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HEPATIC METABOLISM OF PROSTACYCLIN (PGI_2) IN THE RABBIT: FORMATION OF A POTENT NOVEL INHIBITOR OF PLATELET AGGREGATION

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SUMMARY: Metabolism of [9-^{3}H]-PGI_2 was studied in the isolated Tyrode's perfused rabbit liver. Five products, four radioactive and one non-radioactive, were identified in the perfusate: 19-hydroxy-6-keto-$PGF_{1\alpha}$, 6-keto-$PGF_{1\alpha}$, dinor-6-keto-$PGF_{1\alpha}$, pentanor $PGF_{1\alpha}$ and a 6-keto-PGE_1-like substance. The first two, 19-hydroxy-6-keto-$PGF_{1\alpha}$ and 6-keto-$PGF_{1\alpha}$, represented 5% and 45% respectively, of the total radioactivity; the last two accounted for 39%. The presence of dinor and pentanor derivatives of 6-keto-$PGF_{1\alpha}$ indicated that β-oxidation and oxidative-decarboxylation occurs in the liver as the major metabolic pathway of PGI_2. One non-radioactive metabolite which co-migrated with authentic 6-keto-PGE_1 was found to inhibit platelet aggregation, having a potency similar to authentic 6-keto-PGE_1, and its effect can be eliminated by boiling and by alkali treatment. This metabolite, having similar Rf value on TLC and biological behavior as 6-keto-PGE_1, may arise from oxidation of 6-keto-$PGF_{1\alpha}$ via the 9-hydroxyprostaglandin dehydrogenase pathway, as suggested by recovery of tritiated water in the aqueous phase of the perfusate. This material, a potent inhibitor of platelet aggregation, may arise from PGI_2 or its hydrolysis product, 6-keto-$PGF_{1\alpha}$.

INTRODUCTION: Prostacyclin was suggested to be a circulating hormone as it is generated continuously in the lung (1,2). We have demonstrated that less than 15% is metabolized during one passage across the lungs (3); this is probably due to the low affinity of PGI_2 for the transport system of the lung as PGI_2 is rapidly metabolized by the major pulmonary degradative enzyme, 15-hydroxyprostaglandin dehydrogenase (15-OH-PGDH), in a cell-free preparation (3,4).

In this report we have examined the metabolic fate of [9-^{3}H]-PGI_2 in the isolated perfused liver and have identified, in addition to 19-hydroxy-6-keto-$PGF_{1\alpha}$, dinor, and pentanor metabolites of PGI_2, one non-radioactive metabolite having chrom-

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Abbreviations: PGI_2, Prostacyclin; 6-keto-$PGF_{1\alpha}$, 6-ketoprostaglandin $F_{1\alpha}$; 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketoprostaglandin $F_{1\alpha}$; TLC, thin-layer chromatography Me_3Si, trimethylsilyl; MeMOTMSI, methyl-oxime trimethylsilyl; GC-MS, Gas-chromatography mass-spectometry.

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atographic and biological properties of 6-keto-PGE_1. The latter material was relatively stable and was equipotent to PGI_2 as an inhibitor of platelet aggregation (5). Axen et al. (6) first reported that 6-keto-PGE_1 has a potency similar to PGI_2 in inhibiting platelet aggregation. Quilley et al. (7) have demonstrated in the rat that 6-keto-PGE_1, like PGI_2, escapes significant pulmonary metabolism.

MATERIALS AND METHODS: Radiolabeled prostacyclin methyl ester (PGI_2-Me) was synthesized chemically from [9-^{3}H]-$PGF_{2\alpha}$ Me ester (10 Ci/mmole, New England Nuclear, Boston, Mass.) (8). Purity of [9-^{3}H]-PGI_2 Me was established by TLC and GC-MS as previously described (3). The methyl ester of [9-^{3}H]-PGI_2 was converted to the PGI_2 sodium salt the day before use by mild alkaline hydrolysis (9) and diluted with authentic prostacyclin sodium salt (Upjohn) to a specific activity of 1.8 uCi/mmole.

Liver Perfusion: Male New Zealand rabbits were anesthetized with sodium pentobarbital (30 mg/kg i.v.). After mid-line laparotomy, the liver was exposed. The portal vein was cannulated and the liver was flushed with Tyrode's solution, removed from the animal and placed in a thermostatically controlled chamber. The liver was perfused through the portal vein with oxygenated Tyrode's solution (37°C) at a rate of 10 ml/min using a Harvard peristaltic pump, as previously described (10). [9-^{3}H]-PGI_2 (1.8 uCi/mM) was infused into the liver through the cannula inflow for 5 min. Effluents were collected during administration of [9-^{3}H]-PGI_2 and thereafter for an additional 25 min. After recirculating twice, the final perfusate was collected in a container immersed in ice, acidified with 1 N HCl to pH 3.0 and extracted three times with an equal volume of ethyl acetate. Extracts were combined and evaporated to dryness.

Thin-Layer and Radiometric Gas-Chromatography: The dry crude lipid extract was dissolved in a chloroform/methanol/water mixture (86/14/1, v/v/v) and passed through a Sephadex G-25 column (1 x 12 cm) to remove non-lipid contaminants (11). The lipid material was then eluted from the Sephadex G-25 column with the same solvent and dried under N_2. The residue was dissolved in 1 ml of dry acetone; aliquots of 200 ul of the extracts were applied to TLC plates (Brinkman) and co-migrated with authentic 6-keto-$PGF_{1\alpha}$, 6,15-diketo-$PGF_{1\alpha}$, 6-keto-PGE_1, pentanor (γ-lactone), PGE_2 and $PGF_{2\alpha}$ standards (Upjohn). The plates were developed twice with A): iso-octane/ethyl acetate/acetic acid/water (25/55/10/50, v/v) B): benzene: deoxane: acetic acid (60:30:3, v/v) (3, 14). Radioactive products were detected with a Packard radiochromatogram scanner, Model 7230. Zones corresponding to 6,15-diketo-$PGF_{1\alpha}$, 6,15-diketo-13, 14-dihydro-$PGF_{1\alpha}$, 6-keto-PGE_1, and 6-keto-$PGF_{1\alpha}$ (Rf value in solvent A: 0.41, 0.47, 0.36 and 0.23 respectively) were scraped from the TLC plate and extracted with methanol. One portion of the resulting lipid extracts was methyl esterified with diazomethane in ether: methanol (9:1) followed by oxime synthesis with methoxyamine HCl in pyridine (5 mg/ml). Finally, oxime esters were converted to trimethylsilyl (TMSi) ethers by treatment with bis-trimethylsilyl trifluoroacetamide (BSTFA) to form methyloxime trimethylsilyl (MOTMSi) derivatives before analysis with radiometric GC and GC-MS. Portions of the residue were also converted to the TMSi ester, methyloxime, TMSi ether (TMSiMOTMSi) derivatives with methoxyamine HCl and BSTFA containing 1% trimethylchlorosilane (37°C for 2 min). Reagent was removed by evaporation under a stream of nitrogen and the residue dissolved in a small aliquot of acetone before injection. GC was carried out on a Varian 2700 GC coupled to a Packard 894 radioactivity detector for simultaneous recording of mass and radioactivity. GC-MS analyses were carried out with a LKB-9000 mass spectrometer. The 6 ft. 1%-SE-30 on Chromosorb-W (HP) column was kept at 210°C, the flash-heater at 240°C and the separator at 250°C. Electron energy was 22.5 eV.

Bioassay for 6-Keto-PGE_1: The zone corresponding to 6-keto-PGE_1 on TLC plates was collected as described above and dried under N_2. The residue was resuspended in

either 0.9% saline or Tris buffer (pH 8.8, 50mM) and tested for 6-keto-PGE_1-like activity on Kreb's superfused rat stomach strips (RSS) and bovine coronary arteries (BCA) as described previously (12).

Platelet Aggregation: Blood was drawn from volunteers who had not taken aspirin or other drugs for the preceding 10 days. Nine parts of whole blood were mixed with 1 part of 3.8% sodium citrate to a total volume of 5 ml in a plastic tube. The platelet-rich plasma (PRP) was removed with a siliconized pipette after centrifugation of the blood at 150 g for 10 min. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining blood at 12,000 g for 10 min. The final platelet count in PRP was adjusted to 2 x 10^8/ul with PPP. Platelet aggregation studies were performed with 0.5 ml aliquots of PRP stirred at 1,200 rpm at 37° in a dual channel Payton aggregation module and transcribed on a linear recorder (Payton Associates, Buffalo, NY). The concentration of 6-keto-PGE_1, which inhibited platelet aggregation, was determined for each batch of PRP and was used throughout the experiments to inhibit aggregation induced by ADP (6 uM). 6-keto-PGE_1 was stored in dry acetone (1 mg/ml) at -25° and diluted with Tris buffer (50 mM, pH 8.8) before use. The residue of 6-keto-PGE_1 zone was resuspended in Tris buffer (50 mM, pH 8.8) and diluted to the required concentration prior to use with the same buffer.

RESULTS AND DISCUSSION: The radiochromatogram scan of the extract of the liver perfusate revealed one major peak (I) and one minor radioactive peak (II) (Fig. 1, upper panel). The major product, peak I, had the same mobility as 6-keto-$PGF_{1\alpha}$; peak II was similar in mobility to three compounds: 6,15-diketo-$PGF_{1\alpha}$; 6,15-diketo-13, 14-dihydro-$PGF_{1\alpha}$; 9,11, 15-trihydroxy-pentanor-prosta-13-enoic acid and its γ -lactone (pentanor-$PGF_{1\alpha}$ γ-lactone) (13). Total radioactivity recovered in the liver perfusate was 96% of that injected. Of this, peak I represented 50% and peak II 39%. The remaining radioactivity was not extracted from the perfusate; after distillation it was found to be mainly tritiated water (7%, average of 5 distillation experiments from one typical perfusate).

Radiometric GC of the MOTMSi derivative of peak I revealed one major and one minor compound (compounds IA and IB) having a GC peak ratio of 10:1, which co-chromatographed with the corresponding derivatives of 6-keto-$PGF_{1\alpha}$ (45%) and 19-hydroxy-6-keto-$PGF_{1\alpha}$ (5%). After similar derivatization, GC resolved peak II into two major radioactive compounds (IIA, IIB) whose relative retention times corresponded to those of the MOTMSi derivative of dinor-6-keto-$PGF_{1\alpha}$ (compound IIA), and pentanor γ-lactone and free acid (compound IIB). The TMSi-ester derivative of compound IIB revealed two peaks which co-chromatographed with the corresponding derivatives of 9,11, 15-trihydroxy-pentanor-prosta-13-enoic acid and pentanor γ-lactone. Compound IA was identified as 6-keto-$PGF_{1\alpha}$ by GC-MS (9). The mass spectrum of the MOTMSi derivative

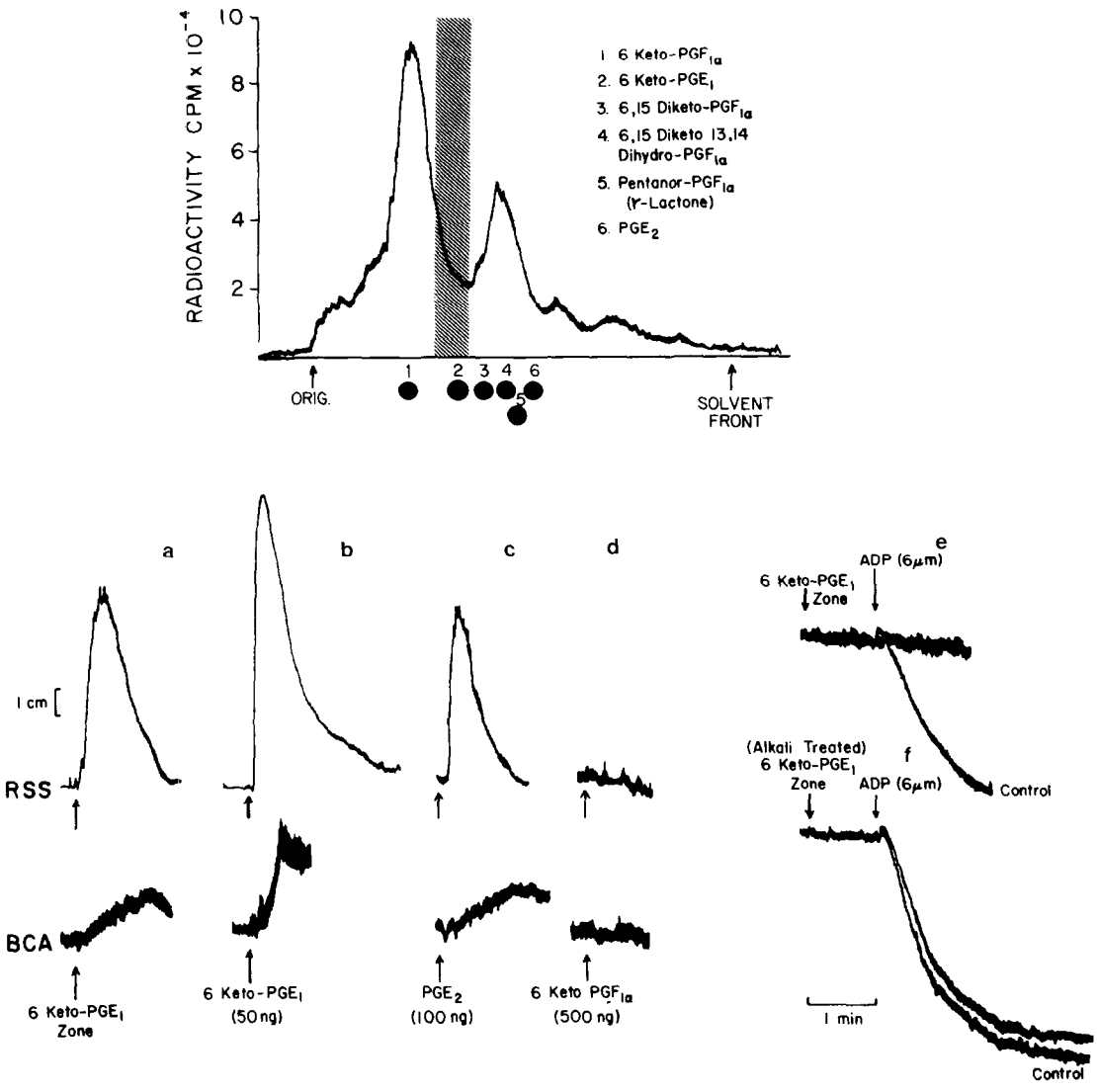

Fig. I (Upper Panel) Radiochromatography scan of radioactive products extracted from the rabbit liver perfusate after infusion of [9-^{3}H]-PGI_2. Radioactive metabolites were extracted, separated and identified as described under "Methods".

(Lower Panel) Biological activity of 6-keto-PGE_1-like substance isolated from TLC using system A. The 6-keto-PGE_1 zone was scraped and eluted from silica gel with chloroform / methanol (1/1, v/v), dried under N_2 and the residue was divided into two parts. One part was suspended in 0.9% saline and tested for 6-keto-PGE_1 like activity on rat-stomach strip (RSS) and bovine coronary arteries (BCA), Fig. 1 a, b, c, d,. The other part of the residue was suspended in 50 mM Tris buffer, pH 8.8, and tested for its effect on platelet aggregation, Fig. 1 e, f.

of compound IB (Fig. 2a) corroborated the structure of 19-hydroxy-6-keto-$PGF_{1\alpha}$. GC-MS of the MOTMSi derivative of compound IIA was identical to dinor-6-keto-$PGF_{1\alpha}$ (14),

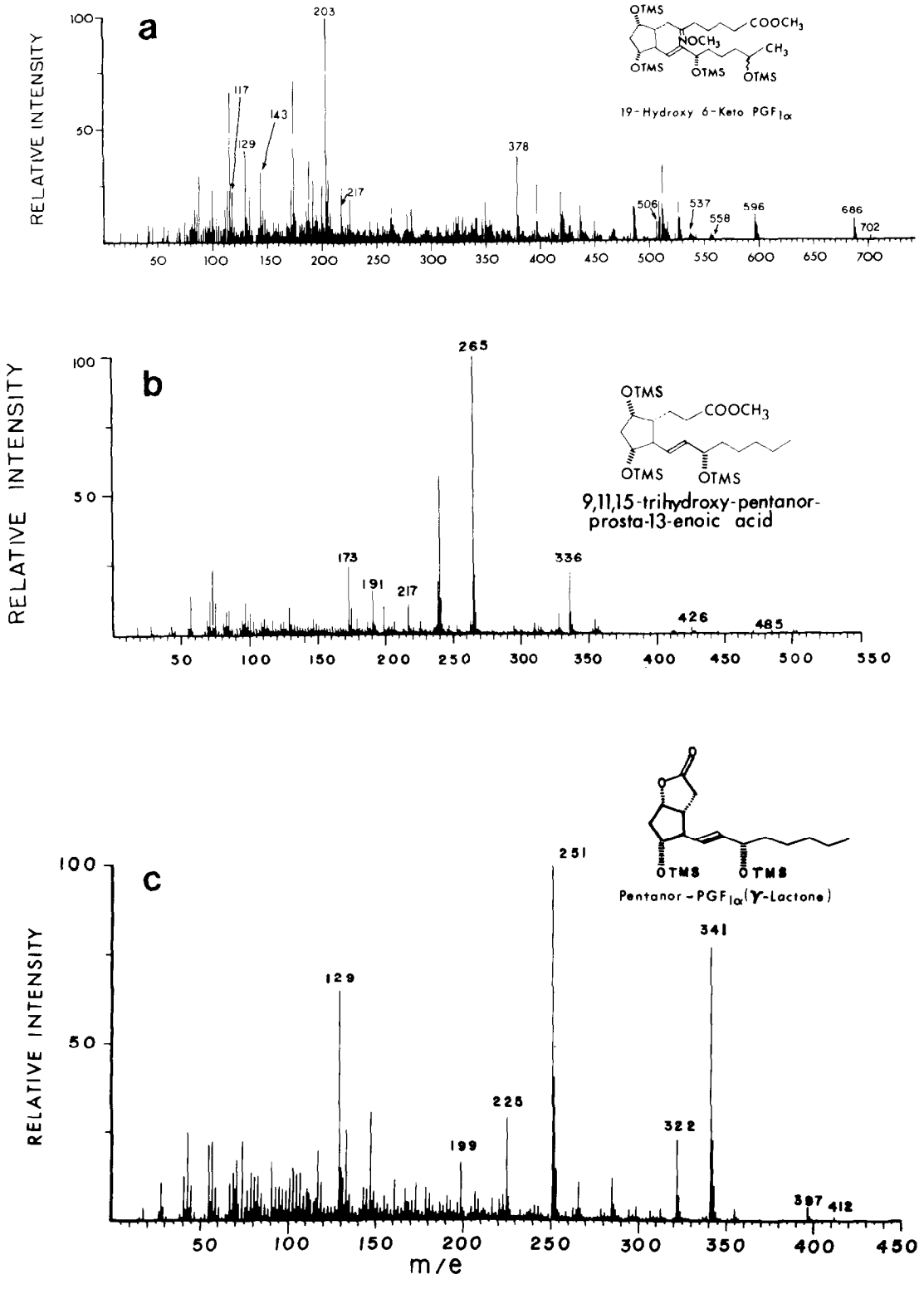
a
RELATIVE INTENSITY
19-Hydroxy 6-Keto PGF1α
b
9,11,15-trihydroxy-pentanor-prosta-13-enoic acid
c
Pentanor-PGF1α(γ-Lactone)
m/e

Figure II.

whereas compound IIB showed the major fragment ions in aggreement with the proposed structures of 9,11, 15-trihydroxy-pentanor-prosta-13-enoic acid (Fig. 2b) and its γ-lactone derivative (Fig. 2c). Confirmation of the structure was obtained by comparison with authentic standards.

About 7% of the remaining radioactivity in the hepatic effluent contained tritiated water after distillation (5 experiments). The 6-keto-PGE_1 zone (Rf value 0.36, solvent system A) was scraped from TLC plates and eluted from the silica gel with chloroform/methanol (1/1, v/v), dried under N_2, and part of the residue suspended in 0.9% saline and tested for 6-keto-PGE_1-like activity on RSS and BCA (Fig. Ia, b, c, d, lower panel). The material in the 6-keto-PGE_1 zone contracted both the RSS and the BCA as did authentic 6-keto-PGE_1. In contrast, PGI_2 relaxed the BCA and produced relatively weak contraction of the RSS. Another part of the residue was suspended in 50 mM Tris buffer, pH 8.8, and tested for its effect on platelet aggregation. A 10 ul aliquot of the suspension, equivalent to approximately 2.5 ng of metabolite (assumed from 7% loss of tritium from the total [9-^{3}H]-PGI_2Na salt infused), inhibited platelet aggregation induced by ADP (6 mM) (Fig. Ie, lower panel); its effect was similar to that of the authentic 6-keto-PGE_1 standard (2.5 ng) (5). Alkali treatment abolished the platelet anti-aggregatory effect of this metabolite (Fig. If, lower panel), an effect similar to that of authentic 6-keto-PGE_1. Based on its chromatographic mobility, musculotropic and platelet anti-aggregatory activity, this substance was tentatively identified as 6-keto-PGE_1. However, material recovered from those TLC zones associated with the other four metabolites, the pentanor and dinor of 6-keto-$PGF_{1\alpha}$ and 19-hydroxy-6-keto-$PGF_{1\alpha}$, as well as their authentic standards, did not inhibit aggregation of human platelets and did not contract the bovine coronary artery and the rat stomach strip.

This study demonstrates that in the isolated perfused rabbit liver PGI_2 is metabolized to two major products by β-oxidation: dinor-6-keto-$PGF_{1\alpha}$ (20%) and pentanor-$PGF_{1\alpha}$ (19%), (total 39%) and two minor products: 19-hydroxy-6-keto-$PGF_{1\alpha}$ (5%), and a

Fig. II Mass spectrum of methyl-o-methyl-oxime trimethylsilyl ether derivative of 19-hydroxy-6-keto-$PGF_{1\alpha}$ (compound 1B, Fig. IIa upper panel); trimethylsilyl ether derivative of 9,11,15-trihydroxy-pentanor-prosta-13-enoic acid. (Compound IIB, Fig. IIb middle panel) and trimethylsilyl ether derivative of 2,6-pentanor-$PGF_{1\alpha}$ γ-lactone (compound IIC, Fig. IIC lower panel)

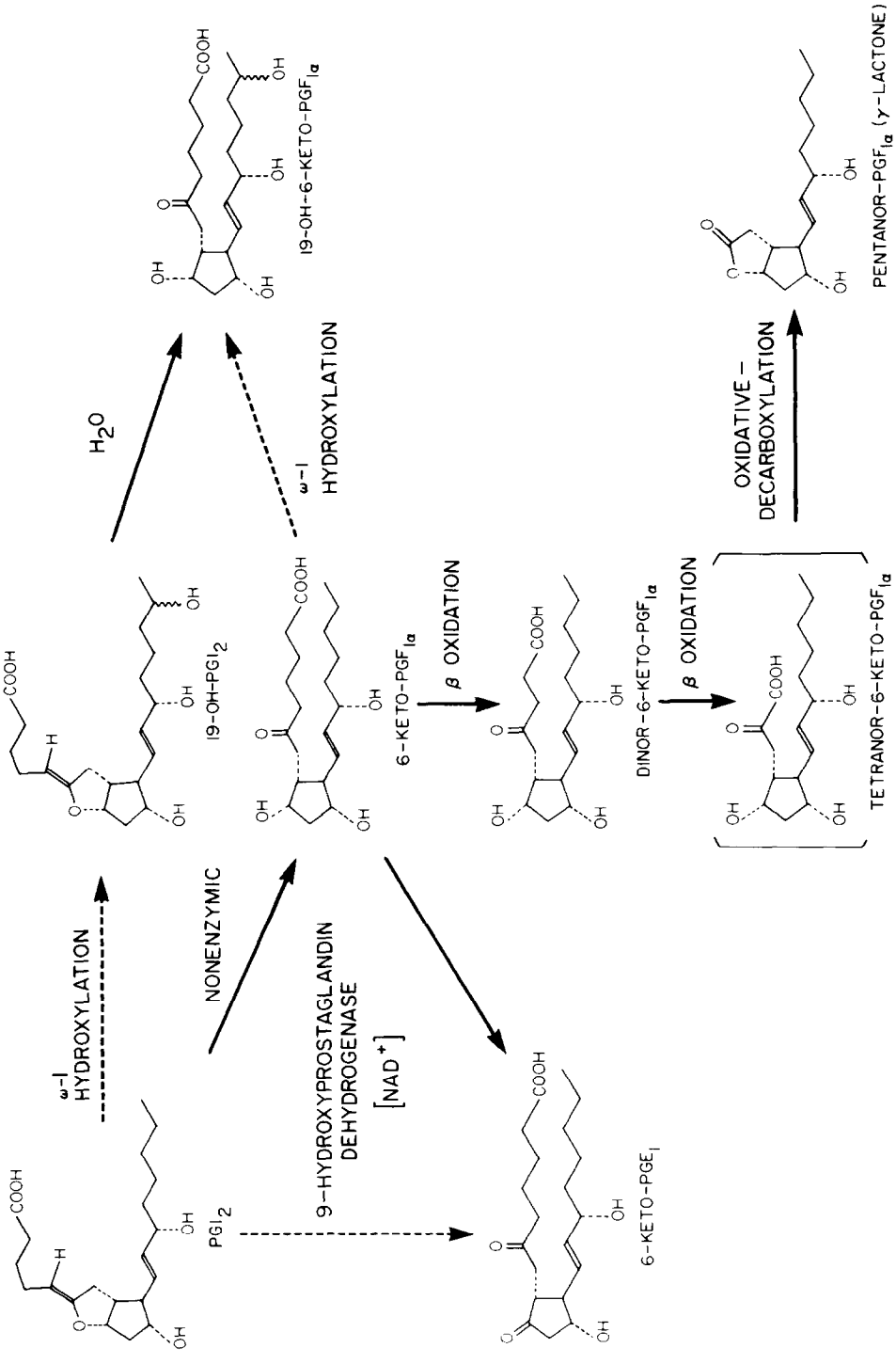

Fig. III Proposed metabolic pathways of prostacyclin (PGI_2) in the rabbit liver.

6-keto-PGE_1-like metabolite (7%). Almost one-half of the remainder represented the hydrolysis product of PGI_2, 6-keto-$PGF_{1\alpha}$ together with a small amount of 19-hydroxy-6-keto-$PGF_{1\alpha}$.

The results suggested that metabolism of prostacyclin in the liver involved 1) β-oxidation 2) β-oxidation followed by oxidative decarboxylation and 3) ω-1 hydroxylation of 6-keto-$PGF_{1\alpha}$ (Fig. 3). Due to the low 15-OH PGDH activity in the liver (15), 6,15-diketo-$PGF_{1\alpha}$ was not found in the perfusate. Isolation and identification of pentanor-$PGF_{1\alpha}$ suggested that tetranor-6-keto-$PGF_{1\alpha}$ (Fig. 3) was further transformed by the one-carbon oxidative decarboxylation pathway to form pentanoic acid which subsequently cyclized to the γ-lactone. Oxidative decarboxylation of α-keto acids (e.g., tetranor-6-keto-$PGF_{1\alpha}$) has been described in detail by Davies, Levis and their co-workers (16,17). A small amount of 19-hydroxy-6-keto-$PGF_{1\alpha}$ (compound IB) was detected in the perfusate, which possibly derives from 6-keto-$PGF_{1\alpha}$ via ω-1 hydroxylation (18).

The isolated perfused liver may also convert exogenous PGI_2 into a substance having the biological properties of 6-keto-PGE_1. This metabolite co-migrated with 6-keto-PGE_1 (authentic standard) on TLC, and had similar potency to PGI_2 in inhibiting platelet aggregation (5,6,19). It may arise from oxidation of 6-keto-$PGF_{1\alpha}$ via the 9-hydroxyprostaglandin dehydrogenase (9-OH-PGDH) pathway, as suggested by the recovery of tritiated water from the aqueous phase of the perfusate due to the loss of [9-^{3}H] (20,21). Since 9-OH-PGDH activity had been demonstrated in the rat liver (22), this enzyme may be responsible for oxidation of the 9-OH group of 6-keto-$PGF_{1\alpha}$ to a ketone, a function similar to the enzymic activity which converts $PGF_{2\alpha}$ directly to PGE_2 (20). This 6-keto-PGE_1-like substance generated by the liver is a relatively stable biologically active metabolite which could act synergistically with PGI_2 as it escapes metabolism by the lung (7). Further, this study indicates that the liver is a major site of PGI_2 clearance.

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