Peroxisomal chain-shortening of prostaglandin $F_{2\alpha}$

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Abstract We have recently reported that prostaglandin $F_{2\alpha}$ can be chain-shortened by isolated rat liver peroxisomes. In the present study it is further established by cell fractionation experiments that the enzymes involved in this reaction are localized to peroxisomes. Under the conditions employed, the highest activity was found in the light mitochondrial fraction. Further fractionation of the light mitochondrial fraction by sucrose density gradient centrifugation showed that the prostaglandin oxidation activity comigrated with peroxisomal marker enzymes. Di(2-ethylhexyl)phthalate treatment resulted in a tenfold increased capacity for the conversion of prostaglandin $F_{2\alpha}$ into tetranorprostaglandin $F_{1\alpha}$. The reaction was not inhibited by KCN. The reaction was further characterized with respect to cofactor requirements. The prostaglandin oxidation was found to be completely dependent on NAD, CoA, ATP, Mg^{2+} and was stimulated by FAD. Incubation of prostaglandin E_2 with peroxisomes resulted in conversion into several products. After alkaline hydrolysis, one of these was identified as tetranorprostaglandin B_1. —**Diczfalusy, U., and S. E. H. Alexson.** Peroxisomal chain-shortening of prostaglandin $F_{2\alpha}$. *J. Lipid Res.* 1988. **29:** 1629–1636.

Supplementary key words DEHP-induction • rat liver peroxisomes • gas–liquid chromatography–mass spectrometry

Prostaglandins are biologically active compounds derived from C_{20} polyunsaturated fatty acids (1). The enzymes required for prostaglandin biosynthesis are present in almost all mammalian tissues. However, isomerases and synthases converting the prostaglandin endoperoxide intermediate into different prostaglandins are unevenly distributed throughout the body, leading to different prostaglandins in different tissues. Prostaglandins act locally and are inactivated close to the site of action. Reactions rendering the prostaglandin molecule biologically inactive and transforming it into a more easily excretable form include oxidation of the 15-alcohol group, saturation of the 13, 14-double bond, omega-oxidation, and β-oxidation (2). Prostaglandin metabolites excreted in the urine are usually chain-shortened by two or four carbon atoms (3–6). It has been reported that prostaglandins can be β-oxidized by the carnitine-dependent system in mitochondria (7, 8). A β-oxidation system, which acts mainly to chain-shorten fatty acids, has been demonstrated in peroxisomes (9–11). We have recently shown that prostaglandin $F_{2\alpha}$ can be chain-shortened to its tetranor-metabolite in isolated rat liver peroxisomes (12). In the present work this reaction is further characterized and additional proof is given for its cellular localization to peroxisomes. Furthermore, incubation of prostaglandin E_2 with isolated peroxisomes was also found to yield a tetranor metabolite.

MATERIALS AND METHODS

Chemicals

Prostaglandin $F_{2\alpha}$ (Tris salt) was purchased from Sigma (St. Louis, MO) and [9-^{3}H]prostaglandin $F_{2\alpha}$ was obtained from New England Nuclear (Dreieich, Germany). All other reagents were of analytical grade.

Cell fractionation and preparation of peroxisomes from rat liver

Male Sprague Dawley rats (150–200 g) were fed ordinary lab chow or lab chow supplemented with 2% (w/w) of di(2-ethylhexyl)-phthalate (DEHP) for 1–2 weeks. The rats were injected with Triton WR-1339 (85 mg/100 g body weight) 3 1/2 days before sacrifice (13) and fasted overnight before they were killed. The livers were homogenized in 0.25 M sucrose containing 3 mM imidazole, pH 7.4, 0.1% ethanol, and 1 mM EDTA. The homogenates were centrifuged as described (12) to obtain nuclear (N), heavy mitochondrial (H), and light mitochondrial (L) fractions. The 25,300 *g* supernatant was centrifuged at 100,000 *g* for 45 min to obtain microsomal (P) and high-speed supernatant (S) fractions. Peroxisomes were isolated by sucrose density gradient centrifugation of the light mitochondrial fraction as described (12) except that the gradients were subjected to an integrated centrifugation of 8.66×10^{10} rad^2/sec in a Beckman VTi 50 rotor (corresponding to a centrifugation at 45,000 rpm for 65 min). Catalase (14), cytochrome-*c*-oxidase (13), ester-

Abbreviations: DEHP, di(2-ethylhexyl)phthalate; HPLC, high performance liquid chromatography; DTT, dithiothreitol.

ase (15), acid phosphatase (16), acyl-CoA oxidase (17), 3-hydroxyacyl-CoA dehydrogenase (18), and protein (19) were measured as described.

Incubation procedure

Incubations (total volume 1.0 ml) were performed in 0.085 M Tris-buffer, pH 8.0, supplemented with the following cofactors (final concentrations): 2.5 mM ATP, 0.5 mM CoA, 10 mM $MgCl_2$, and 35 μM FAD. The prostaglandin $F_{2\alpha}$ concentration was 60 μM and 2 μCi [9β-^{3}H]prostaglandin $F_{2\alpha}$ (15 Ci/mmol) was added to each incubation. One hundred μg peroxisomal protein was added and the incubation mixture was preincubated for 15 min at 37°C. The reaction was initiated by the addition of 10 μl 20 mM NAD and the incubation was continued for 30 min.

Alkaline hydrolysis of CoA-esters

Three hundred and fifty μl 0.5 M KOH was added to the incubation mixture and the sample was incubated at 60°C for 60 min.

Extraction of prostaglandins

The sample was placed on ice and acidified to pH 3 with 1 N HCl. The acidified sample was applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA). Five ml water was passed through the Sep-Pak cartridge followed by 5-ml portions of 15% ethanol in water, petroleum ether, methyl formate, and methanol. Prostaglandins were eluted in the methyl formate fraction (20).

Chromatographic isolation of oxidation products

The methyl formate fraction from the extraction step was taken to dryness under a slow stream of nitrogen and the residue was dissolved in 50 μl methanol. Five μl of this extract was injected onto a Pecosphere 3X3C C18 reversed phase HPLC column (33 × 4.6 mm, Perkin-Elmer Corp., Norwalk, CT). The mobile phase was methanol–water–acetic acid 50:50:0.01 (v/v/v) and the flow rate was 1.3 ml/min. Two fractions per min were collected for 15 min. The fractions were analyzed for radioactivity using an LKB 1217 Rackbeta liquid scintillation counter.

Preparative incubations with prostaglandin E_2 and isolated peroxisomes

The same incubation conditions as for prostaglandin $F_{2\alpha}$ were used except that the incubation was scaled up tenfold. Prostaglandin E_2 was dissolved in an α-cyclodextrin solution as previously described (21). The reaction mixture was extracted as described for prostaglandin $F_{2\alpha}$. HPLC of the extract was performed on a Waters Radial-Pak Resolve C18 5 μ-column (8 × 100 mm). The mobile phase was methanol–water–acetic acid 60:40:0.01 (v/v/v) and the flow rate was 1.8 ml/min. The UV-absorption was monitored at 280 nm. Fractions of 1.8 ml were collected and a small aliquot was removed from each fraction for radioactivity determination. The UV trace of the most polar radioactive peak in the chromatogram showed two components absorbing at 280 nm. The two components were collected separately and were then reacted with 50 μl of a 10 mg/ml solution of O-methoxyamine hydrochloride in pyridine at room temperature overnight to convert keto groups to methoximes. Finally, hydroxyl groups were derivatized to trimethylsilyl ethers by treatment with trimethylchlorosilane–hexamethyldisilazane–pyridine 1:2:3 (v/v/v).

Radio gas–liquid chromatography

Radio gas–liquid chromatography was performed as previously described (22). A packed methyl silicone column (1% OV-101 on Chromosorb WHP, 80–100 mesh, 150 cm × 2 mm) was used isothermally at 190°C. Retention times were converted to C-values (23) using saturated fatty acid methyl esters as reference compounds.

Gas–liquid chromatography–mass spectrometry

Gas–liquid chromatography–mass spectrometry was performed on an LKB 2091 instrument equipped with a 30-m capillary column (DB 1301, 0.25 mm × 30 m, J&W Scientific, Folsom, CA) operated isothermally at 250°C. The electron energy was 22.5 eV.

RESULTS

Fractionation of liver homogenates

The intracellular localization of prostaglandin oxidation activity was studied after fractionation of a liver homogenate. **Fig. 1** shows the distribution of prostaglandin oxidation activity after differential centrifugations. The highest relative specific activity of prostaglandin oxidation was found in the light mitochondrial fraction, coincident with sedimentable catalase, a peroxisomal marker enzyme. Substantial catalase activity was also found in the supernatant fraction. The relatively low recovery (42%) of prostaglandin oxidation activity is most likely due to selective leakage of some of the peroxisomal enzymes, e.g., the β-oxidation enzymes (24, 25). 3-Hydroxyacyl-CoA dehydrogenase, a β-oxidation enzyme present in mitochondria and peroxisomes, was evenly distributed between the heavy and light mitochondrial fractions. The other marker enzymes showed the expected distribution. All enzymes studied showed a relative specific activity close to one in the nuclear fraction, indicating incomplete homogenization.

The light mitochondrial fraction was further fractionated on a linear sucrose density gradient. The distribution of prostaglandin oxidation activity, the activities of acyl-CoA oxidase, 3-hydroxyacyl-CoA dehydrogenase,

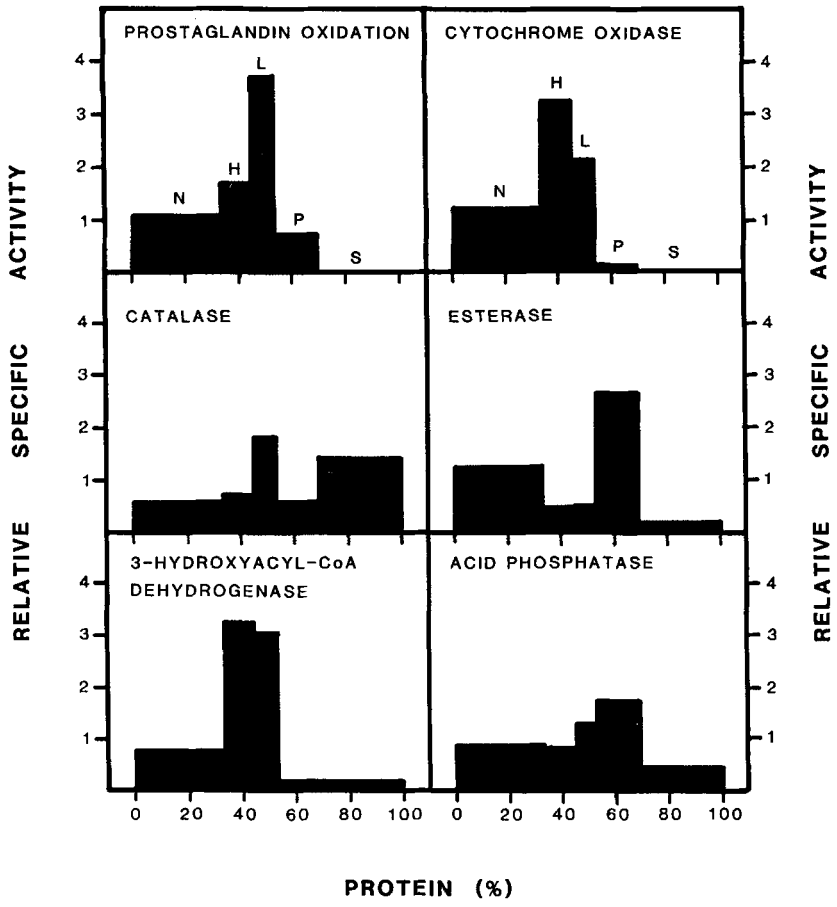

Fig. 1. Enzyme distribution after subcellular fractionation of a liver homogenate from DEHP-treated rats by differential centrifugation. The homogenate was fractionated into nuclear (N), heavy mitochondrial (H), light mitochondrial (L), microsomal (P), and supernatant (S) fractions. The relative specific activity was calculated as percent enzymatic activity divided by percent protein in each fraction. The recovery after differential centrifugation of the homogenate was: protein, 127%; prostaglandin oxidation, 42%; cytochrome oxidase, 88%; catalase, 84%; esterase, 103%; 3-hydroxyacyl-CoA dehydrogenase, 88%; and acid phosphatase, 127%.

and marker enzymes, as well as protein, in the gradient fractions are shown in **Fig. 2**. The distribution of protein and 3-hydroxyacyl-CoA dehydrogenase showed two peaks, the low density peak coinciding with cytochrome oxidase and the high density peak coinciding with catalase. This shows that peroxisomes and mitochondria are well separated. The distribution profiles of prostaglandin oxidation and acyl-CoA oxidase activities were similar and in good agreement with catalase activity. The specific activity of prostaglandin oxidation was about 20 times higher in the peak peroxisome fractions as compared to the homogenate. Microsomes (esterase) and lysosomes (acid phosphatase) were found at lower densities than peroxisomes. However, esterase showed a broad distribution throughout the gradient. The peak peroxisome fraction (fraction 4) contained 4.9% of the total gradient esterase activity and the light mitochondrial fraction contained 4.3% of the homogenate esterase activity. Fraction 4 was essentially free from mitochondrial or lysosomal contamination. The gradient fractions with the highest catalase activities were used for further experiments.

Optimization of reaction conditions for prostaglandin oxidation

The reaction products of the prostaglandin $F_{2\alpha}$ oxidation were isolated by reversed phase HPLC. **Fig. 3** shows HPLC chromatograms from three prostaglandin $F_{2\alpha}$ incubations with and without peroxisomes. Fig. 3A shows a blank incubation without peroxisomal protein and Fig. 3B shows a typical chromatogram from an incubation with 100 μg of peroxisomal protein from untreated rats. Fig. 3C shows a chromatogram from an incubation of prostaglandin $F_{2\alpha}$ with peroxisomes from DEHP-treated rats. The most polar peak (fractions 4–6) in the chromatogram was identified as tetranor-prostaglandin $F_{1\alpha}$ by

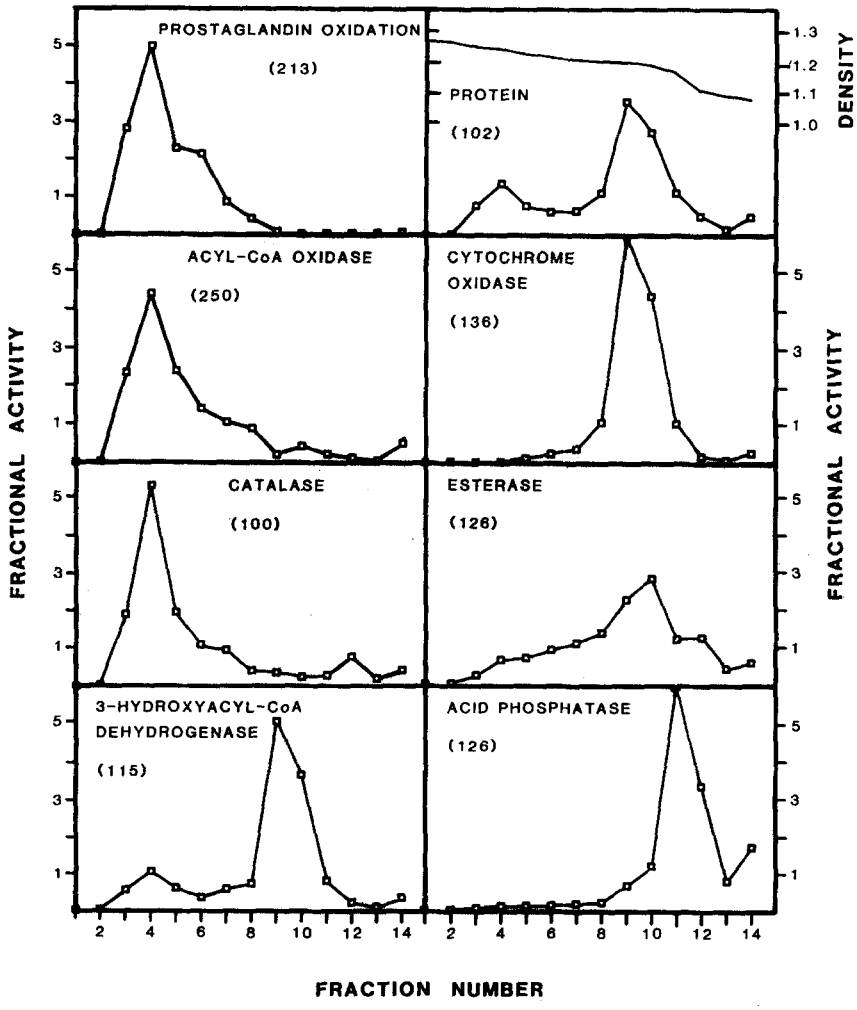

Fig. 2. Enzyme distribution after isopycnic centrifugation of a light mitochondrial fraction in sucrose. The light mitochondrial fraction was prepared from livers of DEHP-treated rats. The numbers in parentheses show the recovery of the enzyme in the gradient.

radio gas-liquid chromatography and gas-liquid chromatography-mass spectrometry (12). The main peak (fractions 24-26) in the chromatogram is unconverted substrate. The relative conversion to tetranor-prostaglandin $F_{1\alpha}$ was calculated from the amount of radioactivity found in the most polar peak in the chromatogram. The different reaction parameters were varied in order to optimize the reaction conditions. As shown in **Fig. 4**, the relative conversion was linear with protein up to 150 μg per incubation. After an initial lag phase, the reaction was linear with time between 5 and 45 min. Maximal reaction rate was observed above 60 μM prostaglandin $F_{2\alpha}$. The reaction showed an absolute requirement for added ATP, Mg^{2+}, CoA, and NAD. The pH optimum of the reaction was about 8. FAD had a stimulatory effect at concentrations up to 10 μM (40%, not shown). Addition of 2 mM DTT or 0.05% Triton X-100 had no stimulatory effect on the reaction. Inclusion of 1 mM KCN in the incubation medium stimulated the reaction slightly.

Induction of peroxisomal chain-shortening of prostaglandin $F_{2\alpha}$ by DEHP

The chain-shortening of prostaglandin $F_{2\alpha}$ to its tetranor-metabolite was measured in peroxisomes from untreated and DEHP-treated rats. The two peak peroxisome fractions from sucrose density gradients from untreated and DEHP-treated rats were incubated with prostaglandin $F_{2\alpha}$. DEHP-treatment resulted in a tenfold induction of the specific activity of the chain-shortening reaction (**Table 1**). There was a very small loss in activity in DEHP-induced peroxisomes that had been kept frozen at – 70°C for several weeks, whereas activity in peroxisomes from untreated rats was detectable only in fresh, unfrozen preparations.

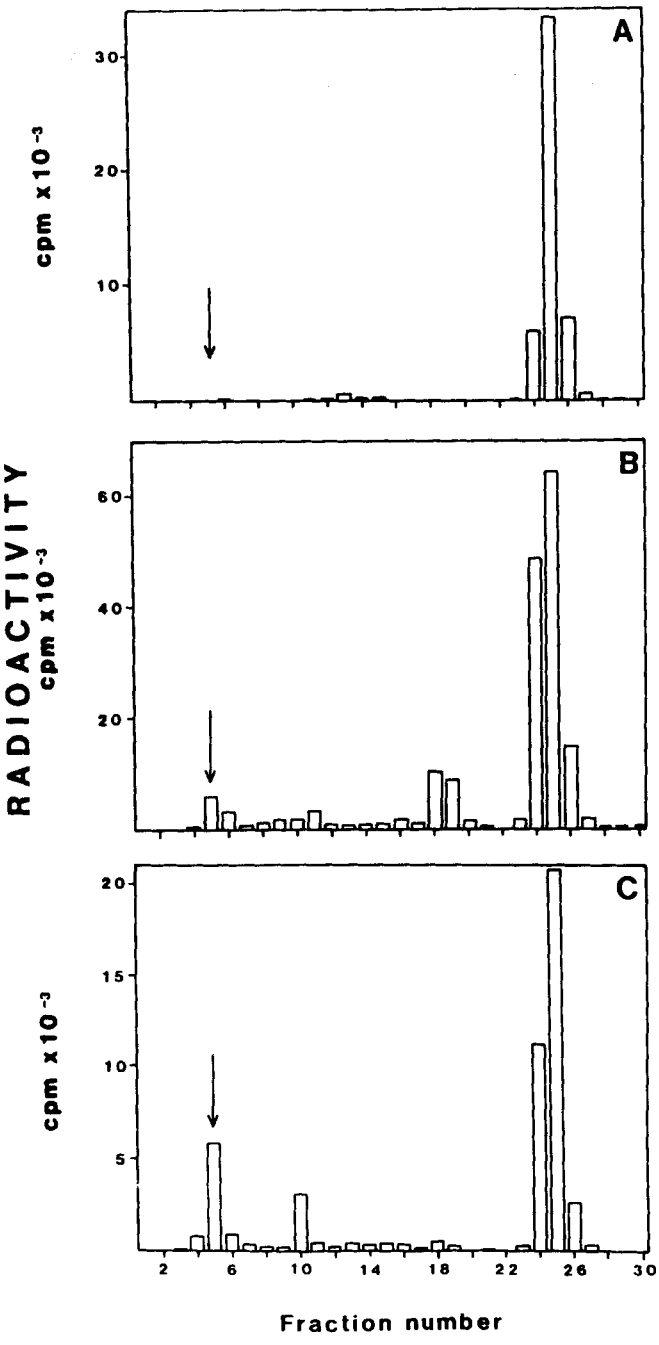

Fig. 3. Reversed phase HPLC chromatograms of extracts from incubations of prostaglandin $F_{2\alpha}$ with and without isolated rat liver peroxisomes. A: Blank incubation without peroxisomes; B: incubation with peroxisomes from untreated rats; C: incubation with peroxisomes from DEHP-treated rats. The major peak (fractions 24–26) is the unconverted substrate (prostaglandin $F_{2\alpha}$). The arrows indicate the position of the chain-shortened metabolite (tetranor-prostaglandin $F_{1\alpha}$).

Incubation of prostaglandin E_2 with isolated peroxisomes

In order to see whether prostaglandins other than prostaglandin $F_{2\alpha}$ were chain-shortened by peroxisomes, prostaglandin E_2 was incubated with isolated peroxisomes. As seen in **Fig. 5**, the HPLC chromatogram of the extracted sample from such an incubation shows great similarities with the corresponding chromatogram from a prostaglandin $F_{2\alpha}$ incubation (Fig. 3C). The most polar peak from a preparative incubation (corresponding to fractions 5–7 in Fig. 5) showed two components absorbing at 280 nm. UV spectra of both compounds had absorbance maxima at 280 nm in ethanol. UV absorbance at 280 nm is characteristic for prostaglandins with a B-type ring which is formed from the E-type ring by alkaline treatment (23). After derivatization for gas–liquid chromatography, the material was analyzed by radio gas–liquid chromatography and gas–liquid chromatography–mass spectrometry. The more polar of the two components had a C-value of 22.3 as determined by radio gas–liquid chromatography. This component was not characterized further. The less polar of the two compounds had a C-value of 20.5. The mass spectrum of this compound showed the molecular ion (M) at an m/e of 395. Ions were also present at m/e 380 (M–15, loss of $\cdot CH_3$), 364 (M–31, loss of $\cdot OCH_3$), 324 (M–71, $\cdot(CH_2)_4CH_3$), 293 (M–(31 + 71)), 274 (M–(31 + 90), loss of $\cdot OCH_3$, and trimethylsilanol), 174 and 173. Both the C-value and the mass spectrum, as well as the absorbance maximum of this compound, fully conform to earlier reported data for 11-hydroxy-5-keto-tetranorprosta-4(8),9-dienoic acid (tetranor-prostaglandin B_1) (26). Prostaglandin E_2 had thus been chain-shortened by four carbon atoms.

DISCUSSION

We have shown in a previous paper that prostaglandin $F_{2\alpha}$ can be chain-shortened to tetranor-prostaglandin $F_{1\alpha}$ by isolated rat liver peroxisomes (12). The results indicated that the chain-shortening reaction could take place in peroxisomes, and with the incubation conditions used, no activity was detected in mitochondria or microsomes. It should be pointed out that the incubation conditions, used also in the present work, were suitable for detection of peroxisomal β-oxidation, and no attempts were made to assay a mitochondrial β-oxidation under optimal conditions. Thus the relative importance of the peroxisomal and the mitochondrial enzymes for degradation of prostaglandins is still unknown. In the present study we have examined the subcellular localization of the chain-shortening reaction in more detail. The highest relative specific activity of prostaglandin oxidation was found in the light mitochondrial fraction obtained after differential centrifugation of a rat liver homogenate. The poor recovery of 42% of prostaglandin oxidation activity after fractionation of the homogenate is most likely due to the inability to detect any activity in the cytosolic fraction. It has been reported that acyl-CoA oxidase (the assumed rate-limiting enzyme) and 3-ketoacyl-CoA thiolase show

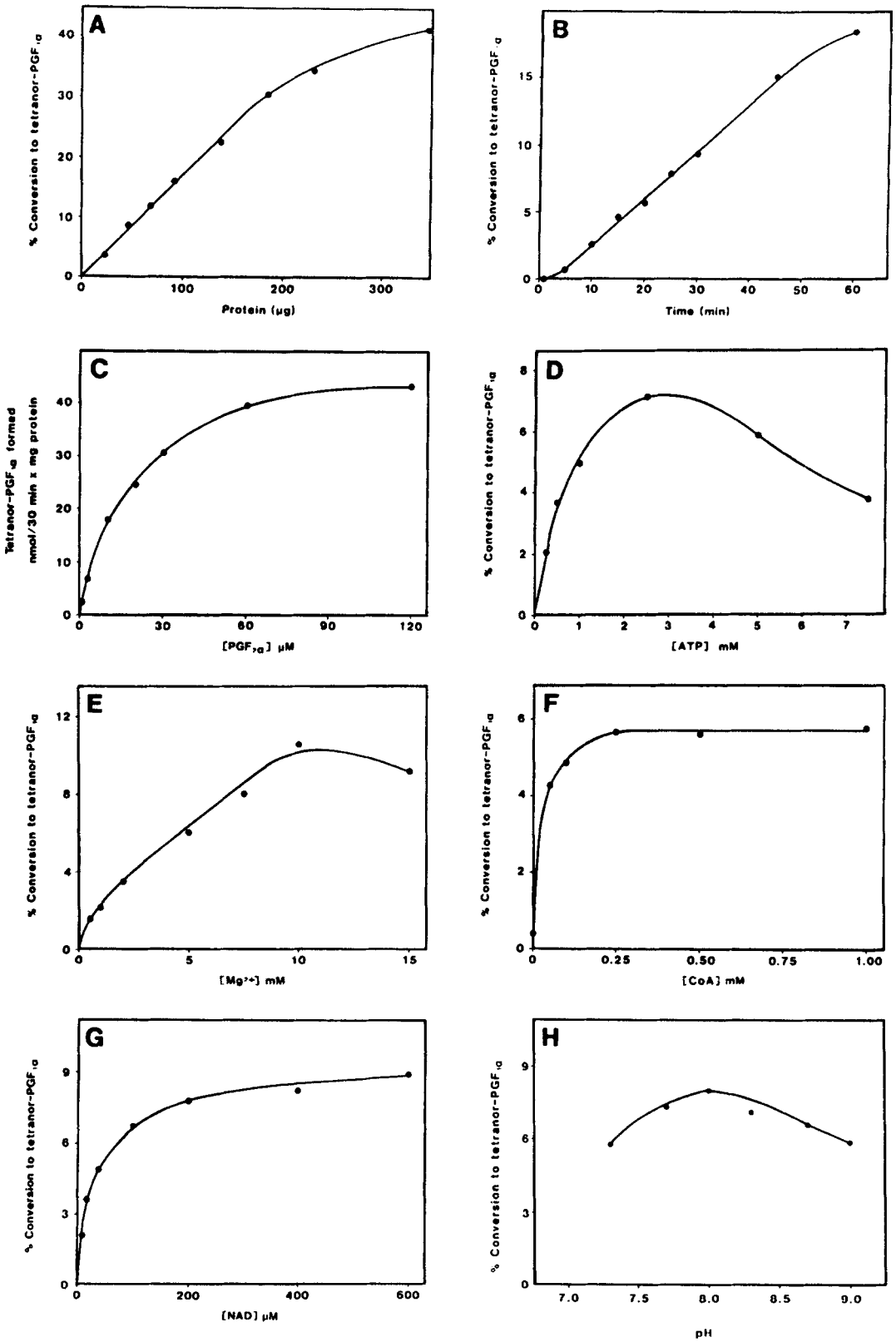

Fig. 4. Peroxisomal prostaglandin $F_{2\alpha}$ oxidation. Dependence on protein (A), time (B), substrate concentration (C), ATP (D), Mg^{2+} (E), CoA (F), NAD (G), and pH (H). Incubations were performed in 0.085 M Tris-buffer, pH 8.0, containing 35 μM FAD, 5 mM Mg^{2+}, 200 μM NAD, 0.5 mM CoA, and 100 μg peroxisomal protein. In A and B the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) concentration was 30 μM and in D-H 60 μM. In A-F the ATP concentration was 5 mM and in G and H 2.5 mM. Otherwise, one parameter was varied at a time as shown in the figures. Tris-buffer (0.085 M) was used for the pH curve (H).

a much higher degree of leakage than the hydratase-dehydrogenase bifunctional protein (24, 25). The bifunctional protein that leaks out has been shown to bind to microsomes (27, 28). We have measured the prostaglandin oxidation as the formation of a tetranor-metabolite which requires the whole set of β-oxidation enzymes. Therefore, a selective leakage of only some of the β-oxidation enzymes will not allow the whole reaction sequence to take place.

The light mitochondrial fraction, enriched in peroxisomes, was then further fractionated by sucrose density gradient centrifugation. The distribution of the prostaglandin oxidation activity showed an excellent correlation with the distribution of the peroxisomal enzymes catalase and acyl-CoA oxidase, the latter enzyme being exclusively localized to peroxisomes. The high recoveries of prostaglandin oxidation and acyl-CoA oxidase in the gradient (Fig. 2) are probably due to underestimation of these activities in the light mitochondrial fraction. These results show that the prostaglandin oxidation reaction is entirely peroxisomal when measured by the current assay. This is further supported by the finding that the reaction was dependent on exogenous NAD and was insensitive to KCN, characteristics of β-oxidation in isolated peroxisomes. In addition, the conversion of prostaglandin $F_{2\alpha}$ to tetranor-prostaglandin $F_{1\alpha}$ was induced about tenfold by feeding rats DEHP, a potent inducer of peroxisomal β-oxidation (29). Peroxisomes from DEHP-treated rats were found to metabolize prostaglandin E_2 at a rate similar to prostaglandin $F_{2\alpha}$. However, the most polar radioactive peak in the HPLC chromatogram from such an incubation (Fig. 5, fractions 5–7) contained several products. One of these, isolated after alkaline hydrolysis, was identified as 11-hydroxy-5-ketotetranorprosta-4(8),9-dienoic acid (tetranorprostaglandin B_1). Although alkaline treatment of E prostaglandins is known to result in conversion into B prostaglandins, we cannot exclude that some of the B prostaglandin was formed prior to the hydrolysis step. Tetranor-prostaglandin B_1 has been identified in rat urine after intravenous injection of prostaglandin E_2 (26). This metabolite of prostaglandin E_2 was thus chain-shortened

TABLE 1. Effect of DEHP on peroxisomal chain-shortening of prostaglandin $F_{2\alpha}$

Gradient Fraction	nmol Product/min × mg Protein
DEHP-4	1.47
DEHP-5	1.21
Control-4	0.12
Control-5	0.04

The gradient fractions from the DEHP-treated rats were incubated as described in Materials and Methods. In the incubations with the gradient fractions from the untreated rats (Control-4 and Control-5) 500 μg and 375 μg protein were used, respectively, and the incubation time was prolonged to 60 min to achieve a similar relative conversion in the incubations as compared to incubations with DEHP-induced peroxisomes.

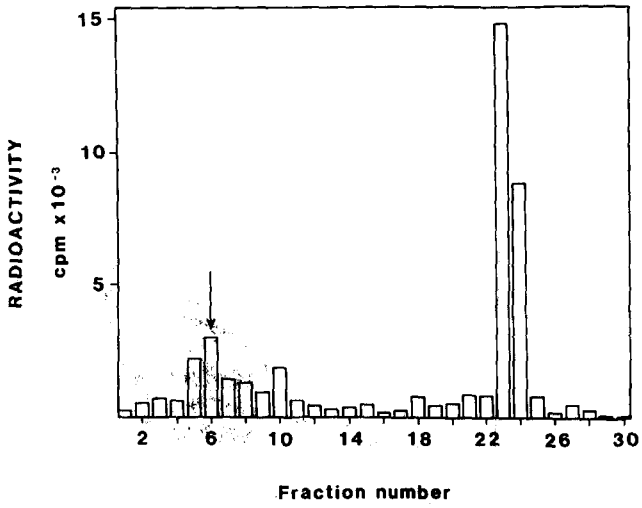

Fig. 5. Reversed phase HPLC chromatogram of an extract from an incubation of prostaglandin E_2 with isolated liver peroxisomes from DEHP-treated rats. The major peak (fractions 23–24) corresponds to unconverted substrate and the arrow indicates the position of the tetranor-metabolite.

by four carbon atoms, analogous to the in vitro oxidation of prostaglandin $F_{2\alpha}$. The product pattern after incubation of prostaglandin E_2 with peroxisomes appeared to be more complex than the corresponding prostaglandin $F_{2\alpha}$ pattern. The structural identification of the other products is currently in progress. It is evident that the reaction product profile for prostaglandin $F_{2\alpha}$ differed in incubations with control peroxisomes compared to DEHP-induced peroxisomes. The most striking difference was the appearance of a slightly more polar peak than prostaglandin $F_{2\alpha}$ (fractions 18–19, Fig. 3B), which was the major product peak in these incubations. Structural elucidation of the currently unidentified metabolites would give additional information about peroxisomal metabolism of prostaglandins. The complex product patterns may indicate that other reactions than β-oxidation may take place in peroxisomes.

After submission of the present report, an article was published by Schepers et al. (30) describing the β-oxidation of prostaglandin E_2 in rat liver peroxisomes. Their results agree well with ours. They reported, however, a higher specific activity of prostaglandin oxidation in isolated peroxisomes than we found. Their higher specific activity may be explained by the fact that they measured total acid-soluble radioactivity from [1-^{14}C]prostaglandin E_2, whereas we have expressed the specific activity as the formation of only one of the products formed, the tetranor-metabolite of prostaglandin $F_{2\alpha}$. In addition, they found that added microsomes stimulated the reaction.

The skillful technical assistance of Ms. Elisabeth Thorsén is gratefully acknowledged. U. D. was supported by a grant from Svenska Sällskapet för Medicinsk Forskning and S. A. was supported by Magnus Bergvalls stiftelse.

Manuscript received 23 February 1988 and in revised form 17 June 1988.

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