Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 24-32

LIPID PEROXIDATION DEPENDENT ALDRIN EPOXIDATION IN LIVER MICROSOMES, HEPATOCYTES AND GRANULATION TISSUE CELLS

B. Lang and P. Maier

Institute of Toxicology, Swiss Federal Institute of Technology and University of Zurich, CH-8603, Switzerland

Received May 6, 1986

SUMMARY: Lipid peroxidation activity was determined in liver microsomes, hepatocytes and cultured granuloma cells by measuring ethane and pentane production with an improved capillary gas chromatographic method. Lipid peroxidation initiated by ferrous ions and NADPH produced significantly more hydrocarbons at 4 % O_2 than under athmospheric (21 % O_2), hyperoxic or hypoxic conditions. In liver microsomes ferrous ions and ascorbic acid stimulated the non-enzymatic lipid peroxidation and concomitantly the epoxidation of aldrin. The results demonstrate that epoxidation of aldrin can be triggered by the iron initiated lipid peroxidation.

Oxidation and oxygenation reactions play an important role in the activation of xenobiotics to toxic, mutagenic and carcinogenic derivatives (1,2). In most cases the metabolic conversion of these compounds is catalyzed by mixed function oxidases (MFO), but peroxidative processes such as prostaglandin synthesis (3,4) or lipid peroxidation (5) can also be involved. A free radical mechanism has been postulated for the enzymatic and autoxidative peroxidation of endogenous polyunsaturated fatty acids (6,7).

Xenobiotics might initiate lipid peroxidation and/or serve as a cofactor during the peroxidase reaction becoming epoxidized in the process. These epoxides are often unstable and highly reactive, and therefore difficult to extract and to quantify. In order to circumvent this problem we used aldrin which is epoxidized to the stable product dieldrin (8) and can be detected in picogram amounts by gas chromatography. The low detection limit is important when extrahepatic tissues and cells with expected low activities are investigated(9-11).

Lipid peroxidation was investigated in subcellular fractions (liver microsomes) and intact cells (freshly isolated hepatocytes and cultured granulation tissue cells) by measuring the volatile hydrocarbons ethane and

0006-291X/86 $1.50

pentane (12-14). The analysis was improved by the use of capillary gas chromatography. The influence of tissue oxygen partial pressure in the propagation of lipid peroxidation (14,15) was investigated. Results obtained by simultaneous measurements of ethane, pentane and dieldrin formation suggest that epoxidation of aldrin in vivo can be mediated by lipid peroxidation.

MATERIALS AND METHODS

Chemicals:

Aldrin (1,2,3,4,10,10- hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-exo-dimethanonaphthalene) and dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octa-hydro-1,4-endo-5,8-exo-dimethanonaphthalene), purity > 99 %, were from Riedel-de-Haen, Seelze, FRG. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP were purchased from Sigma Chemical Co, St. Louis, MO, USA. H_2O_2 (30 %), ascorbic acid and n-hexane (zur Rückstandsanalyse) were from E. Merck, Darmstadt, FRG. Ethane and pentane standards were from Pan Gas, Lucerne, Switzerland.

Animals and freshly isolated cells:

Animals used were random bred Sprague Dawley male rats (210 - 250 g, SIV 50 Ivanovas Kissleg, FRG) kept under standard conditions (12 h light-dark cycle, diet No 890 from NAFAG, Gossau, Switzerland).

Hepatocytes were obtained by liver perfusion according the method of Berry and Friend (16) modified by Bieri et al. (17).

The granulation tissue was initiated by the injection of 25 ml germ-free air into the subcutaneous scapulaer area of the rats. After 4 days the growing tissue was dissected out and dissociated enzymically into single cells with collagenase-dispase according to standardized protocols (18).

Microsome preparations:

The preparation was performed at 4° C. The liver was dissected out, chopped and homogenized in a glass/teflon Potter homogenizer with a 0.15 M KCl-solution (4 % w/v) at pH 7.4. The homogenate was centrifuged at 9000 x g for 20 min and the supernatant again at 100'000 g for 30 min. The microsomal pellet was suspended in 0.15 M KCl (pH 7.4) to a protein concentration of 10 mg/ml and stored at -70° C. The protein content was determined by the method of Bradford (19) using bovine serum albumin as a standard.

Lipid peroxidation and ethane and pentane measurements:

Liver microsomes: 0.2 mg microsomal protein / ml was incubated in 100 mM phosphate buffer, pH 7.4 containing 1 mM glucose-6-phosphate and 0.1 mM NADP. Incubations were performed under controlled pO_2 in 14 ml glass flasks sealed with rubber caps. Oxygen tension of the stirred incubation medium was

determined by an intravascular oxygen sensor (Kontron Ltd. Zürich, Switzerland) and adjusted with nitrogen or oxygen to the desired O_2 concentration. The enzymatic lipid peroxidation was started by the addition of 7 U/ml glucose-6-phosphate dehydrogenase and Fe^{2+} ($FeCl_2$). The nonenzymatic reaction was carried out with heat denaturated microsomes (100° C, 10 min) and started with ascorbic acid and oxygen- free $FeCl_2$ solution.

Granulation tissue cells: 0.5 x 10^6 freshly isolated cells were cultured to confluency in 25 cm^2 flasks (total volume 65 ml) with 10 ml Dulbecco's modified medium supplemented with 10% fetal calf serum and 5 µg/ml gentamycin (12.5% CO_2; 5% or 19% O_2; pH 7.4; 36.5 °C; Rh 95%). Oxygen tension was controlled as described above. The reaction was started by the addition of the oxygen- free $FeCl_2$ solution to a final concentration of 50 µM.

Hepatocytes: Within four hours after isolation, 2 - 4 x 10^6 freshly isolated hepatocytes were incubated in 1 ml William's E medium without serum for 2 hours in sealed 14 ml glass flasks with adapted pO_2. Aldrin epoxidation was started with Fe Cl_2.

Analysis: 1 ml samples of the headspace from the incubation vials were analysed by a Carlo Erba HRGC 5160 capillary gas chromatograph equipped with a flame ionisation detector. The hydrocarbons were separated by a 18 m x 0.32 mm glass capillary column (PS - 255 as stationary phase, film thickness 3.1 µm). The carrier gas was hydrogen with a linear velocity of 0.17 m/sec. The oven temperature was 40° C and the injector and detector temperature was 160° C. The peak integration was performed by a Hewlett-Packard Integrator 3390A. For the quantification of the hydrocarbon gases a standardized curve for ethane and pentane (10 - 200 pmol) was used. The limit of detection is 10 pmol ethane or pentane. n-butane was taken as internal standard.

Lipid peroxidation-dependent aldrin epoxidation:

After a preincubation of denaturated liver microsomes for 2 min with iron ions and ascorbic acid a previously determined optimal concentration of 50 µM aldrin was added. The reaction was stopped by the addition of 5 ml ice cold n-hexane. The dieldrin formed was extracted into the n-hexane phase. The aqueous phase was separated by centrifugation and the organic phase stored at -20° C until analysis.

Analysis: Quantitative analysis of dieldrin was carried out again with a Carlo Erba gas chromatograph (model fractovap 4160) equipped with a ^{63}Ni-electron-capture detector HT-25 and a temperature programmer LT Mod. 430. The temperature program was 60° C for 1 min at injection, subsequently 50° C/ min up to 185° C and 10°C/min up to 240° C. The compounds in the hexane extract were separated on a glass capillary column (19 m x 0.32 mm) coated with SE-54 as the stationary phase, (film thickness 0.15 µM). The carrier gas

was hydrogen (0.2 kg/cm^2, linear velocity 0.5 m/s) and the make-up gas nitrogen (1.2 kg/cm^2). The temperature of the detector was 275° C. The dieldrin content was determined by measuring peak heights calibrated with known amounts of dieldrin and by peak analysis with a Hewlett-Packard Integrator Model 3390 A using external dieldrin standards.

RESULTS

The use of glass capillary gas chromatography allows the conjugated dienes to be separated and n-butane to use as an internal standard (Fig.1).

The pO_2 in the incubation mixture had a significant influence on the ethane/pentane formation by the NADPH-dependent lipid peroxidation in liver microsomes (Fig 2). Highest levels of hydrocarbons were found under tissue pO_2 levels (4% O_2). Only small amounts of pentane and ethane were detectable under anoxic (1% O_2) or hyperoxic conditions (21% O_2). The two hydrocarbons were formed in approximately equal amounts.

With intact cells at high pO_2 (21%) only hepatocytes showed a slight pentane formation (Fig. 3b). At low pO_2 (5% O_2), hepatocytes produced 18 pmol ethane and 50 pmol pentane (Fig. 3b) and no activity was found in granulation tissue cells (Fig.3c). In the presence of NADPH liver microsomes as a reference produced 30 pmol ethane and 180 pmol pentane/mg protein/h.

The optimal ferrous ion concentration found for the stimulation of lipid peroxidation was 50 µM in liver microsomes. Using this concentration, lipid peroxidation could be stimulated in hepatocytes and granulation tissue cells at low or high pO_2 (Fig.3b and c). Under hyperoxic conditions (21%) the stimulation by Fe^{2+} was significant in granuloma cells only. At 4 % O_2 however the stimulating activity of Fe^{2+} was moderate in hepatocytes and highest in granuloma cells and in liver microsomes(Fig. 3a-c).

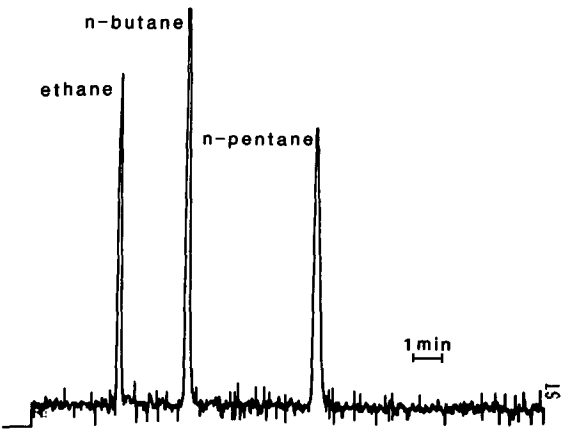


FIG. 1 Capillary gas chromatographic separation of ethane, n-butane and n-pentane.

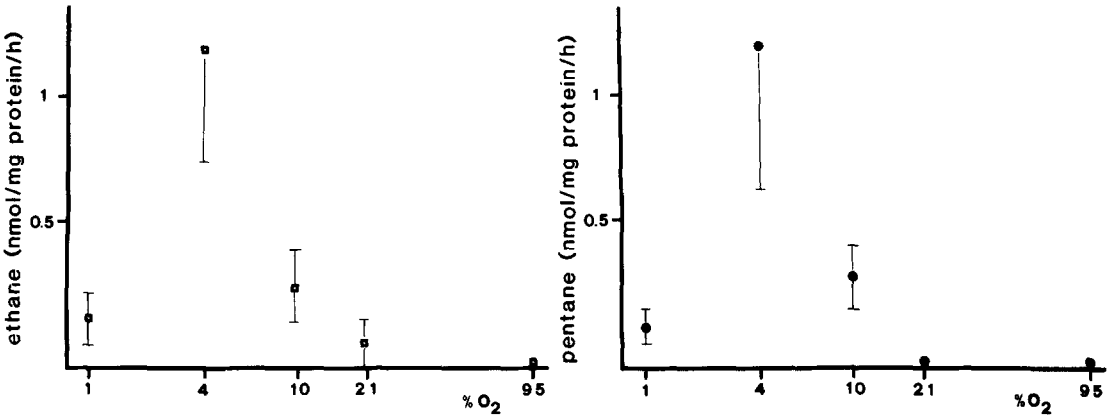
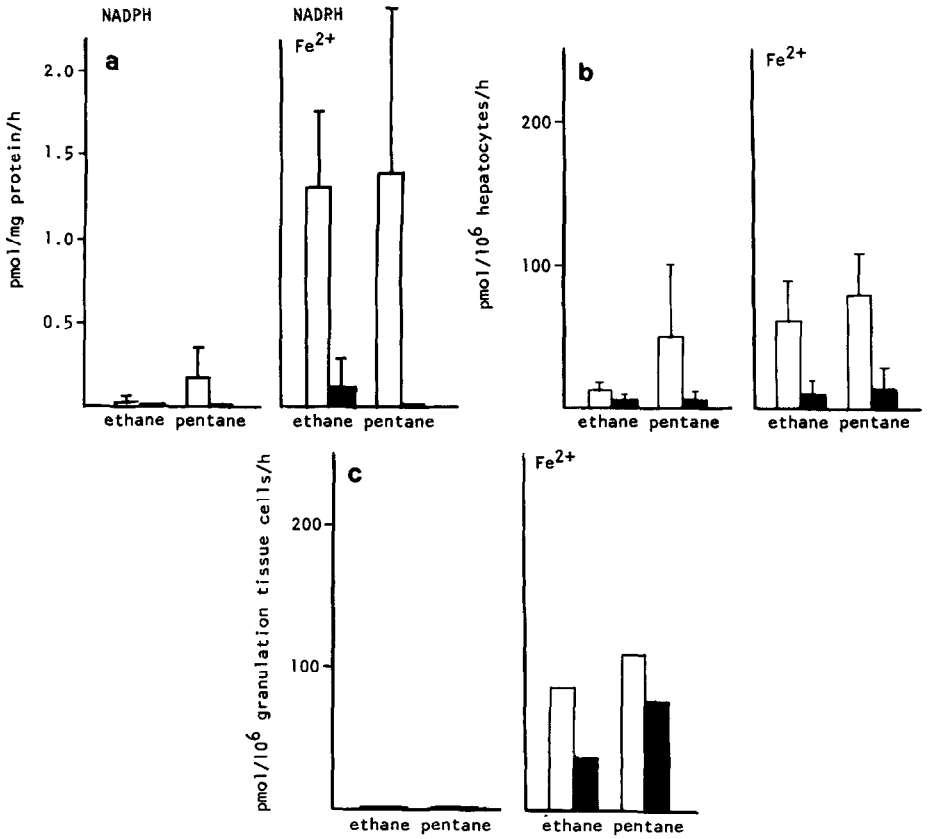
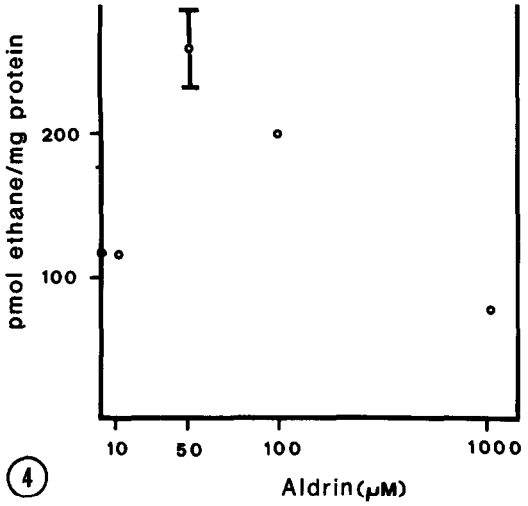


FIG. 2 Influence of pO_2 on ethane and pentane formation in rat liver microsomes. The incubation contained 0.2 mg rat liver microsomes, 1 mM glucose-6-phosphate, 7 U glucose-6-phosphate dehydrogenase , 1mM NADP in 1 ml of 100 mM phosphate buffer, pH 7.4. The incubation occured at 37° C. pO_2 was determined as described in material and methods. The reaction was started by the addition of 50 µM Fe^{2+}. Ethane and pentane analysis was performed after 2 h.
Mean value ± S.E.M., n = 6





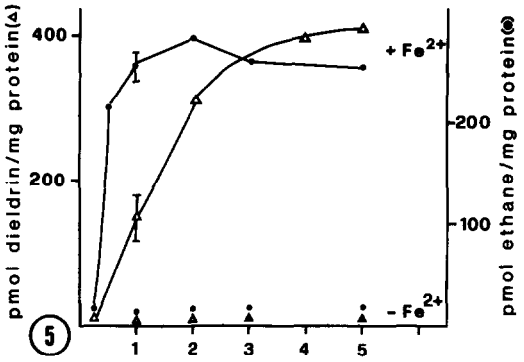


FIG. 4 Influence of aldrin concentration on ethane formation in denaturated rat liver microsomes. 0.2 mg microsomes/ml were incubated in 0.1 mM phosphat buffer (pH 7.4), aldrin, 200 μM ascorbic acid at 37° C and 4 % O_2. The reaction was started by the addition of 20 μM Fe^{2+}. The hydrocarbon analysis was performed after 2 hours. Blank incubations contained no iron.
Mean value ± S.E.M., n = 4, or mean value of two experiments.

FIG. 5 Time dependent ethane and dieldrin formation in denaturated rat liver microsomes. Denaturated rat liver microsomes (0.2 mg/ml) were incubated in 0.1 mM phosphat buffer, 50 μM aldrin, 200 μM ascorbic acid at 37° C and 4 % O_2. The reaction was started by the addition of 50 μM Fe^{2+}. Blank incubations contain no iron.
Mean value ± S.E.M., n = 6, or mean value of two experiments.

To exclude the monooxygenase-dependent aldrin epoxidation present in liver tissue, denaturated liver microsomes were used. This restricts the epoxidation activity to the non-enzymatic lipid peroxidation. The optimal substrate concentration found was 50 uM aldrin (Fig.4). The simultaneous determination of lipid peroxidation products and dieldrin formation showed that with prolonged incubation time, dieldrin production increased concomitantly with ethane and pentane formation (Fig.5). A lag phase of 15 minutes was observed. The absence of iron ions completely depressed lipid peroxidation and dieldrin formation.

FIG. 3 Influence of pO_2 and Fe^{2+} on ethane and pentane formation in rat liver microsomes, hepatocytes and granulation tissue cells.
a) Rat liver microsomes (0.2 mg). The incubation contained the NADPH regenerating system.
b) Hepatocytes ($2 - 4 \times 10^6$) were incubated in 1ml DMEM at 37° C.
c) Granulation tissue cells were cultured at 5 % and 21 % O_2 to confluency. The hydrocarbon analysis was performed after exposure for 2 hours.
Fe^{2+}: 50 μM
□ : 4 % O_2, ■ : 21 % O_2
Mean value ± S.E.M., n = 3 or mean value of two experiments

DISCUSSION

Measurements of hydrocarbon gases as a parameter of lipid peroxidation by the improved capillary gas chromatography analysis resulted in a sensitivity of approximately 10 pmol ethane and pentane. This allows a reproducible analysis to be performed in 1 ml headspace of the chosen incubation mixtures. With intact cells the determination of ethane seems to be more reliable than pentane, most likely because pentane can be degraded intracellularly (20).

Oxygen tension found in vivo in liver was between 4 - 13 % O_2 (21,22) and in the subcutaneous tissue between 2-5% (23). Our results indicate that reduction of O_2-concentration in vitro to that present in tissues (4%) enhanced the ethane and pentane formation in rat liver microsomes and hepatocytes (Fig.3a and b). Thus standard laboratory incubation conditions used in cell culture would not be representative for the in vivo situation. They suppress a xenobiotic mediated lipid peroxidation. Oxygen tension must be low enough to allow the presence of free radicals but high enough (no activity with 1% O_2) to propagate lipid peroxidation (Fig. 2). Similar findings were reported with brain and liver homogenates from mice (14). Therefore adaption of pO_2 to tissue levels in cell culture will be of specific interest to in vitro toxicity testing of xenobiotics.

Based on identical protein aequivalents (10^6 hepatocytes correspond to 1.3 mg protein) lipid peroxidation products formed in the microsomal liver fraction were twice as high as that found in intact hepatocytes. Active radical-scavenging compounds and enzymes in the cytoplasm of intact liver cells on the one hand and easy acces of ferrous ions to membrane lipids in subcellular preparations on the other might be the reasons for this difference. Accordingly, a lower stimulating effect by ferrous iron was found in hepatocytes than in microsomes.

The relative stimulating activity of ferrous ions alone and in combination with low pO_2 was more efficient in the extrahepatic granuloma cells than in hepatocytes (Table 1). Low pO_2 was found in vivo in

Table 1 The relative enhancement of ethane formation in hepatocytes and granulation tissue by Fe^{2+} and oxygen expressed in multiples of the mean value of ethane formed by hepatocytes at 19-20% O_2

Oxygen concentration	4-5% O_2	19-21% O_2
Hepatocytes	2x	1x
Hepatocytes + Fe^{2+} (50uM)	10x	1.5x
Gran.tissue cells	n.d.	n.d.
Gran.tissue cells + Fe^{2+} (50uM)	13x	6x

n.d.: not detectable

subcutaneous tissues (23). Therefore it is most likely that in vivo epoxidation of aldrin in extrahepatic tissues is triggered by an iron triggered lipid peroxidation. The conversion of aldrin to dieldrin might be used as a model reaction for a lipid peroxidation catalyzed epoxidation of xenobiotics.

The contribution of the nonenzymatic lipid peroxidation in denaturated liver microsomes induced by aldrin was highest at 50 uM (Fig. 4). At higher levels (100 -1000 μM) the chemical most likely scavenges oxygen radicals depressing lipid peroxides and subsequently inhibits propagation (25). The simultaneous formation of lipid peroxidation products (ethane) and of dieldrin (Fig. 5) clearly confirm that epoxidation of xenobiotics in extrahepatic cells can be mediated by lipid peroxidation.

ACKNOWLEDGMENTS

This work was supported by a grant of the Swiss National Science Foundation, contract Nr. 3.935 - 0.82.

REFERENCES

1) Miller, J.A. (1970) Cancer Res. 30, 559-576.

2) Dipple, A., C.J. Michejda, and E.K. Weisburger (1985) Pharmac. Ther., 27, 265-296.

3) Eling, T., J. Boyd, G. Reed, R. Mason and K. Sivarajah (1983) Drug Met. Rev., 14, 1023-1053.

4) Marnett, L.J. and T. Eling (1983) in Reviews in Biochemical Toxicology, 5 (Hodgson, Bend, Philpot eds.), 135-172, Elsevier/North-Holland.

5) Dix, T.A., and L.J. Marnett (1983) Science, 221, 77-79.

6) Aust and Svingen (1982) in Free Radicals in Biology, (w.A Pryor, ed.), pp. 1-28, Academic Press, Inc., New York

7) Yagi, K. (1982) (Ed.) Lipide Peroxides in Biology and Medicine, Academic Press, New York.

8) wolff, T. (1980) in Fine Needle Aspiration Biopsy of the Rat Liver (G. Zbinden ed.) pp. 49-59, Pergamon Press, Inc. Oxford.

9) Wiebel, F.J., T. Wolff and M. Lambiotte (1980) Biochem. Biophys. Res. Commun., 94, 466-472.

10) Van Cantfort, J., M. Leonard-Poma, J. Sele-Doyen and J.E. Gielen (1983) Biochem. Pharmac., 32, 2697-2702.

11) Lang, B., K. Frei and P. Maier (1986) (submitted)

12) Tappel, A (1980) in Free Radicals in Biology (W.A. Pryor, ed.) Vol. IV, pp. 1-47, Academic Press, New York.

13) Wendel, A. and D.E. Dumelin (1981) in Methods in Enzymology (Packer, L. ed.) Vol. 77, pp. 10-15, Academic Press, London.

14) Cohen, G. (1982) in Lipid Peroxides in Biology and Medicine (Yagi, ed.) Academic press, 199-222.

15) Kostrucha, J., A. Djovkor and H. Kappus (1984) Abstract P1/37, 6th Internat. Symposium Microsomes Drug Oxidations.

16) Berry, M.N. and D.S. Friend (1969) J. Cell Biol. 43, 506-520.

17) Bieri, F. , P. Bentley, F. Waechter and W. Stäubli (1984) Carcinogenesis, 5, 1033-1039.

18) Bradford, M. (1976) Anal. Biochem., 72, 248-254.

19) Donato, H. (1981) (Sohal, R.S. ed.) pp. 63-81, Elsevier/North-Holland Biomedical Press.

20) Filser, J.G., H.M. Bolt, H. Muliawan and H. Kappus (1983) Arch. Toxicol., 52, 135-147.

21) Kessler, M., H. Lang, E. Sinagowitz, R. Rink and J. Höper (1973) in oxygen transport to tissue, Part (A) (Eds. D.F. Bruley and U.I. Bicher, pp. 351, Plenum Press, New York.

22) Kieczka, H. and H. Kappus (1980) Toxicol. Lett., 5, 191-196.

23) Maier, P., H.P. Schawalder, B. Weibel and G. Zbinden (1986) Mutation Res. submitted.

24) Free, M.J., Schluntz G.A. and R.A. Jaffe (1976) Biology of Reproduction 14, 481- 488.

25) Egan, R.W., P.H. Cale, G.C. Beveridge, L.J. Marnett, and F.A. Kuehl Jr. (1980) Adv. Prostaglandin Thromboxane Res., 6, 153-155.