6,8,9,11,12,14,15-^{3}H(N)	$PGF_{2\alpha}$

(1) The Radiochemical Centre, Amersham, Bucks, England.
(2) Field Instruments Co. Ltd., Richmond, Surrey, England.
(3) New England Nuclear, Boston, Mass. USA.

Efficiency of synthesis, extraction and purification

In Table 2 are shown the yields of product at various stages in the preparation of $PGF_{2\alpha}$. Each operation proceeds at greater than 90% efficiency with the exception of the initial synthesis of PGE_2. Unused radiolabelled arachidonic acid (approx. 12% of the original substrate) may be recovered from the initial column chromatographic fractions and recycled to improve the efficiency of this first step. When purified PGE_2 was used as substrate for PGE_2-9-ketoreductase overall yields of 93 - 98% were obtained.

DISCUSSION

The procedure described above has been routinely used in our laboratories to synthesise and purify [1-^{14}C]-PGE_2 and [1-^{14}C]-$PGF_{2\alpha}$ from [1-^{14}]-arachidonic acid. The method is fast, exceptionally efficient and produces prostaglandins with the "natural" geometry at each optically active centre. The absence of $PGF_{2\beta}$ formation considerably simplifies the purification of the product. Both the enzymes utilised in this synthesis of $PGF_{2\alpha}$ are stable at - 17° and may be stored at this temperature without loss of activity for many months.

When PGE_2 is the desired product from large scale preparations, gradient elution of the column chromatograph may be used to increase the separation of PGE_2 from minor side products. When $PGF_{2\alpha}$ is the desired product, stepwise elution is entirely satisfactory.

In principle the method could also be used to prepare radiolabelled $PGF_{1\alpha}$ and $PGF_{3\alpha}$ from eicosatrienoic acid and eicosapentaenoic acid respectively.

Currently all commercially available radioactive prostaglandins with the exception of [2-^{14}C]-PGE_1 are labelled with tritium. The above method has the advantage of utilising commercially available [1-^{14}C]-fatty acid substrates to prepare [1-^{14}C]-PGE_1, -PGE_2, -$PGF_{1\alpha}$, -$PGF_{2\alpha}$ (see Table 3). Furthermore as the enzymatic reduction, unlike that using borohydride, produces only the 9α isomer, $PGF_{1\alpha}$ and $PGF_{2\alpha}$ may be prepared from radiolabelled PGE_1 and PGE_2 with yields approaching 100%.

The efficiency with which the renal enzyme converts PGE to PGF_α might also be exploited in certain reaction schemes in which unlabelled PGF_α is synthesised via a PGE derivative. For example, the syntheses of $PGF_{2\alpha}$ from (15R)- and (15S)-PGA_2 esters, which are readily obtainable from the gorgonian Plexaura homomalla, have been described (6). The syntheses involve epoxidisation of the PGA_2 derivative to a mixture of isomeric 10,11-epoxides which are reduced to a mixture of isomeric 11-hydroxy PGE_2-esters. These esters are separated by chromatography, and the 11α isomer is reduced to the $PGF_{2\alpha}$ derivative with borohydride and hydrolysed. Use of the renal PGE_2-9-ketoreductase would not only improve the synthesis of the 9α isomer, but might also eliminate the necessity to separate the 11-isomers before reduction of the 9-keto function.

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