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Nonenzymatic Reduction of Prostaglandin H by Lipoic Acid†

Lawrence J. Marnett* and Michael J. Bienkowski

ABSTRACT: Lipoic acid has recently been found to stimulate prostaglandin biosynthesis by sheep vesicular gland microsomes (Marnett, L. J., and Wilcox, C. L. (1977), *Biochim. Biophys. Acta 487,* 222). The increase in oxygenated products is predominantly in the formation of prostaglandin F and its structure has been verified by gas chromatography–mass spectrometry. Endoperoxide trapping experiments employing reduced glutathione show that the conversion of prostaglandin H to prostaglandin F is slow in lipoate containing incubation mixtures. Therefore, the net effect of the addition of lipoic acid to vesicular gland microsomes is the stimulation of prostaglandin endoperoxide biosynthesis. Further experiments reveal that the reduction of prostaglandin H to prostaglandin F by lipoate is nonenzymatic and occurs *after* the termination of biosynthesis in the work-up mixture. The reduction takes place preferentially in the organic phase of a Folch extract (chloroform–methanol–2% formic acid 8:4:3). Authentic prostaglandin H_2 is reduced by lipoic acid to prostaglandin $F_{2\alpha}$ in high yield under these conditions.

The conversion of PGH[1] to PGF can occur by either of two pathways. In the first, the cyclic peroxide PGH is isomerized spontaneously or enzymatically to the hydroxy ketone PGE. PGE is then reduced by a pyridine nucleotide to PGF (Leslie and Levine, 1973; Hensby, 1974; Lee and Levine, 1974). Both enzymes of this pathway—prostaglandin endoperoxide E isomerase and prostaglandin E 9-ketoreductase—have been purified and characterized (Ogino et al., 1977; Lee and Levine, 1975). In the second pathway, PGH is directly reduced to PGF. No evidence for the existence of a prostaglandin endoperoxide reductase has been found and it has been suggested that agents such as Cu^{2+}–dithiol complexes which selectively stimulate PGF biosynthesis do so by the nonenzymatic reduction of PGH (Chan et al., 1975).

We have recently observed high yields of PGF in incubations of sheep vesicular gland microsomes with eicosatrienoic and arachidonic acids in the presence of lipoic acid (Marnett and Wilcox, 1977). Lipoate stimulates overall prostaglandin biosynthesis two- to fourfold and selectively enhances the biosynthesis of PGF at the expense of other prostaglandins. In view of the uncertainty surrounding the biosynthesis of PGF, we have investigated in detail its formation in the presence of lipoate. Our study conclusively demonstrates that lipoic acid nonenzymatically reduces PGH to PGF and it illustrates in a novel fashion the importance of nonenzymatic transformations of prostaglandin endoperoxides in vitro.

Experimental Section

Materials

Arachidonic acid was kindly furnished by Dr. John Paulsrud, Hoffmann-La Roche. Eicosatrienoic acid and authentic prostaglandins were generously provided by Dr. John Pike, the Upjohn Co. Diclofenac sodium (GP-45840) was a gift of Dr. William Cash, Ciba-Geigy. [1-^{14}C]Eicosatrienoic acid was purchased from New England Nuclear and [1-^{14}C]arachidonic acid from Applied Science Laboratories. α-Lipoic acid (Sigma) was recrystallized twice with hexane/chloroform. The sulfhydryl content of the recrystallized material was below the limit of detection (~0.1%) using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

The microsomal fraction of sheep vesicular glands was

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received April 19, 1977.* This investigation was supported by Research Grant GM 23642-01 from the National Institutes of Health.

[1] Abbreviations used are: PGD_1, 9α,15α-dihydroxy-11-oxo-13-*trans*-prostenoic acid; PGD_2, 9α,15α-dihydroxy-11-oxo-5-*cis*,13-*trans*-prostadienoic acid; PGE_1, 11α,15α-dihydroxy-9-oxo-13-*trans*-prostenoic acid; PGE_2, 11α,15α-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid; $PGF_{1\alpha}$, 9α,11α,15α-trihydroxy-13-*trans*-prostenoic acid; $PGF_{2\alpha}$, 9α,11α,15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; PGG_1, 15α-hydroperoxy-9α,11α-peroxido-13-*trans*-prostenoic acid; PGG_2, 15α-hydroperoxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; PGH_1, 15α-hydroxy-9α,11α-peroxido-13-*trans*-prostenoic acid; PGH_2, 15α-hydroxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; GSH, reduced glutathione; PG, prostaglandin; TMS, trimethylsilyl.

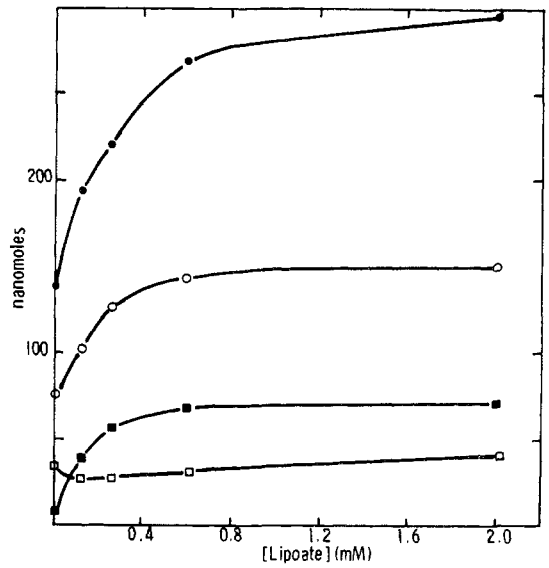


FIGURE 1: O_2 uptake and PG biosynthesis as a function of lipoate concentration. Incubations were performed at 25 °C in the oxygraph cell (1.60 mL). Each incubation contained 600 μg of microsomal protein and 170 nmol of eicosatrienoic acid. Following 1-min incubations the contents of the cell were withdrawn into 7 mL of $CHCl_3$/MeOH (1/1) and worked up as described in the Experimental Section. (●) O_2 uptake; (○) total conversion of eicosatrienoic acid to oxygenated products; (■) $PGF_{1\alpha}$; (□) PGE_1. The standard deviations in triplicate determinations are less than the size of the data points.

prepared as previously described (Marnett and Wilcox, 1977).

Prostaglandin endoperoxides (PGG and PGH) were biosynthesized from arachidonic or eicosatrienoic acid essentially according to published procedures (Hamberg et al., 1974). Vesicular gland microsomes corresponding to 30 g of tissue were suspended in 85 mL of 0.1 M $NaPO_4$ buffer (pH 7.9) at 25 °C. Five milligrams of fatty acid (2.5×10^6 cpm) was added and after 2 min the reaction was terminated by the addition of 5 mL of 0.5 M citric acid. Ether extraction and all subsequent operations were performed at 4 °C. The endoperoxides were purified by chromatography (4 °C) on 2 g of silicic acid (Bio-Rad 100–200 mesh). PGG elutes with ether/hexane (4/6) and PGH with ether/hexane (6/4). Both compounds are reduced to PGF by treatment with 1 mg of triphenylphosphine in 0.1 mL of ether at 25 °C for 1 h (Hamberg et al., 1974). For routine estimation of identity and purity, the endoperoxides were chromatographed at −20 °C on silica gel 60 plates (Merck) with ether/hexane/acetic acid (85/15/0.1) (Miyamoto et al., 1976).

A large scale incubation of arachidonic acid with vesicular gland microsomes and lipoate was carried out to obtain a sufficient amount of product for gas chromatography–mass spectrometry. Twenty milligrams of lipoic acid in 0.2 mL of ethanol was added to a suspension of vesicular gland microsomes (20 g of tissue) in 80 mL of 0.1 M $NaPO_4$ buffer (pH 7.9). The mixture was incubated for 3 min at 25 °C and then 5 mg of arachidonic acid (7×10^5 cpm) was added in 0.5 mL of ethanol. After 2 min the reaction mixture was poured into 350 mL of $CHCl_3$/MeOH (1/1) containing 0.6% formic acid. An additional 150 mL of $CHCl_3$ was added followed by 100 mL of H_2O. After standing at room temperature for 1 h, the organic layer was drawn off and kept at 4 °C overnight. The recovery of radioactivity at this point was 83%. The solvent was evaporated and the residue applied to a 20 × 20 cm silica gel 60 plate and developed using solvents A and B (see below). The zone containing the major radioactive product was scraped from the plate and the labeled material extracted (Wlodawer et al., 1976). It was applied to another 20 × 20 cm silica gel plate and developed with solvent B. Following scraping and extraction the radioactive material was stored in acetone prior to derivatization and analysis. The isolated yield was 850 μg (17%).

Gas chromatography–mass spectrometry was performed with a Pye Unicam Model 104 gas chromatograph interfaced to an AEI-MS 901 by a silicon membrane separator. A 3-ft column of 1% SE-30 on Gas Chrom Q was operated at 210 °C with a flow rate of 60 mL He/min. The separator was maintained at 190 °C and spectra were recorded at 70 eV.

Methods

Effect of GSH Addition to Exhausted Incubation Mixtures. Incubations were performed in the cell of a Gilson Model KM Oxygraph in a total volume of 1.60 mL. Vesicular gland microsomes (750 μg of protein) were preincubated with 0.5 mM lipoate for 3 min at 25 °C in 0.1 M $NaPO_4$ buffer (pH 7.8). Sodium eicosatrienoate (60 μg, 3×10^4 cpm) was added and allowed to react for various times. O_2 uptake ceased after 2 min. GSH was added to a concentration of 1 mM and 1 min later the reaction mixture was withdrawn into a syringe containing 7 mL of $CHCl_3$/MeOH (1/1). Work-up and product identification was as previously published (Marnett and Wilcox, 1977).

Kinetics of "$PGF_{2\alpha}$ Decay" in Incubation Mixtures. Vesicular gland microsomes (1 mg of protein) were suspended in 0.1 M $NaPO_4$ buffer (pH 7.8) in the oxygraph cell and lipoate added to a final concentration of 1 mM. After a 3-min preincubation, sodium arachidonate was added (50 μg, 2.5×10^4 cpm). When O_2 uptake had ceased (1 min at 32 and 38 °C), GP-45840 (diclofenac sodium) was added to a final concentration of 10 μM. The mixture continued to stir for various times and was withdrawn into a syringe containing 7 mL of $CHCl_3$/MeOH containing 0.6% formic acid. During the time intervening the addition of GP-45840 and termination of reaction there was no apparent O_2 uptake. The rest of the work-up was as above.

Incubation of PGH_2 with Lipoic Acid. The organic layer of a mixture of $CHCl_3$/MeOH/2% formic acid (8/4/3) was separated and stored (Folch et al., 1957). PGH_2 (30 μg, 1.6×10^4 cpm) was added to 6.2 mL of this solution in the presence or absence of lipoate (0.1 mM) and allowed to stand at room temperature for 60 min. After standing overnight at 4 °C, the solvent was evaporated and the residue applied to 0.25-mm silica gel plates and developed sequentially with solvents A and B.

Solvent A is benzene/dioxane/acetic acid (80/20/2). Solvent B is the organic phase of ethyl acetate/hexane/benzene/acetic acid/H_2O (50/15/10/5/50). Thin-layer plates were scanned for radioactivity with a Berthold LB 2760. Protein was determined according to Lowry et al. (1951).

Results

Figure 1 displays the effect of increasing concentrations of lipoic acid on O_2 uptake and prostaglandin biosynthesis by microsomal suspensions of sheep vesicular glands. At 25 °C the increase in prostaglandins biosynthesized from eicosatrienoic acid can be accounted for by the increase in products chromatographing with $PGF_{1\alpha}$. When arachidonic acid is employed similar increases are observed in the amounts of material chromatographing with $PGF_{2\alpha}$. The $PGF_{2\alpha}$ has been tentatively identified by gas chromatography as its methyl ester-trimethylsilyl ether and *n*-butyl boronate derivatives (Marnett and Wilcox, 1977). We have prepared the methyl

TABLE I: Effect of Adding GSH to Lipoate Containing Incubation Mixtures after the Cessation of PG Biosynthesis.

Temp (°C)	Time[a] (min)	GSH[b]	PGD_1[c]	PGE_1	$PGF_{1\alpha}$	Total[d]
25	4		24	41	45	162
25	4	+	19	101	7	175
37	2		28	46	46	186
37	2	+	24	94	10	190
37	5		18	86	18	190
37	5	+	22	92	12	192

[a] Time between initiation of biosynthesis and addition of GSH (when added). [b] The addition of 1 mM GSH is indicated by +. [c] All product yields are in nmol. [d] Total conversion of eicosatrienoic acid to oxygenated products.

ester trimethylsilyl ether derivative of the major product obtained from arachidonic acid in the presence of lipoate and subjected it to gas chromatography–mass spectrometry. The mass spectrum displays diagnostic peaks at *m/e* 584 (M; molecular ion), 513 (M − 71; loss of $(CH_2)_4CH_3$), 443 (M − 141; cleavage between C-7 and C-8), 217 (TMS—O^+=CH—CH=CH—OTMS), and 199 (side chain attached to C-12). The spectrum is identical with that of the methyl ester–tris-(trimethylsilyl) ether of an authentic sample of $PGF_{2\alpha}$.

The PGF produced from eicosatrienoic acid and arachidonic acid appears to arise directly from endoperoxide intermediates and not by reduction of PGE (vide infra). The addition of GSH is known to stimulate the conversion of PGH to PGE (Ogino et al., 1977; Van Dorp et al., 1964). If the conversion of PGH to PGF is rapid in the presence of lipoate, then the subsequent addition of GSH to biosynthetically "exhausted" incubation mixtures should produce little change in the high yields of PGF observed. If, however, the conversion of PGH to PGF in the presence of lipoate is slow, the subsequent addition of GSH should convert any unreacted PGH to PGE. Incubations were performed at 25 °C and 37 °C in which labeled eicosatrienoate was added to a microsomal suspension containing 0.5 mM lipoate. At various times GSH was added to a concentration of 1 mM and the reaction terminated 1 min later. Table I compares the amounts of prostaglandins formed with and without a "GSH chase." As Figure 1 suggests large amounts of $PGF_{1\alpha}$ are produced in the presence of lipoate. If, however, GSH is added to lipoate containing incubation mixtures, the major product observed following termination is PGE_1. Very little $PGF_{1\alpha}$ is formed. The total yield of oxygenated products is only slightly greater in GSH containing incubation mixtures indicating that the increased yields of PGE_1 are not the result of fresh biosynthesis stimulated by the addition of GSH. The obvious conclusion to be reached from these experiments is that at 25 °C lipoate stimulated incubation mixtures contain significant amounts of PGH_1 4 min after biosynthesis has stopped. The endoperoxide can be rapidly converted to PGE_1 by the addition of GSH or more slowly to $PGF_{1\alpha}$ by lipoate.

A more subtle implication of the experiments summarized in Table I is that the lipoate dependent conversion of PGH_1 to $PGF_{1\alpha}$ occurs subsequent to the termination of the incubation. To test this hypothesis the following experiments were performed. Sodium arachidonate was added to microsomal suspensions containing lipoate in an oxygraph cell. After O_2 uptake had ceased, diclofenac sodium (10 μM) was added to inhibit further biosynthesis. The incubation mixtures were then allowed to stir in the oxygraph for varying times before withdrawing the contents into acidic $CHCl_3$/MeOH. The normal fate of PGH_2 at neutral pH is decomposition to a mixture of

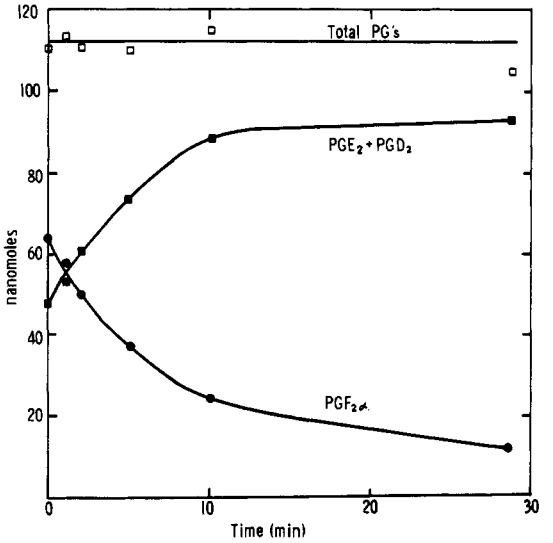


FIGURE 2: Dependence of product formation on the incubation time. The abscissa represents the time elapsed between the inhibition of biosynthesis by diclofenac sodium and termination of reaction. Incubations performed at 32 °C.

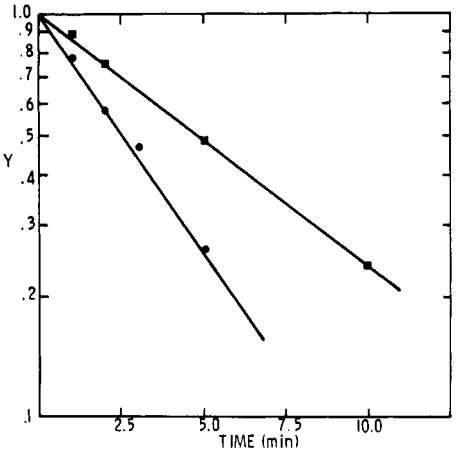


FIGURE 3: Decrease in the yield of $PGF_{2\alpha}$ as a function of the time elapsed between the inhibition of biosynthesis by diclofenac sodium and the termination of reaction: (●) at 38 °C; (■) 32 °C. $Y = [PGF_{2\alpha}]_t - [PGF_{2\alpha}]_\infty / [PGF_{2\alpha}]_0 - [PGF_{2\alpha}]_\infty$. Defined in text.

PGE_2 and PGD_2 (Hamberg et al., 1974; Nugteren and Hazelhof, 1973). If the reduction of PGH_2 to $PGF_{2\alpha}$ by lipoate occurs during the work-up, then increasing the time prior to work-up should lead to a decrease in the formation of $PGF_{2\alpha}$. Figure 2 displays the effect of increasing the time between the cessation of biosynthesis and work-up of the incubation mixture. As expected, the yield of $PGF_{2\alpha}$ decreases with increasing contact time while the yields of PGE_2 and PGD_2 increase. The total yield of prostaglandins remains constant.

The decrease in the yield of $PGF_{2\alpha}$ with increasing contact time can be used as an indicator of the half-life of the species from which it is arising. Figure 3 is a plot of $[PGF_{2\alpha}]_t - [PGF_{2\alpha}]_\infty / [PGF_{2\alpha}]_0 - [PGF_{2\alpha}]_\infty$ vs. the time between the cessation of biosynthesis and the termination of reaction. $[PGF_{2\alpha}]_t$ represents the yield of $PGF_{2\alpha}$ at a given time, $[PGF_{2\alpha}]_0$ represents the yield of $PGF_{2\alpha}$ if the reaction is terminated immediately after the cessation of biosynthesis, and $[PGF_{2\alpha}]_\infty$ represents the yield of $PGF_{2\alpha}$ at very long times. We presume that $[PGF_{2\alpha}]_\infty$ represents the amount of $PGF_{2\alpha}$ formed in the aqueous incubation mixture. The linearity of

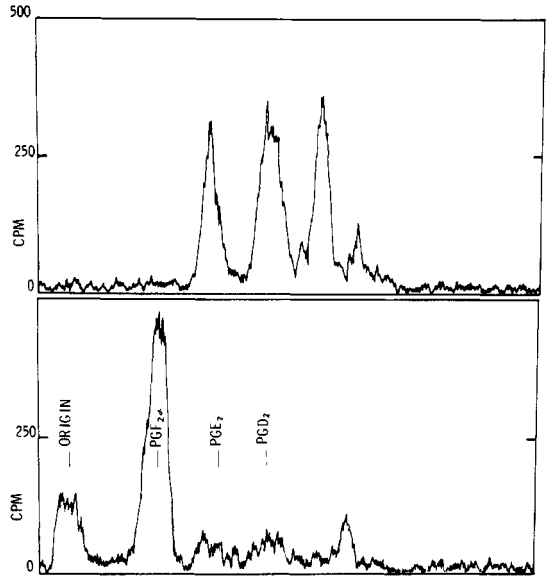


FIGURE 4: Radioactivity profile of the products formed from PGH_2 in the organic phase of a Folch extract in the absence (upper trace) and presence (lower trace) of lipoic acid.

these logarithmic plots is consistent with pseudo-first-order decay of a trappable intermediate in the microsomal preparation. The half-lives of this intermediate at 38 °C (2.5 min) and 32 °C (5 min) are consistent with literature values for the decomposition of the cyclic peroxide of PGG and PGH (Hamberg and Samuelsson, 1973; Nugteren and Hazelhof, 1973).

The experiments summarized in Table I and Figures 2 and 3 suggest that the conversion of PGH_2 and $PGF_{2\alpha}$ in the presence of lipoate not only is nonenzymatic but also occurs during the work-up rather than during the incubation. The method employed in the work-up is basically a Folch extraction of acidic lipids (Folch et al., 1957) with one minor modification. The volume of the incubation mixture was 1.6 mL (oxygraph cell). The reactions were terminated by the addition of 7 mL of $CHCl_3$/MeOH (1/1) rather than the normal 7 volumes (11.2 mL). This modification results in the immediate separation of organic and aqueous layers of approximately equal volumes. Therefore, the formation of $PGF_{2\alpha}$ could possibly occur in either of these layers. Table II shows the effect of adding a small amount of formic acid to the $CHCl_3$/MeOH. Without acid both lipoate and prostaglandins remain in the aqueous phase until the addition of 2% formic acid later in the work-up. Under these conditions the major product is PGE_2. If the $CHCl_3$/MeOH contains 0.6% formic acid, the lipoate and prostaglandins are immediately extracted into the organic phase. In this case the major product is $PGF_{2\alpha}$. Therefore, the nonenzymatic reduction of PGH_2 by lipoate occurs in the organic phase of the work-up mixture. Table II also shows the effect of terminating the reaction by the addition of 7 volumes of $CHCl_3$/MeOH. The initial mixture is homogeneous and phase separation does not occur until later in the work-up. The major product isolated is PGE_2.

These experiments strongly suggest that PGH_1 and PGH_2 are reduced by lipoic acid in the organic phase of an acidic Folch extract. We have, therefore, prepared authentic PGH_2 and incubated it in this organic phase in the presence and absence of lipoic acid. Figure 4 is a comparison of the radioactivity profiles of the products obtained following separation by thin-layer chromatography. In the absence of lipoic acid, three major zones of radioactivity are evident. The two most

TABLE II: Effect of Work-up Conditions on PG Biosynthesis in the Presence of Lipoate.[a]

Termination solvent	PGD_2[b]	PGE_2	$PGF_{2\alpha}$
7 mL of $CHCl_3$/MeOH	19	39	17
7 mL of $CHCl_3$/MeOH	20	19	42
0.6% formic acid/ 7 vol of $CHCl_3$/MeOH	20	42	15

[a] Incubations were performed at 28 °C for 2 min and contained 170 nmol of arachidonic acid. [b] Product yields are in nmol.

polar compounds cochromatograph with PGE_2 and PGD_2, respectively. The amount of radioactivity cochromatographing with $PGF_{2\alpha}$ is 1% of the total on the plate. When lipoic acid is present in the solution prior to the addition of PGH_2, a major product is observed which cochromatographs with $PGF_{2\alpha}$. This zone contains 56% of the radioactivity on the plate. The zone at the origin contains 20% of the radioactivity and the remainder is distributed evenly over the plate.

Discussion

The existence of a reductase capable of converting PGH to PGF has yet to be demonstrated. The ability of a number of agents such as epinephrine and Cu^{2+}-dithiol complexes to stimulate PGF biosynthesis has been attributed to nonenzymatic reduction of the unstable peroxy bridge (Chan et al., 1975). Wlodawer et al. have provided evidence, however, that a macromolecular, mercuribenzoate inhibitable factor reduces PGH_2 to $PGF_{2\alpha}$ in bovine uterus microsomes (Wlodawer et al., 1976). The reducing activity is not stimulated by GSH or NADPH nor is it abolished by heating for 10 min in a boiling water bath. The presence of this activity in the uterus is significant because $PGF_{2\alpha}$ has been identified as the luteolytic agent synthesized in this tissue (Pharris et al., 1972).

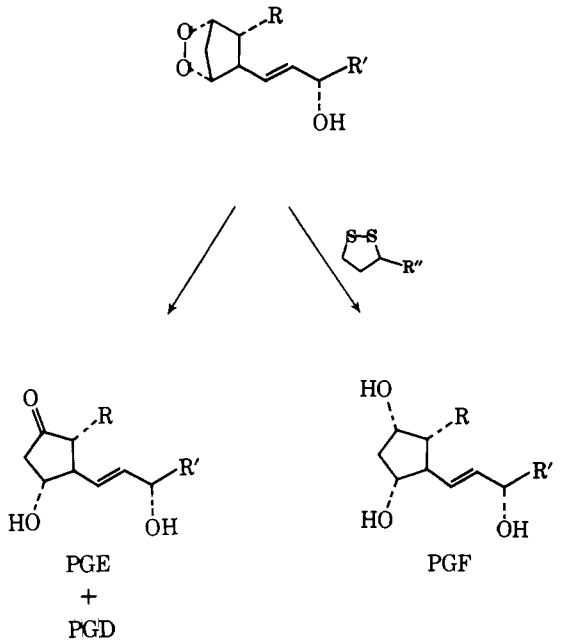
The present study establishes that the high yield of PGF produced in vesicular gland microsomes in the presence of lipoate is due to a nonenzymatic reduction of PGH. Furthermore, the reduction occurs slowly in aqueous solutions with the result that appreciable concentrations of endoperoxide intermediates build up as a consequence of the stimulation of fatty acid oxygenation by lipoate. The net effect of lipoic acid on prostaglandin biosynthesis is, therefore, stimulation of the production of endoperoxide intermediates. The endoperoxides exhibit marked pharmacological properties (Needleman et al., 1977; Hamberg et al., 1974; Willis and Kuhn, 1973) and are common intermediates in the biosynthesis of prostaglandins (Wlodawer and Samuelsson, 1973), thromboxanes (Hamberg et al., 1975), prostacyclin (Moncada et al., 1976; Johnson et al., 1976), and malonaldehyde (Hamberg and Samuelsson, 1967). Agents such as lipoate which stimulate endoperoxide biosynthesis could have profound effects on physiological events initiated by arachidonic acid or eicosatrienoic acid.

The reduction of cyclic peroxides or dialkyl peroxides by disulfides is a reaction for which there is little precedent. Lipoic acid is known to react with hydroperoxides and peroxy acids (Stary et al., 1975) but the present report seems to be the first of its reaction with dialkyl peroxides. The possibility of a dihydrolipoate contaminant being the actual reducing agent seems remote since the thiol concentration of our lipoate preparations is less than 0.1%. Furthermore, a twofold excess of dihydrolipoic acid to PGH_1 causes no increase in the yield of $PGF_{1\alpha}$ over controls.[2]

[2] M. Bienkowski, unpublished results.

The reduction of PGH by lipoic acid occurs primarily in the organic layer of the work-up mixture. This is clearly demonstrated by the kinetic experiments summarized in Figures 2 and 3 and by the results of the incubation of authentic PGH_2 with lipoic acid illustrated in Figure 4. One possibility is that the reduction is solvent dependent and does not obtain in water. However, we feel it is more likely that the difference in products observed following reaction in the aqueous or organic phase is attributable to the relative rates of competing processes. It is well established that PGH is unstable in hydroxylic solvents and decomposes to a mixture of PGE, PGD, and hydroxy acids (Hamberg and Samuelsson, 1973; Nugteren and Hazelhof, 1973). The relative ratio of chloroform to hydroxylic solvents is 5.7 in the lower phase and 0.3 in the upper phase. Therefore, the rate of reduction of PGH by lipoate is probably much less than the rate of spontaneous decomposition in the predominantly hydroxylic upper phase. These observations are summarized in Scheme I.

SCHEME I



R = $(CH_2)_6CO_2H$ or $CH_2CH{=}CH(CH_2)_3CO_2H$
R′ = $(CH_2)_4CH_3$
R″ = $(CH_2)_4CO_2H$

Lipoic acid exerts a number of interesting effects when included in incubation mixtures actively synthesizing prostaglandins. It stimulates the overall rate of O_2 incorporation into fatty acid (Marnett and Wilcox, 1977) and it inhibits the nonspecific cooxidation of xenobiotics (Marnett et al., 1975). Since the reduction of the peroxy bridge in PGG or PGH by lipoate does not appear to occur in the incubation mixture, it can be concluded that this functionality is not important in retarding the rate of biosynthesis or in initiating xenobiotic cooxidation.

Acknowledgments

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