THE INVOLVEMENT OF FREE RADICALS AND LIPID PEROXIDATION IN INFLAMMATION

Pharmacological implications

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THE INVOLVEMENT OF FREE RADICALS AND LIPID PEROXIDATION IN INFLAMMATION: PHARMACOLOGICAL IMPLICATIONS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. J. SPERNA WEILAND
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
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Peter Cornelis Bragt

geboren te Tilburg

krips repro meppel

PROMOTOR : PROF. DR I. L. BONTA
CO-REFERENTEN : PROF. DR. J. F. KOSTER
PROF. J. H. P. WILSON

Het onderzoek dat leidde tot dit proefschrift werd verricht op de afdeling Farmacologie der Medische Faculteit van de Erasmus Universiteit Rotterdam, onder supervisie van prof. dr. I. L. Bonta.

Voor mijn ouders

Voor Diana

....Standing by the crib of one's own baby, with that world-old pain of compassion and protectiveness toward this so little creature that has all its course to run, the heart flies back in yearning and gratitude to those who felt just so toward one's self. Then for the first time one understands the homely succession of sacrifices and pains by which life is transmitted and fostered down the stumbling generations of men....

Christopher Morley

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en

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The author of this thesis has contributed to the following papers:

1. Cannulated teflon chamber implant in the rat: A new model for continuous studies on granulomatous inflammation.
   P.C. Bragt, I.L. Bonta & M.J.P. Adolfs, *J. Pharmacol. Meths. 3, 51-61 (1980).*

2. In vivo metabolism of {1-^{14}C} arachidonic acid during different phases of granuloma development in the rat.
   P.C. Bragt & I.L. Bonta, *Biochem. Pharmacol. 28, 1581-1586 (1979).*

3. Indomethacin inhibits the in vivo formation of the lipoxygenase product HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid) during granulomatous inflammation in the rat.
   P.C. Bragt & I.L. Bonta, *J. Pharm. Pharmacol. 32, 143-144 (1980).*

4. Dissociation between prostaglandin and malondialdehyde formation in exudate and increased levels of malondialdehyde in plasma and liver during granulomatous inflammation in the rat.
   P.C. Bragt, E.J.P.M. Schenkelaars & I.L. Bonta, *Prostaglandins Med. 2, 51-61 (1979).*

5. Increased lipid peroxidation and decreased hepatic aminopyrine metabolism during carrageenin-induced granulomatous inflammation in the rat.
   M.J.P. Adolfs, I.L. Bonta & P.C. Bragt, *Br. J. Pharmacol. 68, 123P-124P.*

6. Depletion of hepatic antioxidants during granulomatous inflammation in the rat and local anti-inflammatory effects of free radical scavengers.
   P.C. Bragt, J.I. Bansberg & I.L. Bonta, *Agents Actions Suppl. 7, - (1980).*

7. Anti-inflammatory effects of free radical scavengers and antioxidants: Further support for pro-inflammatory roles of endogenous hydrogen peroxide and lipid peroxides.
   P.C. Bragt, J.I. Bansberg & I.L. Bonta, *Inflammation 4, 289-299 (1980).*

These articles have been incorporated in the Appendix of this thesis. Other papers to which the author has contributed:

8. Relationship between aggregation and prostaglandin (PG)-biosynthesis in rat platelet rich plasma (PRP).
   H. Bult, P.C. Bragt & I.L. Bonta, *Thromb. Haemostas. 38, 37 (1977), abstr.*

9. A polyether sponge constituent which is antioxidant, anti-inflammatory and increases prostaglandin concentrations at the inflamed site.
   M.J. Parnham, I.L. Bonta, M.J.P. Adolfs & P.C. Bragt, *Agents Actions 7, 539-544 (1977).*

10. Metabolism of arachidonate in vivo during different phases of granuloma development in the rat.
    P.C. Bragt & I.L. Bonta, in: *Advances in Inflammation Research, Vol. 1 (G. Weissmann, B. Samuelsson & R. Paoletti, eds.), Raven Press New York, pp. 499-502 (1979).*

11. Involvement and effects of prostaglandin E in experimental granulomatous inflammation.
I.L. Bonta, M.J. Parnham & P.C. Bragt, *Int. J. Tiss. React. 1, 51-55 (1979).*

12. In vivo models to study interactions between the arachidonate cascade and granulomatous inflammation.
I.L. Bonta, P.C. Bragt, M.J.P. Adolfs & M.J. Parnham, *Agents Actions Suppl. 7, - (1980).*

13. Anti-rheumatic drugs: Present deadlock and new vistas.
I.L. Bonta, M.J. Parnham, J.E. Vincent & P.C. Bragt, in: *Progress in Medicinal Chemistry, Vol. 17 (G.P. Ellis & G.B. West, eds.), Elsevier/North-Holland Biomedical Press, pp. 185-273 (1980).*

14. Oxidant stress during inflammation: Anti-inflammatory effects of antioxidants.
P.C. Bragt & I.L. Bonta, *Agents Actions 10, in press (1980).*

15. Role of trace elements in hepatic changes during inflammatory conditions.
P.C. Bragt & I.L. Bonta, *Agents Actions Suppl. 8, in press (1981).*

16. Comparison of the effects of inhibitors of cytochrome P-450 on human platelet aggregation and arachidonic acid metabolism.
M.J. Parnham, P.C. Bragt, A. Bast & F.J. Zijlstra, *Biochem. Biophys. Acta, submitted for publication.*

17. Zuurstofradicalen en ontsteking.
P.C. Bragt, *Chem. Weekbl. Mag. in press (Febr. 1981).*

CONTENTS

PREFACE

In 1954, Duboulez and Dumas reported the occurrence of lipid peroxides in the hind limb of a rat as a consequence of injury by heat, X- and ultraviolet radiation. They distinguished between two types of peroxide formation: one as a direct result of injury and the other as a result of the inflammatory process following injury.

Seventeen years later it was discovered that aspirin-like anti-inflammatory drugs inhibited the biosynthesis of prostaglandins, which are formed from essential fatty acids via endoperoxide intermediates. Since this discovery, the interactions between prostaglandin biosynthesis, its inhibition and acute inflammation, have been widely studied, and it appeared that the inhibition of the biosynthesis of prostaglandins was an essential component in the mode of action of anti-inflammatory drugs. Subsequent studies showed that many anti-inflammatory drugs which were highly efficient against inflammation models, were only slightly effective against rheumatoid arthritis. This fact led to the initiation of new investigations with anti-rheumatic drugs, but now with the emphasis on alternative modes of action, i.e. not limited to the inhibition of prostaglandin biosynthesis and not limited to the site of inflammation alone.

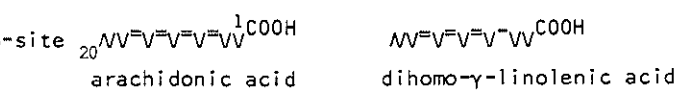
This thesis deals with the suspected involvement of free radicals and lipid peroxidation in various types of chronic inflammation in the rat, which are relevant as models for rheumatoid arthritis in man. Apart from local interactions at the site of inflammation, systemic effects have also been studied. Attention has been focussed on the role of the liver during a remote extra-hepatic inflammation. The underlying supposition is the possibility of a central role of the liver in endogenous modulation of inflammation.

PART I. INTRODUCTION

1. LIPID PEROXIDATION

Lipid peroxidation is the oxidative deterioration of lipids involving free radical mechanisms. Sometimes the term 'autoxidation' is used in the literature to describe the chain of reactions occurring during this deterioration, although, strictly speaking, it is incorrect: during lipid peroxidation, molecular oxygen acts as the oxidant and accepts electrons from the oxidizing lipids to form peroxides with them. In fact, when left by itself (i.e. when atmospheric oxygen is excluded) even a fatty acid with a high degree of unsaturation will be stable for many hours, days and perhaps even years. The term 'deterioration' is also inadequate, in view of the fact that enzyme-catalyzed lipid peroxidation provides an organism with essential, locally acting hormones, the prostaglandins. Thus, conversion is a better term than deterioration.

In this thesis, I shall distinguish between two different types of lipid peroxidation, varying according to the initiating process. The peroxidation of unsaturated fatty acids containing a *cis,cis*-1,4-pentadiene system, for example arachidonic acid (20:4, ω6) and dihomo-γ-linolenic acid (20:3, ω6) (see structures below), which is initiated by a fatty acid



arachidonic acid dihomo-γ-linolenic acid

cyclo-oxygenase (or prostaglandin synthetase) and a lipoxygenase, will be referred to as 'specific' lipid peroxidation. The term 'specific' is used to cover the substrate and product specificity of the peroxidative coversion of the fatty acids involved. On the other hand, the free radical-induced peroxidation of lipids will be referred to as 'unspecific' lipid peroxidation, because almost any lipid substrate is susceptible to free radical attack and numerous products will be formed, although the formation of many products is dependent on certain structural properties of the substrate involved. An example of this is the formation of malonaldehyde (or malondialdehyde) from unsaturated fatty acid precursors. Significant formation of malonaldehyde during unspecific peroxidation only occurs if the substrate has more than two double bonds (in other words, if it is more

than twofold unsaturated). This will be discussed in the section on reaction mechanisms of lipid peroxidation.

This chapter will deal with mechanisms of lipid peroxidation, the different ways in which it can be initiated, the different radicals involved and their eventual scavengers, the problems of the detection of lipid peroxidation in biological systems (especially living organisms) and the consequences of lipid peroxidation for these systems or organisms. Although a wide variety of lipids are susceptible to peroxidation under appropriate conditions (i.e. temperature, oxygen pressure, presence of catalysts), attention will be focussed on polyunsaturated fatty acids (PUFA). The reason for this is twofold: PUFA are of major importance as constituents of all structural membranes of mammals and secondly, PUFA are extremely prone to lipid peroxidation, even at body temperature, on the base of their structural and thermodynamic properties.

1.1. Free radical chemistry of lipid peroxidation

This section is largely based on reviews by Swern (1961), Slater (1972), Pryor (1973), Mead (1976) and Mead and Fulco (1976).

Studies on oxidizing monolayers of PUFA clearly demonstrate that lipid peroxidation consists of a chain of free radical processes. The chain starts with the 'initiation', i.e. the generation of free radicals from nonradical parent molecules (reaction 1). The initiator may be any source

$$RH \rightarrow R\cdot + H\cdot \qquad (1)$$

of sufficient energy, like heat, ultraviolet radiation, X-rays and in this case the rupture of the covalent C-H bond is called 'homolysis'. When the dissociation of the C-H bond is caused by the action of other atoms or molecules, as for example in reactions 2 and 3, this is referred to as

$$RH + O_2 \rightarrow ROO\cdot + H\cdot \qquad (2)$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO\cdot + HO^- \qquad (3)$$

'molecule-induced homolysis'. Reaction 2 may represent the direct attack of singlet oxygen ($^1\Delta_g O_2$; the activated state, which is 92 kJ above the ground state or triplet oxygen, $^3\Sigma_g^- O_2$), while reaction 3 is of great importance in the propagation of NADPH-dependent lipid peroxidation in liver cells.

'Propagation' is the sequence of free radical reactions following the initiation step. During propagation, the total number of radicals either

remains constant or increases and many intermediates will be produced and degraded. Examples of propagation are given in reactions 4 and 5. Also during

$$R\cdot + O_2 \rightarrow ROO\cdot \quad (4)$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot \quad (5)$$

propagation, the chain of a molecule R may break down into several smaller fragments and these fission products may then be partly radical and partly nonradical in nature.

When radicals combine to deliver nonradical species, as for example shown in reactions 6-8, this is called the 'termination' phase. In

$$2R\cdot \rightarrow RR \quad (6)$$

$$ROO\cdot + R\cdot \rightarrow ROOR \quad (7)$$

$$2ROO\cdot \rightarrow O_2 + ROOR \quad (8)$$

the case of adequately chosen compounds and reaction conditions, the end products obtained in the termination phase may be polymeric substances, such as polyethylene, polyvinylchloride or polyethylene terephthalate. The reaction chain may even be broken during the propagation phase, if relatively unreactive radicals are formed. Unreactive radicals are, for example, formed from antioxidants, to yield products with a long half-life. The same effect can be achieved by lowering the temperature during the propagation phase, thus reducing the chances of collision of the radical species. It will be obvious now, that the addition of antioxidants, e.g. tocopherols, propyl gallate and butylated cresols, prevent the propagation phase of lipid peroxidation and thereby the usual formation of stable end products. Slater defines antioxidants in the broadest sense as: 'compounds, which inhibit the oxidative deterioration of other materials, for example lipids'. Using this definition, the antioxidants comprise free radical scavengers, chain-breaking compounds and even chelators of metal ion catalysts. Free radical scavengers react with radical species to form nonradical products. Lipid peroxidation is prevented by the addition of certain scavengers, because the initiating radicals are transformed into less reactive molecules. The boundaries between 'real' antioxidants and 'real' scavengers are not very sharp. Thus, scavengers are not necessarily antioxidants as some radicals may have reducing properties, rather than oxidizing ones. Analogously, an antioxidant is not necessarily a radical scavenger, for instance the metal

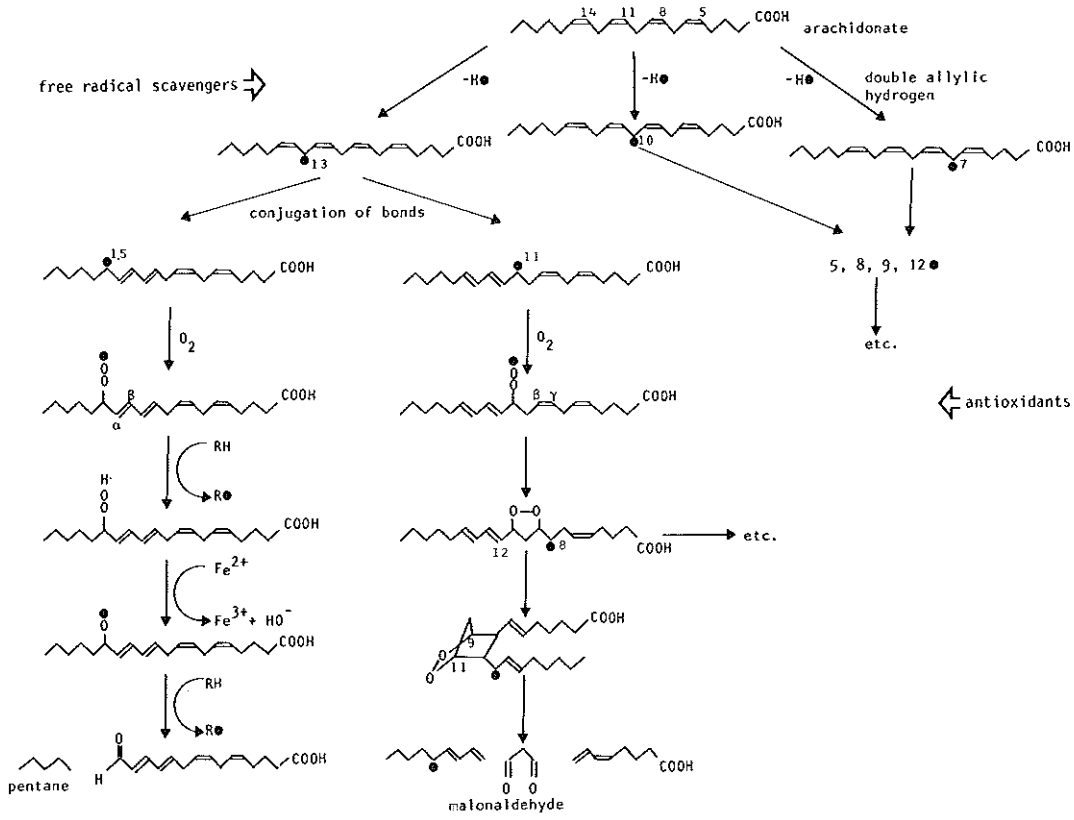
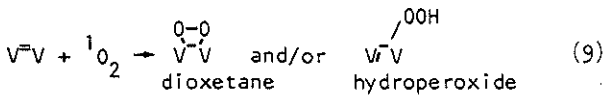


Figure 1. Mechanism of free radical-induced lipid peroxidation. This scheme has been composed from data in the literature. See text for further details.

chelator, EDTA.

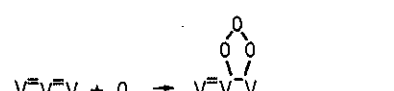
In summary, it is possible to fit the pieces of the puzzle of lipid peroxidation into a scheme of reaction pathways. The scheme should explain the formation of the well-known stable end products of lipid peroxidation and also the levels of interaction of free radical scavengers and antioxidants. A generalized scheme of reactions has been composed from data in the literature and is presented in Figure 1. This figure shows that the initial step in the peroxidation of PUFA (e.g. arachidonic acid) is the abstraction of a hydrogen atom (H·) from preferred positions of the fatty acid. The positions, which are the most liable to hydrogen abstractions are the methylene groups, interposed between the unsaturated bonds and these are localized at the carbon atoms 7, 10 and 13 of the arachidonate molecule. Potent molecular initiators of lipid peroxidation are the radical species, superoxide radical ($\cdot O_2^-$, Kellogg and Fridovich, 1975) and the hydroxyl radical (HO·, Lai and Piette, 1978), and also the nonradical species, singlet oxygen ($^1\Delta_g O_2$ or $^1O_2$, Baird et al, 1977) and ozone (O_3, Goldstein et al, 1968).

As the superoxide radical is considered to be relatively unreactive toward other molecules (Koppenol and Butler, 1977), it is conceivable that $\cdot O_2^-$ has to be transformed into one of the other species mentioned, before it is able to attack lipids. Singlet oxygen attacks double bonds directly and the intermediates formed may be either hydroperoxides or dioxethanes (see Rabek and Ranby, 1978) as shown in reaction 9. Room temperature is probably



(9)

sufficient to cause the cleavage of these intermediates, resulting in free radicals and concomitant lipid peroxidation. The exact mechanisms of ozone-induced lipid peroxidation are still obscure, but recent investigations (Pryor, 1978) suggest that the initiation of peroxidation may proceed via reactions 10-11. The decomposition of the trioxide intermediate into



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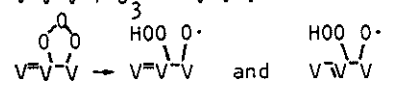
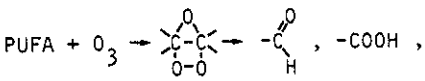


(11)

radical fragments (11), which can act as initiators, explains the fact that conjugated dienes are observed during ozone-induced lipid peroxidation. Besides leading to the formation of trioxides, ozonolysis of olefines also leads to the formation of a type of ozonides named after Criegee. A Criegee ozonide decomposes to aldehydes and acids, but not to radicals, as shown in reaction 12.



(12)

Criegee ozonide

After the initial abstraction of hydrogen atoms from the positions 7, 10 and 13 of the arachidonate molecule (Figure 1), the reaction sequence proceeds with a rearrangement of double bonds to conjugated diene fragments. It will be clear from the figure that there will be 6 possible tetraenes containing a conjugated diene fragment. The tetraenes have a radical site at the positions 5, 8, 9, 11, 12 and 15, respectively. The following step is the attachment of molecular oxygen to the radical site of the conjugated diene. If a hydrogen atom is picked up from another fatty acid molecule, the resulting molecule is a relatively stable hydroperoxide, characterized by its -OOH group. Cyclization will occur when the peroxy-radical group (-OO·) has an adjacent double bond in a β,γ-position. The resulting cyclic peroxides may undergo further cyclization to prostaglandin endoperoxide-like compounds. The latter may decompose, due to heat or H^+, to several fragments, including malonaldehyde, a component which can be very sensitively detected by its reaction with 2-thiobarbituric acid (see Chapter 1.4). The theoretical ratio of formation of hydroperoxides versus cyclic peroxides is 2:4. However, in the presence of metal catalysts (ferrous ions), the hydroperoxides decompose to several fragments and among these, alkanes (ethane in the case of ω3-fatty acids and pentane in the case of ω6-fatty acids) are present (Donovan and Menzel,1978). The importance of alkanes for the detection of lipid peroxidation in vivo, will be considered in Chapter 1.4. The peroxidation of fats results in numerous stable end products, many of which contribute to the observed 'rancidity', as they flavour the original fat by their presence. Among these end products are hydroperoxides, endoperoxides, alkanes, alkenes, aldehydes, alcohols and carbon dioxide (Loury, 1972). Even epoxides and carbon monoxide have been detected under certain circumstances in peroxidizing biological

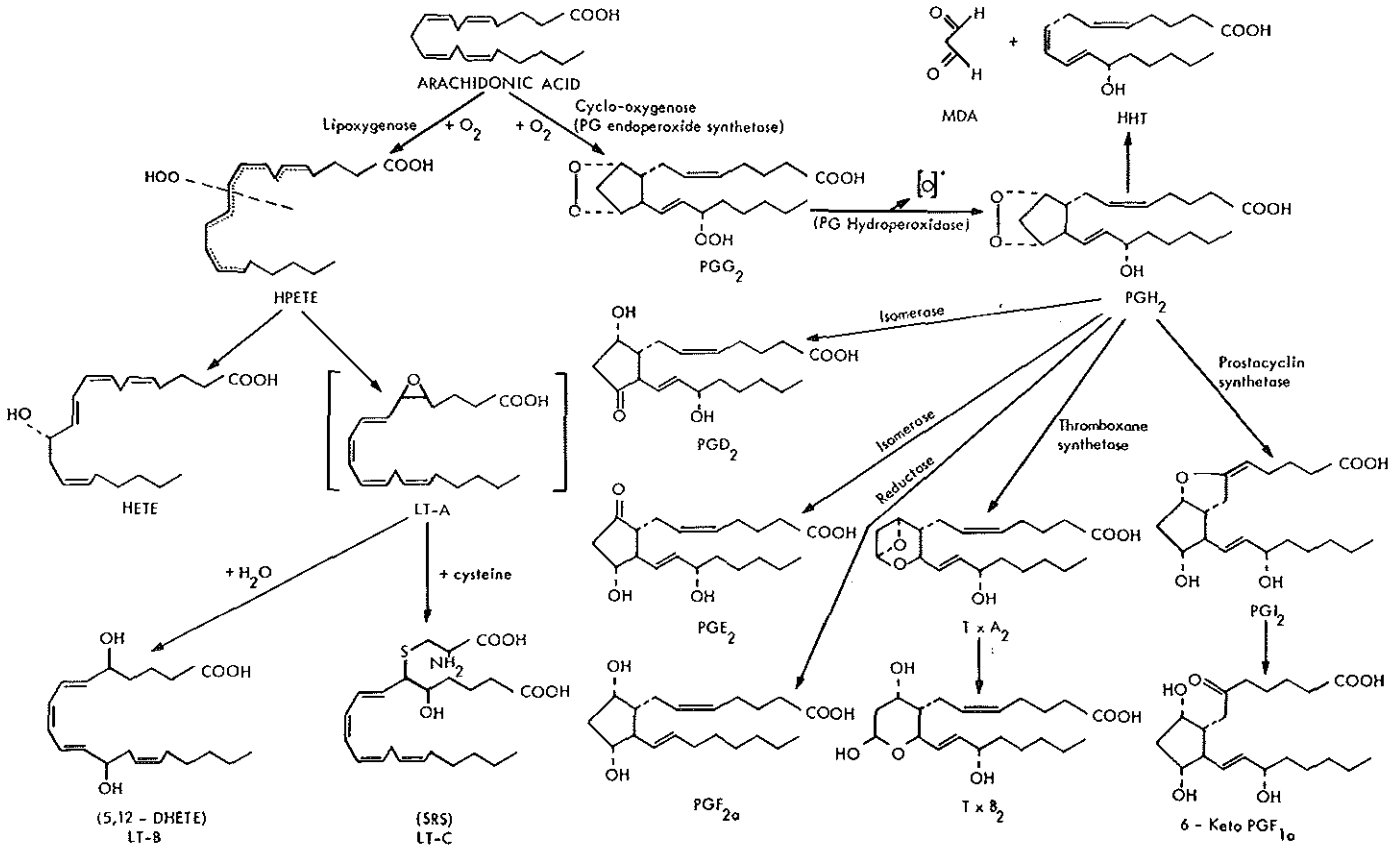


Figure 2. Multiple pathways of arachidonate metabolism (courtesy of M.J. Parnham). See text for details.

systems (Sevanian et al, 1979; Wolff and Bidlack, 1976).

1.2. Enzyme-catalyzed lipid peroxidation

a. Biosynthesis of prostaglandins and related substances

Virtually all mammalian cells have the capacity to biosynthesize prostaglandins, the lipid autacoids (local hormones), which were named by Von Euler in Sweden (1935). He believed that the substances causing vaso-depression and contraction of the uterus originated from the prostate gland. The effects on the uterus of substances in fresh human semen actually led to the discovery of prostaglandins (Kurzrok and Lieb, 1930). This chapter surveys the biosynthesis of prostaglandins and related lipids and the interactions with lipid peroxidation of different origin. For general aspects of the mechanisms of prostaglandin biosynthesis and (patho-)physiological effects, other than related to inflammation, the reader is referred to excellent reviews by Hinman (1972), Gibson (1977), Hemler and Lands (1977), Vapaatalo and Parantainen (1978) and Lands (1979).

The chemical structure of the prostaglandins is derived from the hypothetical 'prostanoic acid', with 20 carbon atoms and a cyclopentane element as elements of the skeleton. The autacoids are synthesized *de novo* from specific fatty acid precursors, namely, dihomo-γ-linolenic acid (8,11,14-eicosatrienoic acid), arachidonic acid (5,8,11,14-eicosatetraenoic acid) and 5,8,11,14,17-eicosapentaenoic acid, with the formation of prostaglandins (PG's) of the 1, 2 and 3 series, respectively. The enzyme system responsible for the *bis*-dioxygenation of the free fatty acids is cyclo-oxygenase (PG-synthetase). Cyclo-oxygenase is localized in the microsomal fraction of cells (100,000 g), consists of four subunits of 70,000 daltons and requires heme for maximal activity. The substrates are obligatory free fatty acids, which have to be de-esterified before they are converted to prostaglandins. Arachidonic acid, the most abundant precursor in mammals, is esterified mainly in the membrane phospholipids. Other, less important stores are the triglycerides (e.g. adipose tissue) and cholesterol esters (e.g. adrenal tissue). The distribution of arachidonate in the phospholipids of human platelets is: 47% in the phosphatidyl ethanolamine fraction, 28% in the phosphatidyl inositol fraction, and 13 and 12% in the phosphatidyl serine and choline (lecithin) fractions, respectively (Blackwell et al, 1977).

As a result of the actions of acylhydrolases (e.g. phospholipase

A_2, which splits off the fatty acid from the 2'-acyl position in phospholipids), the polyenoic fatty acids are liberated from their stores and once in the free form, become substrates for cyclo-oxygenases and lipoxygenases. The multiple pathways of the metabolism of arachidonate are given in Figure 2 (courtesy of M.J. Parnham). Lipoxygenase inserts one oxygen molecule into the fatty acid, resulting in a hydroperoxy intermediate (HPETE). Depending on the type of lipoxygenase, the hydroperoxy moiety may be localized on the 5, 8, 12 and 15 position (see Tobias and Hamilton, 1979). The hydroperoxy fatty acid is converted to the corresponding hydroxy derivative by the action of a peroxidase, which may be part of the lipoxygenase itself.

The heme-requiring *bis*-dioxygenase, cyclo-oxygenase, inserts two oxygen molecules into one substrate molecule, resulting in the formation of the prostaglandin endoperoxide, PGG_2, which is converted to PGH_2 by a peroxidase with an affinity for the 15-hydroperoxy moiety of PGG_2. In aqueous solutions, PGH_2 has a half-life of 4 to 5 min. at 37°C. In cells, further conversion of PGH_2 is assured by the presence of isomerases, reductases and synthetases, which, depending on the kind of tissue, catalyze the formation of the 'classical' PG's, PGE_2, $PGF_{2\alpha}$ and PGD_2, the thromboxanes, TXA_2 ($T_{\frac{1}{2}}$ = 30 s at 37°C) and its decomposition product, TXB_2. Prostacyclin (PGI_2, $T_{\frac{1}{2}}$ = 10 min. at 20°C) and its stable metabolite, 6-keto$PGF_{1\alpha}$, are also formed. Presumably also as a result of a thromboxane synthetase, the hydroxy-fatty acid, 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malonaldehyde are formed. Thus, malonaldehyde is not only formed as a result of the free radical-induced peroxidation of lipids (see Chapter 1.1), but also enzymatically during the biosynthesis of prostaglandins, via the endoperoxide intermediates, PGG_2 and PGH_2.

Recently, it has been shown that the formation of 'slow-reacting substance of anaphylaxis' (SRS-A), the lipid autacoid released from lungs on treatment with cobra venom or on immunological challenge (Feldberg and Kellaway, 1938; Kellaway and Trethewie, 1940), is dependent on a lipoxygenase with a specificity for the 5-position of the arachidonate molecule (Murphy et al, 1979). The originally postulated structure of SRS, given in Figure 2, is a 5-hydroxy-fatty acid derived from arachidonic acid with cysteine substituted at the 6-position. However, the structure of the substituent, which is thioether-linked at this position, is subject to controversion. A recent paper (Morris et al, 1980) revealed the presence of a cysteinyl-glycine substituent, which might be the ultimate result of

proteolytic cleavage of a complete glutathione molecule linked to the 6-position. It is speculative to consider SRS's as detoxified hydroperoxides, in analogy with hepatic glutathione conjugation. It is conceivable that different SRS's exist depending on the kind of synthesizing cells. Recently, the name 'Leukotrienes' has been introduced for the class of derivatives from 7,9,11,14-eicosatetraenoic acid (Borgeat and Samuelsson, 1979). Besides the formation of 5-HPETE, the intermediate in the biosynthesis of leukotrienes (LT's), hydroperoxy moieties are also found to be inserted at positions 8, 12 or 15 of the arachidonate molecule, resulting in 8-HPETE, 12-HPETE and 15-HPETE, respectively (see Tobias and Hamilton, 1979). Figure 3 shows the products formed as a result of the lipoxygenase activities in different cells.

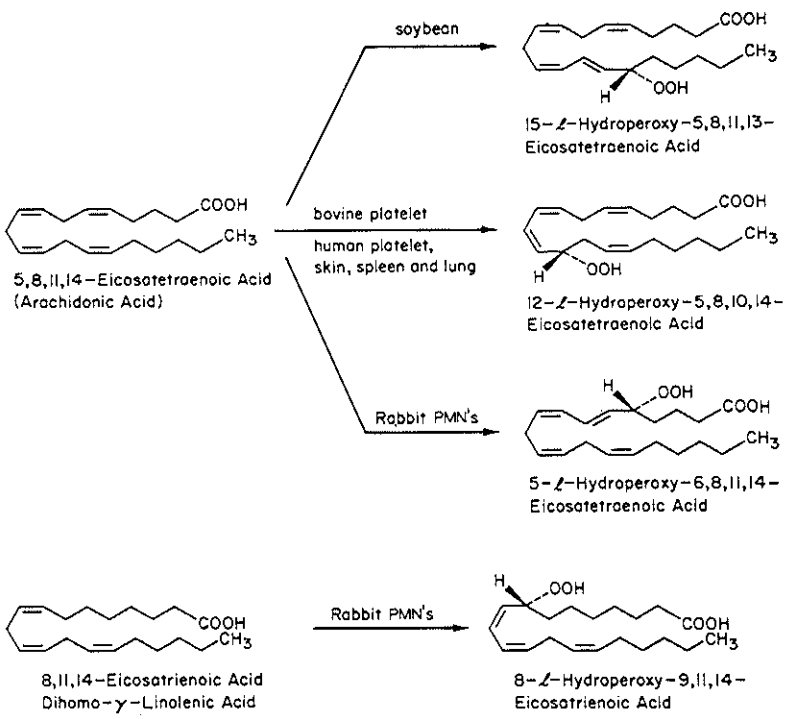
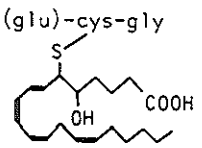


Figure 3. Formation of hydroperoxy intermediates from fatty acid precursors, depending on the type of cell involved (adapted from Tobias and Hamilton, 1979).



SRS-A
proposed structure(s)

b. Interactions between prostaglandin synthetase-dependent and -independent lipid peroxidation

The formation of prostaglandin endoperoxides is not merely limited to the enzymatic actions of cyclo-oxygenase in various tissues, since it has been shown that during the autoxidation of PUFA, prostaglandin endoperoxide-like compounds are formed (Pryor and Stanley, 1975; Pryor et al, 1976). Furthermore, the nonvolatile precursor of malonaldehyde formed from PUFA containing more than two double bonds, is an endoperoxide (Pryor et al, 1976; see Figure 1). In view of the fact that, from a trienoic acid, 128 stereoisomers of the endoperoxide could possibly be formed during lipid peroxidation, it is extremely likely that at least a few of them bear a close resemblance to the prostaglandin endoperoxides, PGG and PGH (Pryor and Stanley, 1975). In addition to the formation of prostaglandin endoperoxide-like compounds during PUFA autoxidation, hydroperoxides are also formed. It has for example been shown that the precursor of SRS-A, namely 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) is formed when arachidonic acid is exposed to atmospheric oxygen for 48 h (Porter et al, 1979). This emphasizes the point of view according to which SRS's are regarded to be detoxified products of hydroperoxides due to conjugation with glutathione or its fragments.

Another interaction between cyclo-oxygenase-dependent and -independent lipid peroxidation is the fact that cyclo-oxygenase requires a peroxide activator to induce its activity (Cook and Lands, 1976). Evidence for this was obtained by the observed inhibition of cyclo-oxygenase activity on the addition of glutathione peroxidase. This enzyme, which requires reduced glutathione (GSH) as an electron donor, catalyzes the conversion of hydrogen peroxide (or organic hydroperoxides) to water (or the corresponding

hydroxy derivatives). A direct implication of the cyclo-oxygenase requirement for a peroxide activator is the regulating role on the biosynthesis of prostaglandins of peroxides (hydroperoxides, hydrogen peroxide, superoxide anions) or factors controlling the cellular levels of peroxides (GSH + glutathione peroxidase, catalase, superoxide dismutase, vitamin E; see Chapter 1.3). Very low levels of peroxide may be sufficient to trigger the biosynthesis of prostaglandins, and PGG_2, once formed, can take over the activation of cyclo-oxygenase by hydroperoxides (Hemler et al, 1979). These possibilities have been summarized in Figure 4. In view of the fact that

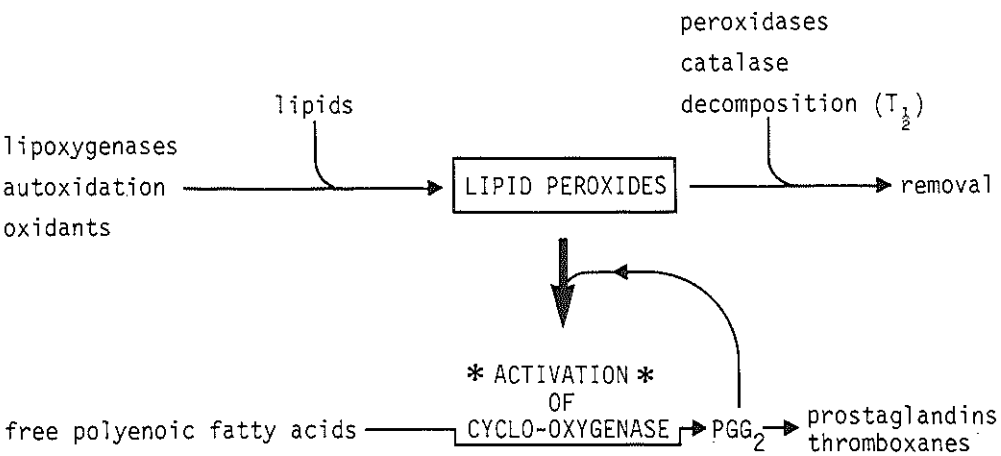
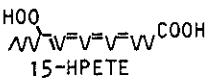


Figure 4. Requirement of cyclo-oxygenase for a peroxide activator (adapted from Hemler et al, 1979). Lipid peroxides are formed as a result of fatty acid autoxidation or lipoxygenation and destroyed by spontaneous or enzyme-catalyzed decomposition. The prostaglandin endoperoxide, PGG_2 may replace lipid peroxide as an activator.

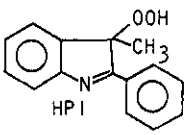
lipoxygenases and cyclo-oxygenase are distributed throughout the tissues in close proximity the generation of peroxide activators for cyclo-oxygenase may be one of the physiological roles of the lipoxygenases. In addition, the lipoxygenase pathway may function as a kind of 'overflow' pathway for the metabolism of polyenoic fatty acids, since it has been reported

that platelet lipoxygenase deficiency leads to a decreased treshold for aggregation induced by arachidonic acid (Okuma and Uchino, 1977).

The enzyme catalyzing the formation of prostacyclin (PGI_2) from the prostaglandin endoperoxide PGH_2, prostacyclin synthetase, is very sensitive to inhibition by peroxides. For instance, 15-hydroperoxy arachidonate (15-HPETE) and 3-hydroperoxy-3-methyl-2-phenyl-3H-indole (HPI) are potent



15-HPETE



HPI

inhibitors of prostacyclin synthetase (Gryglewski et al, 1976; Terashita et al, 1979). The effects of peroxides as inhibitors of prostacyclin synthetase are not very specific, as 9-, 11- and 12-HPETE are all potent inhibitors (IC_{50} ranges from 3-10 μM) of human platelet cyclo-oxygenase (Siegel et al, 1979a). This inhibition is most probably due to the peroxidase activity of cyclo-oxygenase, which is also responsible for the liberation of 'oxygen-centered' radicals as a result of catalyzing the conversion of PGG to PGH. During this conversion, cyclo-oxygenase is inactivated (Egan et al, 1976). In contrast, the biosynthesis of prostaglandins is increased in the presence of free radical scavengers such as lipoic acid (Marnett and Wilcox, 1977; Egan et al, 1978), 2-aminomethyl-4-*t*-butyl-6-iodophenol (MK-447), phenol (Kuehl et al, 1977) and sodium iodide (Egan et al, 1978). In later chapters (2.1 and 2.2) it will be discussed that some free radical scavengers have anti-inflammatory properties, despite the fact that they stimulate (or do not affect) the biosynthesis of prostaglandins and related substances.

c. Inhibitors of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism

Inhibitors of prostaglandin biosynthesis may act at a number of distinct levels. These levels of action of some frequently used inhibitors (Flower, 1974; Gibson, 1977; Needleman, 1978; Nickander et al, 1979; Higgs et al, 1979; Shen, 1979) are shown in Figure 5. The phospholipase A_2-catalyzed de-esterification of arachidonic acid and dihomo-γ-linolenic acid is inhibited by mepacrine, chloroquine and glucocorticoids, such as hydrocortisone and dexamethasone. These inhibitors interfere with the activation of phospholipase A_2, rather than antagonizing its effects. It has been shown

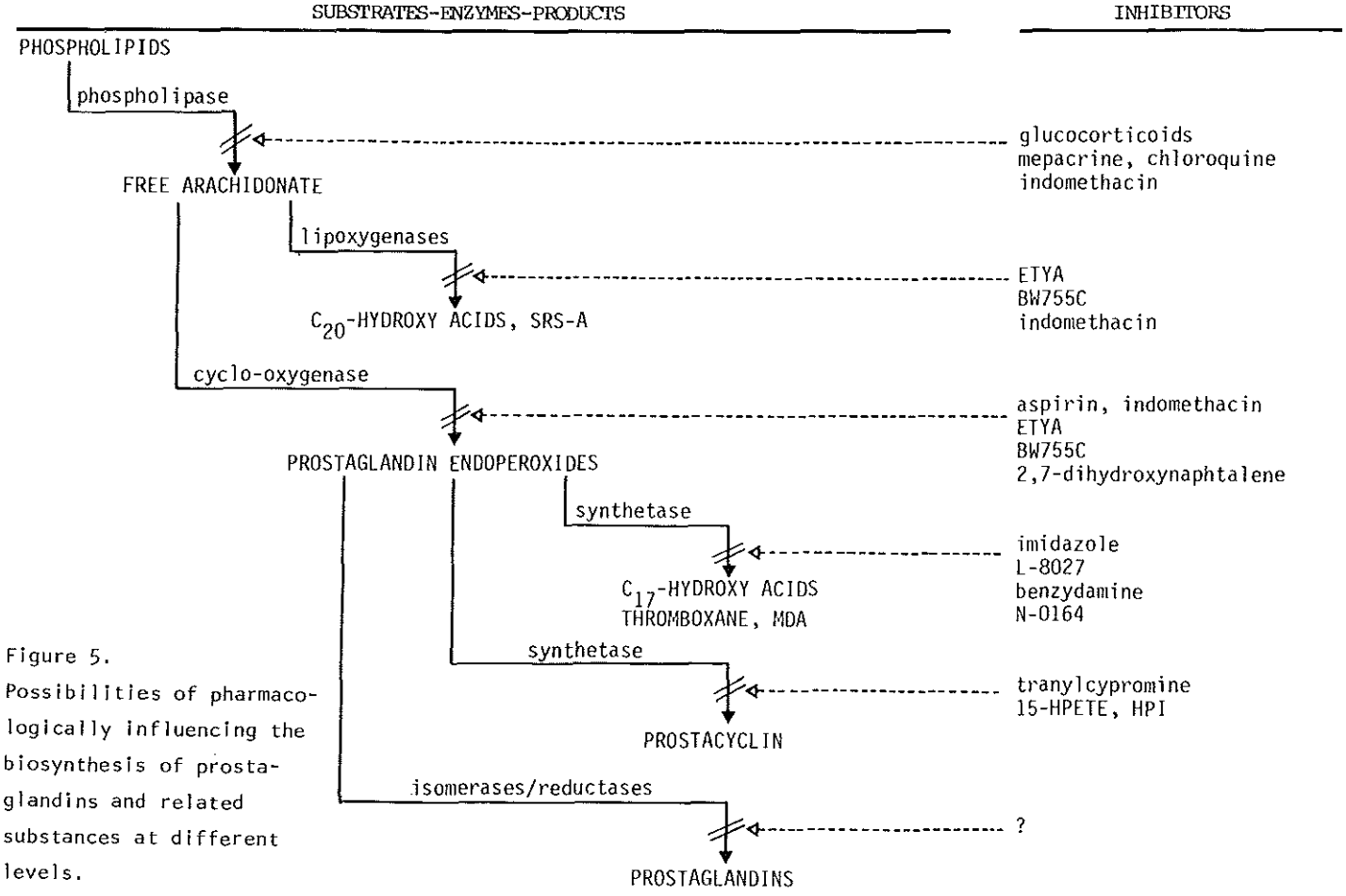


Figure 5.
Possibilities of pharmacologically influencing the biosynthesis of prostaglandins and related substances at different levels.

that the inhibition of phospholipase activation by corticosteroids depends on the synthesis of an inhibitory protein and this may be an explanation of the the slow onset of action of the inhibition by steroids (Danon and Assouline, 1978). Recently, it has been shown that indomethacin inhibits phospholipase A_2 from rabbit polymorphonuclear leukocytes (noncompetitive, K_i = 12 μM) but not that from snake venom or that from pig pancreas (Kaplan et al, 1978).

The conversion of free polyenoic fatty acids by cyclo-oxygenase is inhibited by nonsteroidal anti-inflammatory drugs (NSAID), among which, aspirin and indomethacin. Another inhibitor of cyclo-oxygenase is 2,7-dihydoxynaphthalene. Besides inhibiting cyclo-oxygenase, 5,8,11,14-eicosatetraynoic acid (ETYA, an acetylenic analogue of arachidonic acid), 3-amino-1{*m*-(trifluoromethyl)phenyl}2-pyrazoline (BW755C) and 1-phenyl-3-pyrazolidone (phenidone) are about equipotent in the inhibition of lipoxygenase. So far, no specific inhibitor of mammalian lipoxygenases has been discovered, although α-tocopherol (vitamin E) is a specific inhibitor of soybean lipoxygenase in comparison to cyclo-oxygenase from bovine seminal vesicles (Panganamala et al, 1977). Equally, no inhibitors are known which selectively act on the conversion of PGH_2, via isomerases and a reductase, to PGE_2, PGD_2 and $PGF_{2\alpha}$, respectively.

Apart from the possibilities of pharmacologically influencing the activity of cyclo-oxygenase and subsequent prostaglandin formation, there is some evidence that the biosynthesis of prostaglandins may be endogenously inhibited. A fraction of human, mare, dog, rabbit or rat serum, associated with haptoglobin, has been shown to inhibit the biosynthesis of prostaglandins by bovine seminal vesicle microsomes (Saeed et al, 1977). On intravenous administration, the serum fraction was also active in vivo against arachidonate- and bradykinin-induced bronchoconstriction in the guinea pig. This bronchoconstriction is dependent on prostaglandin formation.

The thromboxane synthetase, catalyzing the formation of TXA_2 from the prostaglandin endoperoxide, PGH_2, and (at least in platelets) also the formation of the C_{17}-hydroxy fatty acid, HHT, in combination with malonaldehyde, is inhibited by imidazole, benzydamine, 1'-(*iso*propyl-2-indolyl)-3-pyridyl-3-ketone (L-8027) and sodium *p*-benzyl-4-{1-oxo-2-(4-chlorobenzyl)-3-phenyl}propylphenyl phosphonate (N-0164).

As already discussed in Chapter 1.2.b., the synthetase catalyzing the formation of prostacyclin (PGI_2) from PGH_2, is inhibited by the per-

oxides 15-HPETE and HPI. Another inhibitor is the monoamine oxidase inhibitor and antidepressant, tranylcypromine.

Many of the inhibitors described are frequently used as tools to investigate the involvement of the different arachidonate metabolites in physiology and pathology, e.g. of inflammation. However, these investigations are often hampered by the unspecificity of the inhibitors used. For instance, the inhibitors of thromboxane synthetase are nearly always inhibitors of cyclo-oxygenase. Indomethacin-effects are not limited to an inhibition of merely cyclo-oxygenase, as this drug may also inhibit phospholipase activation in certain cells (Kaplan et al, 1978) and, in addition, the peroxidase which catalyzes the decomposition of the hydroperoxy fatty acid, HPETE, to the corresponding stable end product of the lipoxygenase pathway, HETE (Siegel et al, 1979b).

d. Hepatic lipid peroxidation dependent on NADPH or reducing agents and iron

The first report dealing with lipid peroxidation during hepatic microsomal NADPH oxidation, dates from 1963 (Hochstein and Ernster, 1963). It was shown in vitro that the oxygen consumption by microsomes during NADPH oxidation, was paralelled by an increased malonaldehyde formation. The production of malonaldehyde (see Figure 1) suggested the involvement of microsomal lipids as substrates for the peroxidizing system. Indeed, it was shown in 1968 (May and McCay, 1968) that NADPH oxidation, catalyzed by rat liver microsomes, resulted in a loss of arachidonate from membrane phospholipids and that arachidonate was a main target of lipid peroxidation. The role of NADPH-cytochrome P-450 reductase, a flavoprotein catalyzing the reduction of ferric cytochrome P-450 to its reduced congener, was clearly demonstrated by the inhibition of NADPH-dependent lipid peroxidation in the presence of antibodies against the flavoprotein (Pederson et al, 1973).

The microsomal cytochrome P-450-dependent hydroxylation of drugs is dependent on electrons donated by NADPH and possibly NADH. Initially, the drug substrate is attached to the ferric form of cytochrome P-450 and an electron is accepted from NADPH via the NADPH-cytochrome P-450 reductase. As a result, NADPH is oxidized to $NADP^+$. The reduced (ferrous) form of cytochrome P-450 in a complex with the substrate, binds oxygen and subsequently, activation of oxygen occurs after the acceptance of a second electron either from NADPH, via the flavoprotein reductase or from NADH, via

cytochrome b_5. One oxygen atom dissociates from the complex and reacts with two protons resulting in the formation of water, whereas the other oxygen now binds to the substrate resulting in hydroxylation of the latter, which then dissociates from the cytochrome catalytic site. The whole sequence of electron transfers is given in Figure 6 (Coon, 1978). Interestingly,

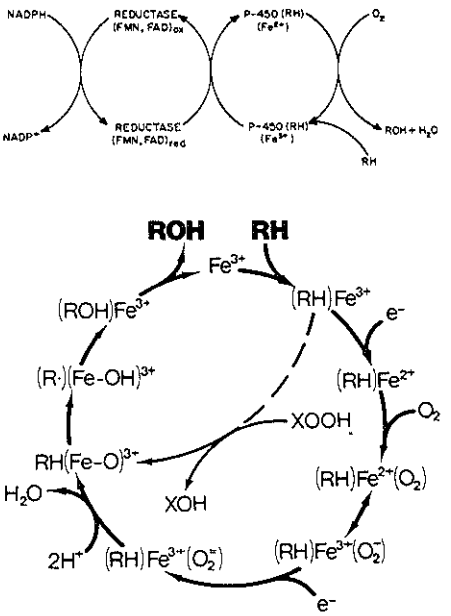


Figure 6. Drug hydroxylation via the cytochrome P-450 catalytic chain (from Coon, 1978). The upper part of the figure shows the electron flows from NADPH to substrate (RH) and water. The lower part shows the detailed reaction sequence of cytochrome P-450. Note that the ferric cytochrome-substrate complex can directly give the hydroxylated substrate, without accepting electrons from NADPH or NADH, when cytochrome P-450 acts as a peroxidase with organic peroxides (XOOH).

cytochrome P-450 can act as a peroxidase and during the catalysis of the reduction of an organic hydroperoxide (XOOH) to the corresponding alcohol

(XOH), drug hydroxylation takes place without an electron flow from NADPH or NADH via cytochrome P-450 reductase and cytochrome b_5, respectively (O'Brien, 1978).

Besides depending on the flavoprotein NADPH-cytochrome P-450 reductase, NADPH-induced microsomal lipid peroxidation requires the presence of iron in combination with ADP or (pyro)phosphate. EDTA in low concentrations inhibits lipid peroxidation and this is the result of the chelation of iron (Wills, 1969a). As a result of lipid peroxidation, the activity of microsomal enzymes such as glucose-6-phosphatase and cytochrome P-450 is strongly depressed (Wills, 1971; Levin et al, 1973). The heme moiety of cytochrome P-450 is especially liable to degeneration during lipid peroxidation (Levin et al, 1973). The catabolism of heme in general, during lipid peroxidation may be responsible for the observed formation of carbon monoxide in peroxidizing microsomal lipids (Wolff and Bidlack, 1976). Inactivation of cytochrome P-450 leads to decreased hepatic drug metabolism as has been reported to occur for the demethylation of aminopyrine and ethylmorphine in vitro (Kamataki and Kitagawa, 1973). In contrast, as a result of their biotransformation, aminopyrine and codeine inhibit lipid peroxidation in microsomes (Wills, 1969b).

Recently, a study on the mechanism of NADPH-dependent microsomal lipid peroxidation has been published which explains most of the observations described above (Svingen et al, 1979). The reactions, given in Figure 7, consist of an initiation step, followed by propagation. The initiation is the ADP-perferryl ($ADP\text{-}Fe^{3+}\cdot O_2^-$) catalyzed formation of lipid hydroperoxides (LOOH). Evidence for such a mechanism was obtained by the observations that the reactions were inhibited by the superoxide scavenger, superoxide dismutase (SOD) and the antioxidant, butylated hydroxytoluene (BHT). The subsequent propagation of lipid peroxidation is dependent on the breakdown of lipid hydroperoxides preformed during initiation. This breakdown resulting in the formation of radical fragments and singlet oxygen ($^1O_2$), is catalyzed by cytochrome P-450 or by NADPH in combination with iron chelates of EDTA or DPTA. The reactive fragments react with lipids (LH) of the microsomal membrane system to form stable end products of lipid peroxidation (e.g. malonaldehyde) and again lipid hydroperoxides. The latter are decomposed and a cyclic chain is completed. During the catalysis of hydroperoxide breakdown, cytochrome P-450 is degraded. Arachidonic acid, on addition to mouse liver microsomes, inhibits the N-demethylation of ethyl-

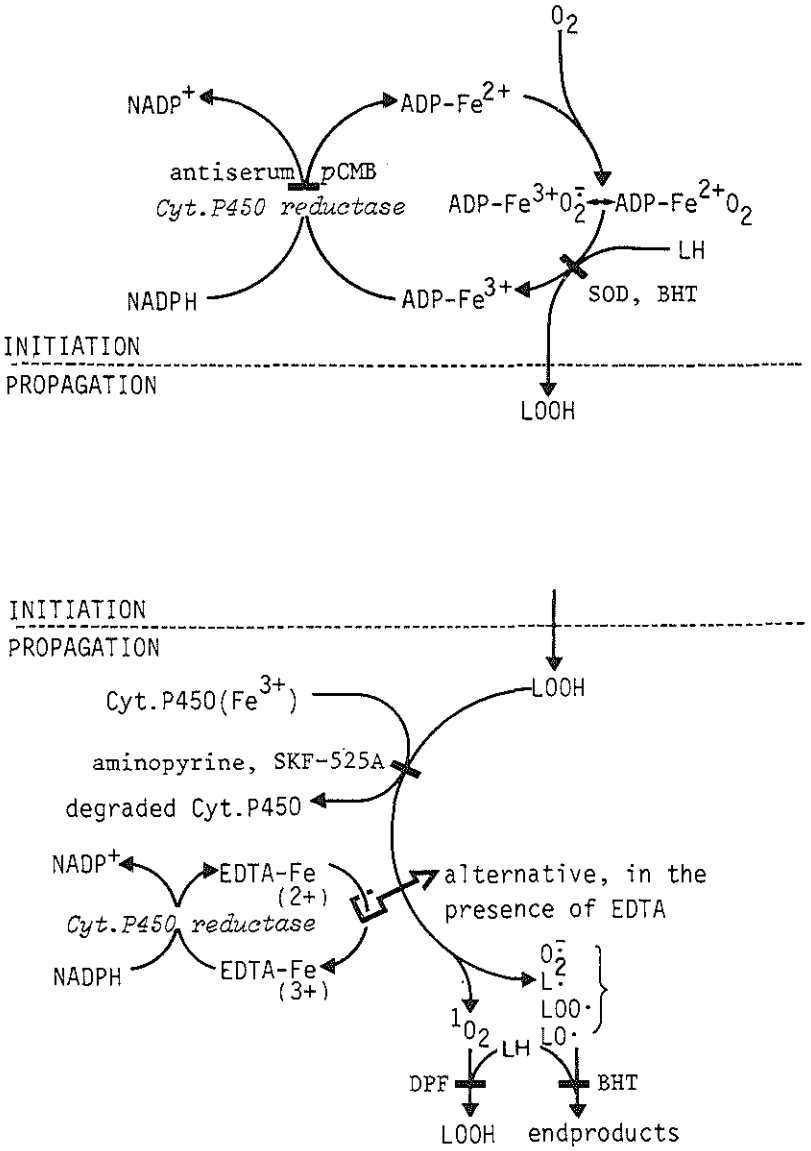


Figure 7. NADPH-dependent microsomal lipid peroxidation as proposed by Svingen et al (1979). Initiation leads to the formation of lipid hydroperoxides (LOOH) which are subsequently metabolized during propagation. During propagation, malonaldehyde is formed.

morphine and the hydroxylation of hexobarbital, both dependent on cytochrome P-450. Repeated intraperitoneal administration of arachidonate evoked biochemical and histological symptoms of hepatotoxicity and this is likely to be due to reactive metabolites from arachidonate peroxidation in vivo (Pessayre et al, 1979). As no details about the purity of arachidonate were given, it is possible that preformed hydroperoxides and aldehydes have been injected.

The inhibition of lipid peroxidation by drugs which depend on cytochrome P-450 metabolism, is the result of the cytochrome acting in the peroxidase mode, during which the drug is hydroxylated via a short-circuited pathway with peroxides as 'oxygen donors' and without the intervention of NADPH as an electron donor (see: Lu, 1976; Coon, 1978; O'Brien, 1978 and Paine, 1978). The possibility that the inhibition of microsomal lipid peroxidation by drugs is due to antioxidant activities of these substances should also be taken into consideration (Miles et al, 1980), a possibility which is a hiatus in the paper of Svingen and coworkers. The experimental drug, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525A), an inhibitor of cytochrome P-450-dependent electron flow, acts as an inhibitor of the propagation and subsequent malonaldehyde formation, whereas the fact that BHT inhibits malonaldehyde formation proves the free radical nature of product formation during propagation (see Figure 7). It must be emphasized that the effects of SKF-525A on malonaldehyde formation are strongly dependent on the type of cells under investigation. Our own studies on human thrombocytes indicate that SKF-525A inhibits platelet aggregation due to inhibition of thromboxane (the pro-aggregatory principle from human platelets) formation, but stimulates malonaldehyde formation (Parnham, Bragt, Bast and Zijlstra, to be published).

The involvement of singlet oxygen-mediated propagation is only of minor importance, as the singlet oxygen scavenger, 2,5-diphenylfuran, inhibited the formation of malonaldehyde by less than 30 percent. The system of NADPH-induced lipid peroxidation is, as far as is known at present, only relevant for in vitro investigations with liver homogenates, isolated hepatocytes and microsomal preparations, although the amount of NADPH in isolated hepatocytes (6.63 nmoles NADPH versus 0.13 nmoles $NADP^+$ per 10^6 cells) is high enough to sustain lipid peroxidation induced by the addition of ADP and Fe^{3+} (Högberg et al, 1975).

Besides NADPH-induced lipid peroxidation in liver microsomes,

lipid peroxidation is also very rapidly induced in the presence of the combination of iron ions with ascorbic acid (vitamin C), in microsomal preparations as well as in mitochondria and nuclei. Incubation with this combination of systems rich in membrane structures leads to lipid peroxidation, which increases in time until a plateau is reached. This kind of peroxidation is enhanced by vitamin E deficiency of the donor animal and inhibited in preparations from scorbutic (vitamin C deficient) animals (see Mead, 1976). Lipid peroxidation dependent on ascorbate is not inhibited on heat-induced protein denaturation nor by treatment with sulphydryl reactants, such as *p*-chloromercuribenzoate. Furthermore, it is pronounced at pH 6 and very moderate at pH 7.4 (Wills, 1966). Whereas NADPH-dependent lipid peroxidation requires free iron in combination with ADP, ascorbate-dependent lipid peroxidation does not require ADP and is even operational in the presence of ferritin-bound iron (Wills, 1966). The latter phenomenon is believed to be due to the ability of ascorbate to release iron from its storage sites on the ferritin molecule, after reduction (Mazur et al, 1955).

The detailed mechanism of ascorbate-induced lipid peroxidation is still obscure. Possibly, ascorbate acts as a ferri-reductant in analogy to the superoxide anion in the iron-catalyzed Haber-Weiss reaction (reaction 13). The nett, uncatalyzed, reaction is too slow to play any significant

$$\begin{array}{l} Fe^{3+} + \cdot O_2^- \rightarrow Fe^{2+} + O_2 \\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^- \\ \hline \cdot O_2^- + H_2O_2 \rightarrow O_2 + HO\cdot + HO^- \end{array} \qquad (13)$$

role with respect to lipid peroxidation and the associated effects in biological systems, since $k = 0.13\ M^{-1}s^{-1}$ (Weinstein and Bielski, 1979). The relevance of the lipid peroxidation system depending on ascorbate and iron is not limited to in vitro systems in view of the results with the intraperitoneal injection into mice of the lipid peroxidation inducing combination, iron (28.2 mg Fe^{2+} as chloride)-ascorbate (176 mg $kg^{-1}\ d^{-1}$), obtained by Fujita (1977). This joint administration resulted in enhanced thiobarbituric acid reactivity (indicative of malonaldehyde formation; see Chapter 1.4) of liver samples from mice 1 and 3 days after treatment. Vitamin E deficient diet fed for 3 weeks also resulted in enhanced thio-

barbituric acid reactivity. Furthermore, the administration of the oxidant system resulted in enhanced levels of triglycerides in the plasma (Fujita, 1977) and in the liver (Placer et al, 1971; Fujita, 1977). Vitamin E deficiency also resulted in hepatic triglyceride accumulation (Fujita, 1977). The conclusion could be drawn from these observations that lipid accumulation in the liver ('fatty liver') may be causally related to lipid peroxidation, since CCL_4, the hepatic metabolism of which also leads to enhanced lipid peroxidation, also induces fatty liver (see Judah et al, 1970). As an alternative, both fatty liver and lipid peroxidation may be evoked by the same phenomenon, namely cellular damage. However, the connection between fatty liver development and hepatic necrosis is still obscure (Judah et al, 1970). Recently, it has been reported (Gravela et al, 1979) that inhibition of CCL_4-induced malonaldehyde formation in isolated hepatocytes by SKF-525A and promethazine was not paralelled by protection against depressed lipoprotein excretion. This implies that malonaldehyde is not the lipid peroxide product responsible for triglyceride accumulation in the liver and also that lipid peroxidation per se is not responsible for this phenomenon.

Increased levels of malonaldehyde have been observed in women, during the second and third trimester of pregnancy and as a result of toxemia of pregnancy, which is characterized by some or all of the symptoms of hypertension, albuminuria, edema, convulsions and coma (Ishihara, 1978). Patients with recent cerebral infarctions and hemorrhages also have increased malonaldehyde levels in their serum (Satoh, 1978). Sato and coworkers (1979) reported the occurrence of elevated malonaldehyde levels in the plasma of diabetic patients. They also demonstrated that in diabetic patients with angiopathy the malonaldehyde levels were twice as high as in diabetics without angiopathy. The relationship between lipid peroxidation and degenerative diseases is again demonstrated by the fact that, during acute, fulminant and chronic active hepatitis, respectively, as well as during liver cirrhosis and fatty liver, serum lipid peroxide levels (measured as malonaldehyde) are elevated (Suematsu et al, 1977). Serum malonaldehyde levels in women tend to be lower than in the corresponding age group of male subjects and increase with increasing age, both in male and female subjects (Suematsu et al, 1977).

In conclusion it can be stated that the origin of malonaldehyde in the blood is, thus far, by no means clear. Neither is it known whether malonaldehyde formation with its subsequent appearance in the blood is due

to activation of cyclo-oxygenase (or, more precisely, thromboxane synthetase) in the tissues prone to the disease. The fact, however, that raised plasma levels of malonaldehyde are often associated with the so-called 'acute phase reactant' proteins of hepatic origin, tempts to suggest that concomitant lipid peroxidation also occurs in the liver. The role of acute phase proteins in inflammation will be dealt with in Chapter 2.4.

1.3. Free radical scavengers and antioxidants as inhibitors of lipid peroxidation

Lipid peroxidation can be inhibited at different levels (see Figure 1). Radical scavengers and quenchers of singlet oxygen prevent the attack by their reactive substrates on labile unsaturated compounds, e.g. polyenoic fatty acids. The antioxidants, butylated hydroxytoluene (BHT) and hydroxyanisole (BHA), react with preformed lipid peroxy-radicals whilst preventing propagation of lipid peroxidation and the formation of stable end products, e.g. aldehydes and alkanes.

Scavengers may be used by biochemists and pharmacologists to unravel the involvement of distinct radical species and processes in biological systems, although caution has to be exercised as many scavengers are not specific for one radical. Table 1 summarizes some scavengers and quenchers of reactive oxygen species with the corresponding reaction or quenching constants for a second order reaction. From this table it can be seen that many of the scavengers mentioned react at an almost diffusion-controlled rate with their substrates, i.e. with a magnitude of about 10^{10} $M^{-1}s^{-1}$. Dimethylsulphoxide (DMSO) and thiourea are extremely good hydroxyl radical scavengers (highest k values), whereas superoxide dismutase (SOD) and cupric ions are equally effective in scavenging superoxide. Furthermore, carotene and bilirubin are the most effective singlet oxygen quenchers. The values of the reaction constant k show great variances with changes in pH and temperature and also with the solvent used. Therefore, they cannot be directly compared with each other.

It is a well-known fact that oxygen is toxic to many organisms and this is demonstrated by decreased viability at elevated oxygen pressures. That we are able to survive at a partial oxygen tension of 0.21 atmosphere, is due to our equipment with at least two defence lines against oxygen toxicity, which act at the cellular level. These defence mechanisms deal

Table 1. Bimolecular rate constant of scavengers and quenchers reacting with their reactive substrates

Reactive species	Scavenger/Quencher	$k(M^{-1}s^{-1})$	Authors and year
HO·	thiourea	5×10^9	Anbar & Neta, 1967
	benzoate	3.8×10^9	,,
	butanol	2.2×10^9	,,
	ethanol	1×10^9	,,
	mannitol	$>10^9$	,,
	DMSO	7×10^9	,,
	glutathione	8×10^9	Lin & Armstrong, 1977
	MK-447	n.d.	Kuehl et al, 1977
$\cdot O_2^-$	SOD	2.4×10^9	Fielden et al, 1974
	Cu^{2+}	2.7×10^9	Brigelius et al, 1974
	$Cu(lysine)_2$	6×10^8	,,
$^1O_2$	β-carotene	1.65×10^{10} †	Koka & Song, 1978
	α-tocopherol	1.05×10^8 †	,,
	DABCO	3.11×10^7 †	,,
	bilirubin	2.5×10^9	Lightner & Park, 1978
	ascorbic acid	$\sim2.2\times10^8$	Bodaness & Chan, 1979
	2,5-dimethylfuran	1.4×10^8	Foote, 1971
LOO·	α-tocopherol	5×10^8 §	Packer et al, 1979

†Calculated rate constant. §Reaction constant for reaction with $Cl_3C\text{-}O\text{-}O\cdot$
n.d. not determined.

primarily with the detoxification of reactive intermediates arising accidentally from the respiration chain, the microsomal drug metabolising system and other oxidases (e.g. xanthine oxidase, aldehyde oxidase).

The first line of defence consists of compounds and enzymes which directly react with preformed radicals, thus protecting labile sulphydryl proteins from inactivation and preventing lipid peroxidation (see Chapter 1.1). Scavengers which play a role in vivo and act against $\cdot O_2^-$, HO· and $^1O_2$, are ascorbic acid, reduced glutathione, methionine, lipoic acid, carotenoid pigments and bilirubin. Furthermore, the cytoplasmatic enzyme, superoxide

dismutase (SOD, identical to hepato- and erythrocuprein), with a molecular weight of 34,000 daltons, catalyzes the dismutation of superoxide anions to hydrogen peroxide (see Fridovich, 1974). Mitochondria contain a SOD with manganese as the active centre instead of copper and zinc in the cytoplasmic SOD. Recently, it has been shown that the copper storage protein, ceruloplasmin, is a scavenger of superoxide (Goldstein et al, 1979), although it has been known for several years that ceruloplasmin is the major antioxidant in blood (Al-Timimi and Dormandy, 1977). The ceruloplasmin apoprotein, without copper which is normally present as 6-8 atoms per protein molecule, is ineffective as an antioxidant.

The second line of defence against the potential damaging effects of the products from reactions of free radicals with lipids or with each other, consist of antioxidants and enzymes catalyzing the breakdown of peroxides to less toxic species. Catalase, the peroxisomal heme protein with a molecular weight of 234,000 daltons, catalyzes the conversion of hydrogen peroxide to molecular oxygen and water. Glutathione peroxidase with a molecular weight of 76,000 daltons and containing 4 selenium atoms per protein molecule, catalyzes the conversion of hydrogen peroxide to water and of lipid peroxides to the corresponding alcohols. This enzyme is dependent on the availability of glutathione in the reduced form (GSH), which is oxidized to its disulphide congener (GSSG) during the catalytic action of the peroxidase (Flohe, 1971). Table 2 summarizes some characteristics of the defence enzymes mentioned and data in brackets are representative for the

Table 2. Some characteristics of protecting enzymes.

	Catalase	Glutathione peroxidase	Superoxide dismutase	Ceruloplasmin
Molecular weight	234,000	76,000	32,000 (80,000)	132,000
Subunits	4	4	2 (4)	-
Active centre atom	Fe	Se	Cu/Zn (Mn)	Cu
Number of active centre atoms	4	4	2/2 (4)	6-8
Localization	peroxisomes	cytosol	cytosol (mitochondria)	liver blood

manganese-containing SOD. The antioxidant vitamin E inhibits the propagation of lipid peroxidation and may also act in the first line of defence as a scavenger of singlet oxygen. The protective function of ceruloplasmin may be even more important than that simply mentioned above, if one considers its oxidase activity. By virtue of its capacity to act as a ferro-oxidase, thus oxidizing the ferrous to the ferric form, ceruloplasmin prevents eventual decompartmentation of iron and thereby the possible involvement of iron in lipid peroxidation reactions. Ceruloplasmin may also be active against somatic effects of stress, since it may oxidize catecholamines and serotonin, in analogy with monoamine oxidase (see Gutteridge, 1978).

1.4. Detection of lipid peroxidation

To detect lipid peroxidation in deteriorating edible fats and oils, the sense of smell is often sufficient to determine whether one should or not consume the objects of investigation! However, with biological samples in studies concerning the role of lipid peroxidation in pathology, more reliable and reproducible methods are needed to detect and quantify lipid peroxidation. Many methods have been described and many well-known methods yearly undergo revisions to make them more reliable, reproducible, sensitive, ...etc. One method is to study the loss of fatty acids from a sample as a consequence of peroxidative deterioration and this can be performed in combination with an oxygen electrode to monitor oxygen utilization. Most of the methods commonly used, however, are based on the detection of intermediates or stable end products of lipid peroxidation (see Gray, 1978). The formation of conjugated dienes (see Figure 1) can be detected by the increase in 234 nm UV absorption. The presence of hydroperoxides can be detected by iodometry, either from the formation of iodine with iodide solutions or, as an alternative, in combination with colorimetric analysis of the I_3^--starch complex. Other methods of detection are gas-liquid chromatography of hydrocarbons formed (see Figure 1), of fatty acid hydroperoxides and of volatile aldehydes.

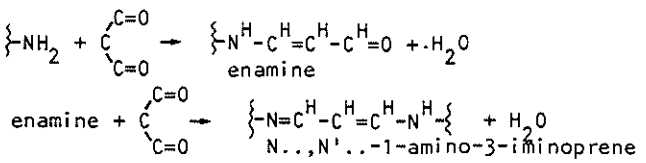
On historical grounds, i.e. based on earlier experiments, I chose the reaction of malon(di)aldehyde (MA or MDA) with 2-thiobarbituric acid, which yields a chromogen with a strong absorbance at 535 nm (molar extinction coefficient at 25°C, 1.56×10^5 $M^{-1}cm^{-1}$). After extraction in butanol, the complex can be detected fluorimetrically, thereby enhancing the specificity of the assay. Because of its great reactivity, malonaldehyde can be detected

by more than ten different spectrophotometric and spectrofluorometric assay methods, although the thiobarbituric acid (TBA) method is one of the most sensitive methods and the malonaldehyde-TBA complex is reasonably stable (Sawicki et al, 1977). A problem arising from the detection of malonaldehyde is the fact that the compound is further metabolized by mitochondrial oxidases present in ample biological samples (Recknagel and Ghoshal, 1966). After intraperitoneal injection of malonaldehyde, serum levels of the compound decrease with time, without a simultaneous increase in urinary malonaldehyde (Placer et al, 1965). This implies that failure to detect malonaldehyde does not necessarily prove absence of its formation or of lipid peroxidation. In addition, it is probably not malonaldehyde itself, which is detected by the TBA method, as it is volatile at low pH values. The principle TBA reactants are supposed to be endoperoxides with a structural resemblance to the prostaglandin endoperoxides (Pryor and Stanley, 1975). Another disadvantage of the determination of malonaldehyde by the TBA method is the lack of available radioactively labelled malonaldehyde for the determination of recoveries. Fortunately, methods for synthesis of ^{14}C-malonaldehyde have been described very recently. One method is based on the hydrolysis of labelled precursor to malonaldehyde and ethanol (Marnett et al, 1979), while the second method is based on the substrate unspecificity of alcohol dehydrogenase. In this case, ^{14}C-labelled 1,3-propanediol is oxidized to malonaldehyde in the presence of an NAD^+ regenerating system for the acceptance of electrons from the diol (Summerfield and Tappel, 1978). Although intensive effort was made to synthesize malonaldehyde according to the latter method, thereby optimalizing and checking the partial reactions carefully, we were unable to obtain more than a few percent of malonaldehyde.

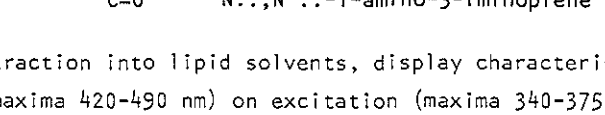
Despite many shortcomings, the TBA assay is the method most frequently used for the detection of lipid peroxidation. Its major advantage as compared to alternative methods is the relative simplicity and the sensitivity of the assay, provided that adequate controls are used. Using the TBA assay, it is now possible to discriminate between preformed malonaldehyde and the presence of malonaldehyde precursors of peroxidic nature (Asakawa and Matsushita, 1979). The discrimination is performed by the addition of an antioxidant with or without ferrous sulphate. In the presence of the antioxidant alone, only preformed malonaldehyde is determined as the antioxidant prevents lipids from further peroxidation. The addition of

ferrous ions, however, catalyzes the breakdown of preformed peroxides to malonaldehyde and other products, whereas the antioxidant present inhibits iron-catalyzed peroxidation of the lipids under investigation. An extensive review on lipid peroxidation, dealing extensively with the benefits and pitfalls of the TBA method has been written by Barber and Bernheim (1967).

Finally, another method has been introduced which is based on the detection of fluorescent products resulting from the reaction of malondehyde with free amino groups of proteins and primary amino phospholipids. The N,N'-disubstituted 1-amino-3-iminoprene moiety, which is the result of the reaction mentioned, is highly fluorescent (Chio and Tappel, 1969; Bidlack and Tappel, 1973). Reactions 14 and 15 show the subsequent steps of the fluorophore formation. The detection is based on the fact that cross-linked



(14)



(15)

polymers, after extraction into lipid solvents, display characteristic emission spectra (maxima 420-490 nm) on excitation (maxima 340-375 nm). The pigment, lipofuscin, which accumulates during aging in heart muscle, liver and brain, has an emission maximum at 470 nm on excitation at 365 nm and its formation may be the result of the interaction of malonaldehyde with proteins and phospholipids (Chio et al, 1969). This observation is an important principle in the 'free radical theory of aging' (see Hocman, 1979). It has to be stressed that the authors of the literature prior to 1967 were ignorant of the enzymatic formation of malonaldehyde during the biosynthesis of prostaglandins (Hamberg and Samuelsson, 1967). Therefore, no dissociation can be made between enzymatic and free radical-induced lipid peroxidation in the old literature. An additional feature is the possibility of artifactually provoked lipid peroxidation (i.e. prostaglandin biosynthesis) in many experiments, which is nowadays prevented by the immediate addition of prostaglandin synthetase inhibitors, before manipulation. Thus, if malonaldehyde is used as a monitor of lipid peroxidation, it is necessary to investigate its actual origin, by treatment with aspirin-like drugs or by measuring stable prostaglandins and thromboxane formed.

1.5. Implications of lipid peroxidation in biological systems

At the biochemical level, it is well-known that lipid peroxidation, via the generation of reactive products (free radicals, singlet oxygen, aldehydes), inhibits many enzymes and damages many compounds of biological importance. For instance, cytochrome c, cytochrome P-450 heme, cytochrome c reductase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and ribonuclease A have been shown to be inactivated or destroyed during, and as a consequence of lipid peroxidation. In addition, lipid hydroperoxides react with cysteine, methionine, hystidine and lysine, whereas malonaldehyde cross-links DNA, serum albumin and many substances with free amino residues (see also Chapter 1.4). In contrast to these inhibitory effects of lipid peroxidation, unsaturated fatty acid peroxides as well as superoxide and hydroxyl radicals, stimulate guanylate cyclase from platelets and liver cells (Hidaka and Asano, 1977; Mittal and Murad, 1977). At the subcellular level, lipid peroxidation leads to lysosome rupture with subsequent release of lysosomal enzymes and to swelling and lysis of mitochondria resulting in an inhibition of the respiratory chain. In addition as a result of membrane alterations, lipid peroxidation interferes with cellular transport processes for instance of calcium. At the cellular level, lipid peroxidation may cause lysis of erythrocytes, whereas peroxidized fatty acids, in the presence of calcium ions, evoke platelet aggregation which is inhibited by indomethacin but not inhibited by substances elevating cyclic AMP. Lipid peroxides may also contract isolated tissues, e.g. the rat stomach fundus, aorta, uterus and coronary arteries of the cat (Roycroft et al, 1977; Trachte et al, 1979). Furthermore, fatty acid hydroperoxides cause a sensation of pain on intradermal injection in man (Ferreira, 1972). Finally, lipid peroxidation has been demonstrated to be involved in the toxic effects of chlorinated hydrocarbons, bleomycin, adriamycin, dapsone, ethanol and UV radiation of the skin, while lipid peroxides are very toxic on oral intake (see Barber and Bernheim, 1967).

1.6. Summary

In Chapters 1.1-1.5, the mechanisms of lipid peroxidation have been outlined. Polyunsaturated fatty acids, e.g. arachidonic acid which is

ubiquitous in mammalian membranes, are particularly susceptible to peroxidative deterioration at the methylene moieties interrupting the double bonds. Free radical scavengers and antioxidants prevent the formation of stable end products as a consequence of lipid peroxidation. Lipoxygenases and cyclo-oxygenase are present in virtually all mammalian cells and these enzymes catalyze the formation of (endo)peroxides from specific polyenoic acids, which are further metabolized to biologically active compounds, namely prostaglandins, thromboxanes and slow-reacting substance of anaphylaxis. The formation of prostaglandins and thromboxanes is inhibited by aspirin-like drugs, whereas arachidonate analogues also inhibit the formation of lipoxygenase-dependent products.

Although the products of enzymatic lipid peroxidation often play physiological roles (e.g. in platelet aggregation, in the cardiovascular system), free radical-induced lipid peroxidation is not benificial for the organism. It may inhibit several enzymes and cause cellular and tissue damage. Furthermore, lipid peroxidation interferes with the metabolism of drugs. To prevent deleterious effects of lipid peroxidation, cells are equipped with defence systems against free radicals and peroxides. Inadequate functioning of theses lines of defence leads to oxidant stress, e.g. in deficiencies of glutathione and vitamin E. The likelihood of the presence of an oxidant stress as a result of inflammation and the anti-inflammatory effects of different free radical scavengers and antioxidants, will be dealt with in the experimental section (Appendix) of this thesis.

2. INFLAMMATION

Inflammation is the reaction of the body on tissue injury of any kind. At the inflamed site, macroscopical changes occur which include redness, oedema, heat, pain and, after a certain period of time, loss of function. These symptoms had already been described in the time of the ancient Greeks. The noxa may be of a chemical, physical or microbiological nature. The acute phase of an inflammatory process is characterized by vasodilatation and increased vascular permeability which are the result of the local release of inflammatory mediators, such as histamine, serotonin, kinins and prostaglandins and activation of the complement system. In carrageenin-induced rat paw oedema, an acute type of inflammation, three different phases can be distinguished. The first phase (0-0.5 h) is characterized by histamine and serotonin release and is followed by the kinin phase (1-3 h) and the phase of prostaglandins (3-6 h) acting as mediators.

Granulocytes are recruited from the blood by chemotactic factors originating from the complement cascade or from the oxidative conversion of fatty acids. Having arrived at the inflamed site, the granulocytes start phagocytozing the materials recognized as 'foreign'. During phagocytosis, which is primarily intended to make the ingested materials harmless to the host, the granulocyte may act as a secretory cell, releasing lysosomal enzymes, prostaglandins, superoxide radicals, hydrogen peroxide and other substances into the extracellular environment. This release amplifies the inflammatory response due to protein breakdown and the generation of new chemotactic principles. Monocytes arrive from the blood, become activated and differentiate into macrophages with a heavy load of lysosomal enzymes which are partially released during phagocytosis. During their stay at the inflamed site, macrophages mature and form a granuloma in which fibroblasts, lymphocytes and even neutrophils may participate. In other words, granulomatous inflammation is a proliferative type of chronic inflammation and it is characterized by continuous infiltration of mononuclear leukocytes from the blood. In addition, macrophages, sometimes in the form of epitheloid cells or fused to form giant cells, constantly undergo divisions within the granuloma. In this respect, granuloma models are analogous to and representative of the cellular proliferation and connective tissue synthesis observed during rheumatoid arthritis in man. This disease is characterized

by lesions in the synovia. The lesions consist of two types, namely an inflammatory lesion with mononuclear cell infiltration below the lining cells and the accumulation of exudate rich in polymorphonuclear leukocytes within the synovial cavity. The second type is the proliferative lesion, characterized by an enormous increase in the amount of connective tissue, in the number of small blood vessels and in the number of mesenchymal cells with phagocytic and synthetic functions. Furthermore, connective tissue activating peptides are released, including lymphokines, which stimulate fibroblast activation and the production of collagenase. This so-called 'pannus' of granulation tissue may lead to joint destruction and fibrosis.

The granuloma represents a dynamic state of cell proliferation and breakdown. Macrophages die, but their enzymes may retain their activities over long periods. Necrosis of tissue may, but does not necessarily occur. At any stage of inflammation, the balance between the various processes involved, may be influenced by the actions of inflammatory modulators (e.g. prostaglandins, anti-inflammatory proteins), resulting in a cessation of the inflammatory response. The inflammation may occasionally fail to achieve chronicity if the damaging agent is rendered harmless within a short period after the induction of inflammation (see Adams, 1976; Willoughby 1978).

The studies performed which have led to this thesis, have been carried out on models of granulomatous inflammation in the rat and some of the results obtained with them will be discussed in terms of their relevance to rheumatoid arthritis.

2.1. Generation of free radicals and other oxidants at the site of inflammation

After the recognition by phagocytes (granulocytes, monocytes) of opsonized 'foreign' materials, a burst of oxygen consumption is observed and an activation of the hexose monophosphate shunt. The oxygen consumed is mainly converted to superoxide anions and hydrogen peroxide. The micro-organism or particle becomes attached to the phagocyte membrane which subsequently invaginates the particle within the phagocyte. When the material is completely surrounded by the cell membrane cavity, the latter splits off to form a phagosome that migrates into the cytoplasm. Concomitantly with the invagination, an NADPH oxidase is activated which univalently

reduces molecular oxygen to superoxide radicals. The flux of radicals is primarily directed against the invader within the membrane sink, but there is evidence that many radicals escape into the extracellular environment (Salin and McCord, 1975; Baehner et al, 1977). Besides the release of the relatively unreactive superoxide radical, there is now considerable evidence that the highly reactive hydroxyl radical is also produced as a result of stimulation of both granulocytes and monocytes (Tauber and Babior, 1977; Green et al, 1979; Weiss et al, 1977). Therefore it is not surprising that phagocytozing cells rapidly loose their motility and phagocytic capacity in vitro (Baehner et al, 1977) and die a premature death (Salin and McCord, 1975), during which the lysosomal enzymes they contain leak into the environment. Premature death of granulocytes is prevented by the addition of either superoxide dismutase or catalase, or of the hydroxyl radical scavenger mannitol (see Chapter 1.3, Table 1) to the medium (Salin and McCord, 1975).

Internally, the phagocyte is well equipped with defence mechanisms against reactive intermediates. The sequence of reactions concerned with this defence are given in Figure 8 (from Roos, 1977). The superoxide radical, generated by a membrane-bound NADPH oxidase, is scavenged by the cytoplasmic superoxide dismutase and converted to hydrogen peroxide. Hydrogen peroxide is utilized by myeloperoxidase to convert halides to hypohalides, which results in chlorination, decarboxylation and proteolysis of the invader's proteins. Catalase deals with the ubiquitous hydrogen peroxide and catalyzes its decomposition to oxygen and water. Glutathione peroxidase also converts hydrogen peroxide to water, but this conversion requires reduced glutathione (GSH) which is oxidized to its disulphide congener (GSSG). GSH is regenerated by glutathione reductase, with NADPH from the hexose monophosphate shunt as an electron donor. The possible generation of singlet molecular oxygen has been offered as an explanation for the chemiluminescence of phagocytozing granulocytes, but this possibility has severely been questioned (see Roos, 1977).

Reduced availability of protecting enzymes and scavengers leads to alterations in the response of granulocytes to 'foreign' materials. Thus, deficiency of glutathione synthetase makes the cell membrane and the microtubular system vulnerable to oxidative damage and the result is impaired phagocytic function in combination with attenuated microbicidal properties (Spielberg et al, 1979). Vitamin E may restore some of the basic functions

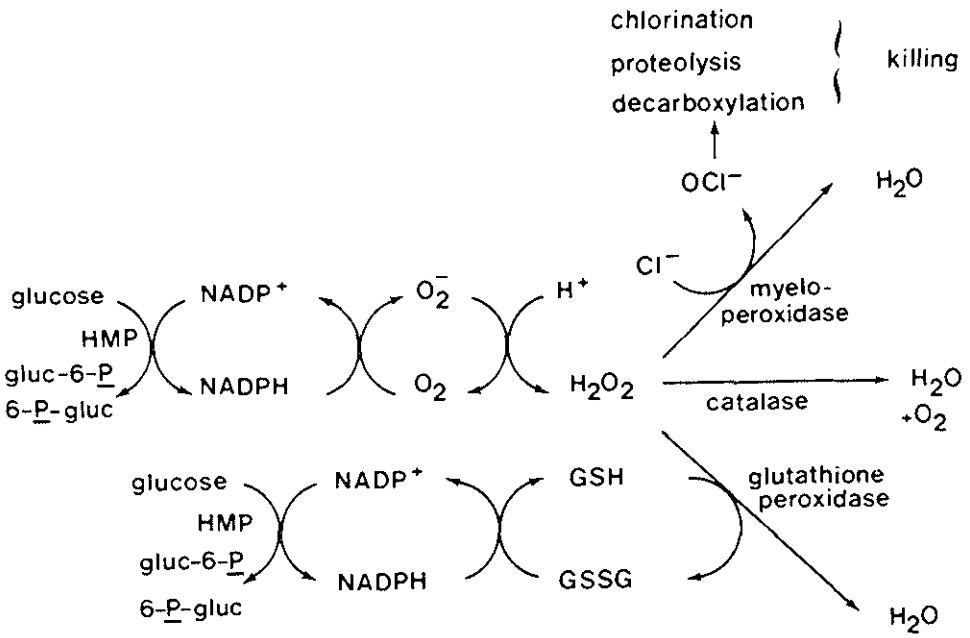


Figure 8. Mechanisms of protection against potentially damaging molecules in phagocytozing cells (from Roos, 1977)

of glutathione synthetase-deficient polymorphs during phagocytosis, such as reducing hydrogen peroxide release to normal values and increasing the bactericidal capacity (Boxer et al, 1979). Glutathione peroxidase deficiency leads to an attenuation of microbicidal capacity, increased H_2O_2 release and a decreased response to chemotactic stimuly in rat granulocytes (McCallister et al, 1980). Vitamin E deficiency leads to increased oxygen consumption, slightly increased H_2O_2 release and again decreased chemotactic activity (Boxer et al, 1979). Lack of ability to produce superoxide, as a result of NADPH oxidase deficiency or lack of activity, leads to chronic granulomatous disease (Curnutte et al, 1974). Patients suffering from the disease are highly susceptible to infections. In contrast, it has been shown that children with rheumatoid arthritis have low superoxide dismutase levels in their polymorphonuclear leukocytes (Rister et al, 1979). This reduced availability of the protecting enzyme may lead to enhanced damage to phagocytozing granulocytes, release of lysosomal enzymes and amplification

Table 3. Inhibition by NSAID of the release and activities of oxygen derived free radicals

Drug	Scavenging of hypoxanthine-xanthine oxidase-derived $\cdot O_2^-$		Generation of $\cdot O_2^-$ by guinea pig macrophages (IC_{50})	Horse radish peroxidase-H_2O_2 catalyzed oxidation of *o*-dianisidine (IC_{50})
	HX-XOD catalyzed oxidation of NADH (IC_{50})	Depolymerization of bovine synovial fluid (% inhibition at 80 µM drug)		
Indomethacin	3×10^{-4} M	96%	1×10^{-5} M	1.4×10^{-5} M
Mefenamic acid	1×10^{-3}	63	7×10^{-5}	8×10^{-4}
Phenylbutazone	Inactive	62	7×10^{-5}	9×10^{-4}
Oxyphenbutazone	Inactive	-	1×10^{-6}	1.8×10^{-4}
Paracetamol	-	74	-	1.9×10^{-4}
Acetylsalicylic acid	Inactive	Inactive	1.5×10^{-4}	Inactive
Diclofenac	Inactive	-	4×10^{-7}	-
Niflumic acid	-	94	-	-
Flufenamic acid	3×10^{-4}	-	8×10^{-5}	-

NSAID: nonsteroidal anti-inflammatory drugs.

HX-XOD: hypoxanthine-xanthine oxidase.

IC_{50}: concentration of drug halving effect.

of superoxide, using another testsystem (Table 3).

The potency of the NSAID in inhibiting superoxide production by maccrophages correlates positively with their potency in inhibiting sheep seminal vesicle (SSV) cyclo-oxygenase (Oyanagui, 1976; (Ham et al, 1972) with an $r_s = 0.70$ ($n = 5$, Spearman rank test). The IC_{50} values for the inhibition of synovial fluid depolymerization (Puig-Parellada and Planas, 1978) and the horse radish/H_2O_2 system (Saeed and Warren, 1965) are also correlated with their IC_{50} values for the inhibition of SSV cyclo-oxygenase with an $r_s = 1.00$ in both cases ($n = 5$ and 4 drugs, respectively). Thus, while these compounds are inhibitors of cyclo-oxygenase and therefore of the biosynthesis of prostaglandins, this activity is closely related to their ability to inhibit the generation and/or activity of free radicals. Whether these two actions are causally linked or not is, as yet, unclear, but both represent feasible mechanisms for their anti-inflammatory actions. In this context, MK-447 is an experimental anti-inflammatory drug, which may act as a hydroxyl radical scavenger. It prevents the pro-inflammatory actions of hydroxyl radicals themselves and shortens the half-life of the prostaglandin endoperoxide PGG_2, which is a potential inflammatory mediator (Kuehl et al, 1976). The effects of MK-447 on the qualitative formation of arachidonate-dependent products has been discussed elsewhere in the review (Bonta et al, 1980). Kuehl and coworkers (1976) have suggested that the free radical formed during the conversion of PGG_2 to PGH_2 and which is scavenged by MK-447, may be more important as an inflammatory mediator than the stable prostaglandins such as PGE_2.

b. The steroidal anti-inflammatory drugs

Prednisone, dexamethasone, triamcinolone and hydrocortisone, when added in a concentration of 80 µM, inhibit the depolymerization of bovine synovial fluid induced by hypoxanthine/xanthine oxidase. The degrees of inhibition are 74, 67, 59 and 39 percent, respectively (Puig-parellada and Planas, 1978). This action is probably not due to a direct inhibition of superoxide generating mechanisms, as dexamethasone was inactive against $\cdot O_2^-$ production by macrophages and the hypoxanthine/xanthine oxidase system (Oyanagui, 1976). However, methylprednisolone (sodium succinate) inhibited the UV-induced peroxidation of liposomes but not in a dose-dependent manner (Seligman et al, 1979). It is possible that membrane-stabilizing

properties are involved in the complex mechanisms of protection by methylprednisolone.

c. Phenothiazines

The sedatives and histamine (H_1) antagonists such as chlorpromazine and promethazine, which have anti-inflammatory properties in several inflammation models (Green et al, 1979), are used commercially in the oil industry as antioxidants. Their antioxidant properties may be due to the fact that the free radical forms of the phenothiazines are relatively stable (Piette et al, 1964) and inhibit the propagation of lipid peroxidation (see Chapters 1.1 and 1.3). Furthermore, chlorpromazine inhibits superoxide production by hypoxanthine/xanthine oxidase and by macrophages (Oyanagui, 1976).

d. Sulphydryl compounds

This chapter emphasizes the radical scavenging and antioxidant properties of penicillamine and related compounds, such as cysteine, cysteamine (β-mercaptoethylamine) and glutathione (γ-L-glutamyl-L-cyteinyl-glycine). Like penicillamine on rheumatoid arthritis, cysteine and glutathione have benificial effects on experimentally induced inflammation (Bailey and Sheffner, 1967; Thomas and West, 1973). The mechanisms of action of these compounds in protecting against ionizing radiation may be explained in two ways: firstly by their sulphydryl-saving properties and secondly, by their heavy metal chelating properties. As already mentioned, the sulphydryl compounds (RSH) are important lines of defence against free radicals and lipid peroxidation, either by reacting directly with free radicals or indirectly, by saving cellular glutathione, which is the specific substrate for the peroxide metabolizing enzyme, glutathione peroxidase.

e. Copper-containing scavengers: ceruloplasmin, superoxide dismutase

The mechanisms by which the chelation of metals protects against radiation damage is still controversial (Thomson, 1962), but a point of unanimity is the crucial role of copper in the production of cellular damage. From the positive correlation between the copper content of certain cells or tissues and their sensitivity toward radiation damage (heart, liver, brains > spleen, pancreas, leukocytes), it has been postulated that the

damage is due to the preferential oxidation by free radicals of copper bound to endogenous ligands, for instance in cytochrome c oxidase. The oxidized copper is then immediately reduced by an electron from the protein ligand which in return also becomes oxidized. This oxidized molecule, whether the ligand decomposes or not, is no longer able to exert its physiological function. The merit of the chelating agents is thought to be the removal of copper from its storage site on the endogenous ligands and the preferential decomposition of the chelating agent. The copper is released on decomposition and rebound to the original endogenous ligand (Anbar, 1964).

Interestingly, the main storage site of copper in mammals, namely ceruloplasmin, with a molecular weight of about 140,000 and containing 8 copper atoms per molecule, has been shown to be a scavenger of $\cdot O_2^-$ (Goldstein et al, 1979) and to have anti-inflammatory properties against urate-induced rat paw edema (Denko, 1979). During rheumatoid arthritis, the enhanced hepatic synthesis of acute phase proteins leads to elevated levels of ceruloplasmin in the serum. Besides maintaining in this way the systemic antioxidant potential, ceruloplasmin possibly protects the inflamed site by virtue of its scavenging and anti-inflammatory properties. In this context it has been shown that ceruloplasmin may be synthesized locally at the site of inflammation (Gitlin et al, 1977). In addition, the protecting enzyme, superoxide dismutase, with a molecular weight of 34,000 daltons and containing 2 atoms of copper and 2 of zinc per molecule, has anti-inflammatory properties in mammals (Huber et al, 1977). Whereas superoxide dismutase protects synovial membranes and fluid against superoxide-induced degradation (McCord, 1974), the levels of this enzyme in polymorhonuclear leukocytes of children with rheumatoid arthritis are decreased (Rister et al, 1979). Based on the idea that the release of superoxide by granulocytes during inflammation may contribute to inflammatory reaction, superoxide dismutase was successfully used to combat osteoarthritis in horses (Huber et al, 1977). However, the efficacy of superoxide dismutase as an anti-inflammatory agent is limited by its rapid degradation after systemic and local injection (McCord et al, 1979), whether enzymatically or by its own product, hydrogen peroxide, which is formed during the dismutation of superoxide.

Ceruloplasmin, superoxide dismutase and the sulphydryl compounds have one profile in common, i.e. the ability to react directly with free radicals and to form strong complexes with copper. On this base, compounds such as cysteamine, cysteine and acetylcysteine might therefore be consider-

ed as candidates for clinical trials against rheumatoid arthritis. Another mode of action of anti-inflammatory agents to be developed may be the stimulation of the synthesis of the acute phase protein, ceruloplasmin, as this protein is an endogenous antioxidant in combination with an anti-inflammatory agent.

f. Other compounds combining anti-inflammatory activity with the scavenging of free radicals

Imidazole has a complex spectrum of action against inflammation and some of the underlying mechanisms are based on interactions with the prostaglandin cascade. In addition, at a concentration of 80 µM, imidazole is as potent as mefenamic acid in inhibiting superoxide-induced degradation of bovine synovial fluid (Puig-parellada and Planas, 1978; see Table 3).

Antioxidant compounds, such as phenol, *nor*dihydroguaiaretic acid (NDGA), N,N'-diphenyl-*p*-phenylenediamine (DPPD), propyl gallate and α-tocopherol (vitamin E), which are used as preservatives in rubber and food technology for example, have anti-inflammatory properties in a wide range of inflammation models in animals (Stuyvesant and Jolley, 1967; Egan et al, 1978; Sobański et al, 1976; Levy and Kerley, 1974 and McDonald-Gibson et al, 1976). Although some of these antioxidants are reasonably good inhibitors of cyclo-oxygenase, vitamin E, for example, is not (Panganamala et al, 1977; confirmed by our own experiments)! This suggests that lipid peroxides which are formed independently of the prostaglandin pathway, may play roles as inflammatory mediators.

Finally, there are some other unusual compounds, such as lipoic acid, sodium iodide, zinc salts, adrenalin and dimethylsulphoxide, which have been reported to be anti-inflammatory in at least one type of (clinical or experimental) inflammation and which have free radical scavenging properties (Oyanagui, 1976; Egan et al, 1978; Dorfman and Adams, 1973 and Wilson, 1977).

In conclusion, the inhibition of the formation of free radicals or the scavenging of newly formed radicals during inflammation may represent a possible mechanism of action of many established anti-inflammatory compounds. Although many investigations have to be carried out in this area, it is possible that the scavenging of free radicals in some types of inflammation may be even more important than the inhibition of prostaglandin biosynthesis, in order to exert anti-inflammatory effects (Kuehl et al, 1976).

This may be so for two main reasons, namely on one hand, the intrinsic tissue damaging effects of free radicals and, on the other hand, the reported anti-inflammatory effects of some arachidonate-dependent products such as PGE_2, in the chronic phase of inflammation.

2.3. Systemic response to inflammation: role of the liver

Two characteristics of the systemic response to inflammation are usually obvious, namely increased body temperature and pain. Other features are not noticeable without the use of an invasive technique to obtain blood or a sample of inflamed tissue. These features of the systemic response comprise the activation of the pituitary-adrenal axis, elevated blood levels of the (positive) acute phase proteins in combination with decreased levels of albumin and reduction of the hepatic drug metabolizing capacity (see Whitehouse, 1977). The acute phase reactant proteins are synthesized in the liver (parenchymal cells) and contain carbohydrate moieties within the molecule. Hence, it is justifiable to define acute phase proteins as 'trauma-induced plasma proteins synthesized by the liver'. An excellent review on the significance of the acute phase reactants has been written by Koj (1974).

The acute phase response is unspecific since it is not limited to a specific disease, e.g. rheumatoid arthritis. However, the response seems to be associated with any kind of tissue damage and with the inflammation parallelling trauma. Thus, increased blood levels of acute phase proteins are found after surgical trauma, in rheumatoid arthritis, in some types of cancer and after myocardial infarction. The acute phase response as a result of inflammation consists of an initial increase in the blood levels of C-reactive protein, which occur rapidly (4-8 h) after the initiation of inflammation. Fibrinogen, haptoglobin, α_1-antitrypsin and ceruloplasmin are slowly (12 h) elevated. The maximum levels of the 'fast' acute phase proteins are reached within 48-72 h, whereas the 'slow' ones reach their maxima within 72-96 h. Other acute phase reactants are transferrin, the iron transport protein, and α_2-fetoprotein. The latter and C-reactive protein are not detectable in blood of normal subjects. Albumin behaves like a 'negative' acute phase protein, since its levels decrease in parallel with a rise in the other reactants. The decrease in albumin represents an important loss of the (low affinity, high capacity) plasma drug-binding capacity, which may lead to significantly elevated concentrations of the free drug in the blood.

The decrease in serum albumin during the acute phase response is also important for the maintenance of the osmotic pressure of the blood (Smith, 1977). A perennial question associated with the acute phase response is the means by which it is induced. It is generally believed that some humoral factor from the site of injury initiates the hepatic synthesis of the acute phase proteins or inhibits the gene repression. The differences in the time-dependent maxima of different acute phase reactants do not necessarily require distinct 'trigger signals' from the inflamed site, as the gene repression may be affected in different ways. The factor responsible for the initiation of the acute phase response is transferable from one animal (sterile exudate) to another and it is heat labile. Histamine, lysosomal proteases and factors obtained from leukocytes have been suspected 'the' triggers but experiments with the perfused isolated liver did not confirm these hypotheses (see Koj, 1974). Recently, it has been reported that PGE_1 induces an elevation in the serum levels of haptoglobin, on intravenous administration to rabbits (Shim, 1976). PGE_2 also induces the acute phase response, as elevated levels of fibrinogen are observed in rabbit plasma 24 h after PGE_2 infusion into the marginal ear veins. $PGF_{2\alpha}$ and PGD_2 were almost ineffective as initiators. The prostaglandin precursors, linoleic acid, dihomo-γ-linolenic acid and arachidonic acid are also initiators of the acute phase response measured as fibrinogen. The effect of arachidonate was not blocked by indomethacin. This, and the fact that oleic and palmitic acid also elicited the acute phase response, implies that prostaglandins are not necessarily involved the initiation by fatty acids (Carlson et al, 1978). In view of the observations that (15S)-15-methyl-prostaglandin E_2 (methyl ester), which is not metabolized by 15-hydroxydehydrogenase, is more potent than PGD_2 and $PGF_{2\alpha}$ but less potent than PGE_2, it is likely that there are several distinct triggering mechanisms. Fatty acids and irritants may initiate an 'unspecific' response, whereas selected prostaglandins may initiate a 'specific' response. This has been confirmed by the observation of Shim (1976) that inhibitors of protein synthesis inhibit the acute phase response triggered by PGE_1, but not that by turpentine. In rabbits bearing the VX_2-carcinoma, enhanced levels of the acute phase proteins, ceruloplasmin and haptoglobin, occur in the blood in combination with the PGE_2 metabolite, 13,14-dihydro-15-keto-prostaglandin E_2, which follows the same time course. Indomethacin inhibited the increased levels of the PGE_2 metabolite as well as those of ceruloplasmin, indicating that locally formed prostaglandins

may have initiated the acute phase reaction on transport to the liver (Voelkel et al, 1978).

With regard to inflammation, it is interesting that the intraperitoneal administration of the prostaglandin precursor, arachidonate, to mice inhibits the hepatic metabolism of type I substrates to cytochrome P-450 and leads to a loss of cytochrome P-450 activity, after prolonged (5 days) administration (Pessayre et al, 1979). This might be a competitive inhibition but it is also conceivable that oxygenated arachidonate metabolites inhibit, and lead to destruction of, cytochrome P-450 (see Chapter 1.2d).

Inflammation leads to a hepatic syndrome, which is obvious from a number of independent observations from different laboratoria. Some of these data are given in Table 4. The enzymes, β-galactosidase and β-N-acetyl-

Table 4. Hepatic changes as a result of remote localized inflammation

Hepatic parameter inhibited	Type of injury	Authors and year
adaptive synthesis of fatty acid synthetase	s.c. turpentine oil in the rat	Shutler et al, 1977
peroxisomal catalase synthesis	,,	Canonico et al, 1977
levels of reduced glutathione	unilateral hind limb tourniquet in rat	Marozzi & Malone, 1968
glutathione-S-transferase	subplantar oleyl alcohol and s.c. M. tuberculosis in tail	Fujihara et al, 1979
β-galactosidase and β-N-acetylglucosaminidase	s.c. turpentine oil in the rat	Kaplan & Jamieson, 1977
ascorbic acid levels	subplantar oleyl alcohol and s.c. M. tuberculosis in tail	Fujihara & Whitehouse, 1977

glucosaminidase, are involved in the catabolism of serum glycoproteins and their inhibition as a result of inflammation, may contribute to the

elevation of the serum acute phase glycoproteins. This is merely a contribution to and as such not the only reason for the elevated levels, as it has clearly been demonstrated (see Koj, 1974) that the enhanced levels are due mainly to the increase of *de novo* synthesis of acute phase proteins in the liver.

Catalase and glutathione participate in the defence system against reactive states of oxygen (see Chapter 1.3). Decreased availability of these substances may lead to oxidative damage to the liver, as it has been shown that depletion of glutathione induces lipid peroxidation in the livers of rats and mice (Younes and Siegers, 1980). During carrageenin-induced paw edema in mice, increased lipid peroxidation has been shown to occur when livers are removed from the animals and subsequently incubated for 3 hours (Sharma et al, 1972). Although different inflammation models have been used, it is likely that a remote localized inflammation, by one way or another, decreases hepatic catalase and glutathione levels and increases lipid peroxidation. The trigger signal may be the same as that responsible for the initiation of the acute phase response.

The exact functions of the acute phase proteins have by no means been elucidated yet. Increased fibrinogen levels may protect against blood loss from damaged vessels at the inflamed site, while C-reactive protein reacts with the C-polysaccharide from pneumococci occasionally infiltrated. α_1-Antitrypsin is an antiprotease, which protects against proteolytic enzymes released at the inflamed site. Haptoglobin combines with hemoglobin and the complex formed has essential peroxidase activity. That means that it oxidizes organic substrates in the presence of hydrogen peroxide as an electron acceptor. Ceruloplasmin is an oxidase, preferentially oxidizing ferrous iron and organic substrates, such as *p*-phenylene diamine, catechols and hydroxy-indoles. This means that ceruloplasmin may act as a monoamine oxidase, thus terminating the actions of (nor)adrenalin and serotonin, which are released during stress and shock. By virtue of its ferro-oxidase activity, ceruloplasmin protects against iron decompartmentalization, since ferrous iron is not bound to the iron stores, ferritin and transferrin.

Another aspect of the acute phase protein ceruloplasmin is that this protein is the main storage site for copper in the body. Enhanced levels of ceruloplasmin result in enhanced serum copper levels. Copper(II) ions, as well as copper bound to amino acids, are scavengers of superoxide radicals (see Chapter 1.3), while copper in combination with zinc, is essential for

the activity of cytoplasmic superoxide dismutase. This enzyme protects against the depolymerization of synovial fluid and hyaluronate in vitro, induced by the xanthine/xanthine oxidase system (McCord, 1974) and has anti-inflammatory properties in many inflammation models (Huber et al, 1977). Many copper chelates also have anti-inflammatory properties against adjuvant arthritis in the rat (Sorenson, 1976), while salicylates may exert anti-inflammatory activities by virtue of forming chelates with copper in vivo (Richardson, 1976). Totally in agreement with aforementioned anti-inflammatory activities of numerous copper compounds, is the reported anti-inflammatory activity of ceruloplasmin against urate-induced rat paw oedema (Denko, 1979). This may be at least one valid explanation for the remission of rheumatoid arthritis during pregnancy, as serum levels of ceruloplasmin gradually rise during this state.

In the light of the subject of this thesis, it is of great importance that ceruloplasmin has the ability to scavenge superoxide radicals, albeit less effectively than superoxide dismutase (Goldstein et al, 1979). It has been known for a long time that ceruloplasmin is responsible for the major part of serum antioxidant activity (see Cranfield et al, 1979). Furthermore, a plasma fraction associated with haptoglobin, is an endogenous inhibitor of the prostaglandin-synthesizing enzyme, cyclo-oxygenase (see Chapter 1.2c). The haptoglobin-hemoglobin complex can deal with hydrogen peroxide released from granulocytes, by virtue of its peroxidase activity, while also the complex of C-reactive protein and hematin has peroxidase activity (see Koj, 1974).

Injection of 2-amino-1,2,4-triazole into rabbits, leading to an inhibition of catalase activity, results in enhanced levels of serum C-reactive protein and when the hepatic catalase levels had returned to normal, the levels of C-reactive protein were lowered in parallel. Furthermore, purified C-reactive protein inhibited catalase synthesis on addition to liver slices in tissue culture (Hokama et al, 1969). From these observations it seems likely that C-reactive protein inversely reflects the hepatic catalase activity. This may be extrapolated to the elevated C-reactive protein levels in patients with rheumatoid arthritis and animal models of inflammation. In adjuvant arthritic rabbits it was possible to restore the decreased hepatic catalase activity to normal (and even supranormal) levels by the intravenous injection of hematin. The induction of catalase activity was marked as early as 3 h after treatment with hematin (0.5 mg/kg), both in

the liver and in the kidneys. Furthermore, the induction of catalase activity was parallelled by a decrease in serum C-reactive protein levels (Hokama et al, 1970). The authors suggest a possible role of C-reactive protein as a dimeric precursor for the tetrameric catalase apoenzyme, which protects against hydrogen peroxide provided that heme is incorporated.

Rat α_2-macroglobulin (foetoprotein), like ceruloplasmin, has also been shown to display anti-inflammatory properties against carrageenin-induced rat paw oedema. Injury (laparotomy) prior to the administration of carrageenin, enhanced the levels of serum α_2-foetoprotein, and the maximum serum concentration was reached 24 h after laparotomy. Subsequently, maximal suppression of paw oedema was observed 24 h after laparotomy, suggesting that the inhibition of oedema formation was a consequence of enhanced levels of foetoprotein (van Gool et al, 1974). Analogously, one of the modes of action of anti-inflammatory glucocorticoids may be their ability to enhance plasma levels of the antiprotease, α_1-antitrypsin and thus, indirectly, glucocorticoids inhibit cellular and tissue damage at the site of inflammation (Pitt and Lewis, 1979).

In addition to the anti-inflammatory proteins which can be recovered from the blood of inflamed animals, a heterogenous group of anti-inflammatory proteins has been shown to occur at the site of inflammation (for a review, see Bonta, 1978). This group may include several acute phase proteins as extra-hepatic synthesis of some acute phase reactants occurs, for instance, α_1-antitrypsin and α_2-macroglobulin synthesis have been observed in human tumours (see Cooper and Milford Ward, 1979). It is conceivable that the protease-antiprotease complexes are trapped from the circulation by macrophages from the tumour, thus providing an apparent extra-hepatic site of synthesis. However, Gitlin and coworkers (1977) reported the ability of human synovial cells to produce ceruloplasmin in vitro, emphasizing the possibility of a close relationship between local anti-inflammatory proteins at the inflamed site and acute phase proteins in the blood.

In view of the properties of the acute phase proteins mentioned in this chapter, it seems justifiable to characterize the acute phase response as being a homeostatic phenomenon. The activities of several proteases are antagonized, the levels of the fibrin precursor are enhanced, thus, protecting against eventual blood loss, free hemoglobin is bound by haptoglobin and the complex displays peroxidase activity. Ceruloplasmin protects against

Fe(II)-catalyzed lipid peroxidation and is a superoxide scavenger. As an oxidase, it may limit the actions of released catecholamines and hydroxyindoles. Like α_1-antitrypsin and rat α_2-macroglobulin, it has anti-inflammatory properties. C-reactive protein is likely to provide precursor peptides for hepatic and renal catalase, the activities of which are diminished as a result of a remote localized injury.

The acute phase response can be affected in several ways, but the individual acute phase reactants do not respond uniformly. Corticosteroid administration leads to enhanced levels of rat α_2-foetoprotein and haptoglobin, but not of ceruloplasmin, while the administration of PGE_1 or PGE_2 results in increased levels of fibrinogen and haptoglobin. Injury and pregnancy inhibit inflammation, presumably as a result of an extra stimulation of the acute phase response. Finally, haptoglobin-like proteins inhibit, in turn, the formation of prostaglandins, which is indicative of the extremely fine-balanced automodulation mechanisms which are operative as a result of and directed against injury of many kinds.

2.4. Summary

Inflammation is characterized by local events, such as redness, oedema and pain, and humoral mediators of inflammation play an important role in the local response. In the sub-acute phase of inflammation, granulocytes and monocytes invade the inflamed area to engulf the inflammagen. During phagocytosis, superoxide anions are generated at sites on the cell membrane of the phagocytes by an NADPH oxidase. Superoxide anions are dismutated to hydrogen peroxide by superoxide dismutase, which, in combination with catalase protects the cytoplasma and its organelles from damage. Nevertheless, superoxide radicals escape into the extracellular fluid and the potent oxidant, the hydroxyl radical, is also formed. This formation may lead to peroxidation of constitutional membrane lipids. Apart from inhibiting the biosynthesis of prostaglandins, many anti-inflammatory drugs act as free radical scavengers, as inhibitors of superoxide production by granulocytes and macrophages and as inhibitors of cell migration to the inflamed site. Systemic effects of inflammation comprise activation of the pituitary-adrenal axis, increased body temperature and alterations in liver function. The activity of many hepatic enzymes is decreased as is the drug handling capacity. In contrast, the liver synthesizes the, 'so-called' acute phase proteins at an enhanced rate. The role of

the acute phase proteins is likely to be a protective one, since distinct acute phase reactants inhibit the biosynthesis of prostaglandins, display peroxidase activity (i.e. they catalyze the breakdown of hydrogen peroxide), reflect hepatic catalase activity, scavenge superoxide radicals and have anti-inflammatory properties. The increased levels of acute phase proteins provides enhanced protection against oxidative events, by virtue of their peroxidase and superoxide-scavenging activities and this may be an indication of the occurrence of a systemic oxidant stress as a result of a remote localized inflammation.

PART II. AIM OF THE STUDIES PERFORMED

In 1954, Duboulez and Dumas showed that lipid peroxidation is involved in acute inflammation. Many years later, it was recognized that after injury prostaglandins and related substances are formed by endoperoxidation of polyunsaturated fatty acids. Thus, dihomo-γ-linolenic acid and arachidonic acid are converted to prostaglandin endoperoxides by cyclo-oxygenase. These endoperoxides are then converted to the stable prostaglandins, thromboxane, prostacyclin, hydroxy-fatty acid and malonaldehyde. Thus, it is possible that the peroxides detected by Duboulez and Dumas were prostaglandin endoperoxides.

The aim of our studies were to investigate whether lipid peroxidation occurs at the site of inflammation independently of the biosynthesis of prostaglandins. This seemed likely, as fatty acid precursors are abundant at the inflamed site and since initiators of lipid peroxidation, namely free radicals, are constantly generated and released by granulocytes and mononuclear phagocytes, during phagocytosis.

Malonaldehyde is a stable end product of the decomposition of endoperoxides formed during lipid peroxidation, which has been used as a monitor of the extent of lipid peroxidation in chemical and biological systems. As locally formed malonaldehyde may originate from the arachidonate cascade and, more specifically, from the thromboxane synthetase pathway, it was planned to investigate time-dependent changes in prostaglandin biosynthesis, for which purpose radioactively labelled arachidonic acid was to be injected into the inflamed site. An inflammation model which allows the accurate injection of drugs and local hormone precursors into the inflamed site, namely the model of cannulated sponge implants in the rat, was in routine use within our laboratory. However, the sponges do not allow for the collection of inflammatory exudate from the granulomata around the sponges, without surgical intervention. Therefore, an analogous model was to be developed which permitted the collection by perfusion of inflammatory exudate containing the radioactively labelled metabolites of arachidonic acid.

In 1972, Sharma and coworkers showed that livers from mice with carrageenin-induced paw oedemata produced significantly more malonaldehyde than livers from control mice, indicating increased hepatic lipid peroxid-

ation as a result of a remote localized inflammation. Another aim of the studies presented in this thesis was to extend the observations of Sharma and coworkers to (semi-)chronic inflammation models in the rat. It was the intention to demonstrate that increased hepatic lipid peroxidation was not due to the resorption of carrageenin at the inflamed site. Therefore, the non-absorbable inflammagen kaolin was used. Furthermore, it was necessary to show eventual increases in the hepatic levels of malonaldehyde ex vivo, i.e. without incubation, as this seemed to be more indicative of in vivo alterations.

Besides hepatic lipid peroxidation as a result of an extra-hepatic inflammation, we were interested in hepatic changes which had been reported to occur in combination with lipid peroxidation under conditions other than inflammation. Therefore, we were to investigate the levels of selected hepatic antioxidants at different stages of an extra-hepatic inflammation, as well as changes in the hepatic drug metabolizing capacity, measured using the model drug, aminopyrine.

A final aim of the studies performed was to pharmacologically influence the inflammatory process by the local administration of antioxidants and free radical scavengers, thereby attempting to dissociate between the distinct states of reduced oxygen which are involved in the inflammatory response.

PART III. GENERAL DISCUSSION AND CONCLUSIONS

The problem of lipid peroxidation in inflammation may be approached by studying either the local site of inflammation or changes which occur in the liver. The majority of investigations in the field of inflammation have been concentrated on the inflamed site. Prostaglandin formation, its interactions with the cyclic nucleotide system, the release and actions of bradykinin, serotonin and histamine, the aspects of chemotaxis and cellular infiltration, the release and actions of lysosomal enzymes, the occurrence of anti-inflammatory proteins at the site of inflammation and the local anti-inflammatory effects of corticosteroids and nonsteroidal anti-inflammatory drugshave all been (and are still) widely investigated.

Fewer investigators have worked on endogenous modulation of inflammation, attention then being focussed on the liver as the site of production of anti-inflammatory proteins, which can be isolated from the blood and the site of inflammation. Very recently, a small number of investigators (Babior et al, 1973; McCord, 1974) started to study the role of free radicals and their products in humoral and cellular systems, which are relevant to inflammation, but their studies have been almost completely confined to in vitro experiments.

The aim of our studies was to investigate the suggested role of lipid peroxidation in inflammation in vivo and its relation to hepatic changes occurring during extra-hepatic inflammation. We focussed our attention initially on the inflamed site, in view of the available know-how within the laboratory on inflammation models, the study of exudation, prostaglandin biosynthesis and granuloma formation. Models of granulomatous inflammation were chosen because of their similarity to the proliferative aspects of rheumatoid arthritis. The proliferative lesion is characterized by an enormous increase in the amount of collagen, which leads to destruction of cartilage and ultimately of the joint itself. The granuloma model and the proliferative lesion have in common the involvement of macrophages as the most important cell type. The model is also representative for prostaglandin biosynthesis, superoxide formation, lysosomal enzyme release and collagen formation and breakdown.

In order to investigate the in vivo metabolism of the polyenoic fatty acid, arachidonic acid, the prostaglandin precursor which is highly

susceptible to peroxidation, we developed a new inflammation model, the model of teflon chamber implants in the rat (see Appendix, Chapter 1). This model shows similarities to other models of granulomatous inflammation, namely with respect to patterns of exudation, granuloma formation and the domination of polymorphonuclear leukocytes in the exudate. The fact that lymphocytes are the second most important cell type in the newly developed chamber model, whereas in the sponge model lymphocytes are hardly present, provides the former with a closer resemblance to rheumatoid arthritis than the model of implanted sponges.

Another similarity of the chamber model with rheumatoid arthritis is the pattern of metabolism of arachidonate (see Appendix, Chapter 2). Thus, injected {1-^{14}C}arachidonate in our rat model was converted mainly to the hydroxy-fatty acids HHT and HETE, and to PGE_2, whereas prostacyclin and thromboxane were formed only in very small amounts. In patients with rheumatoid arthritis, the synovial fluid contained mainly PGE_2, whereas thromboxane was a minor arachidonate metabolite (Trang at al, 1977). Higgs and Salmon (1979) confirmed our results more recently, since they found similar patterns of endogenous arachidonate metabolites in the sponge model, using radioimmuno-assay for the detection of prostaglandins and related substances. Our results and the results of Higgs and Salmon with in vivo models are in marked contrast to the results of Chang et al (1978) in Japan, who found thromboxane and prostacyclin to be the main metabolites from arachidonate in vitro, using homogenate fractions of granulomata removed from rats on day 8 of inflammation. Thus, it is very likely that damage to platelets and vascular walls had interfered with their results and subsequent interpretation.

Another analogy to rheumatoid arthritis, which we observed with the newly developed chamber model, was the lack of improvement of the inflammatory conditions on treatment with nonsteroidal anti-inflammatory drugs, such as indomethacin. Thus, indomethacin inhibited PGE_2 formation but failed to inhibit granuloma formation (see Appendix, Chapter 3). As the inhibition by indomethacin of the different arachidonate metabolites did not occur uniformly, it is possible that different cyclo-oxygenases (or cyclo-oxygenase of different cells) are involved in prostaglandin formation at the inflamed site. This assumption is based on the observation that the cyclo-oxygenase of arterial walls (main product prostacyclin) is less sensitive to inhibition by nonsteroidal anti-inflammatory drugs than platelet cyclo-

oxygenase (main product thromboxane A_2). Another possibility is that the prostaglandin endoperoxide-converting synthetases and isomerases are differently inhibited by aspirin-like drugs.

A possible explanation for the observed lack of inhibition of granuloma formation by indomethacin, is that PGE_2, in the chronic phase of inflammation, may play an inhibitory role in granuloma formation, through the stimulation of the adenylate cyclase of macrophages (Bonta and Parnham, 1978). The inhibition by injected PGE_2 of granuloma formation is potentiated by phosphodiesterase inhibitors and inhibited by the adenylate cyclase antagonist, SQ 22536. At the time of maximal macrophage infiltration in the granuloma, macrophages isolated from granulomata contain minimal amounts of cyclic AMP, which means that the cells are very sensitive to stimulation of their adenylate cyclase by PGE_2. Administration of PGE_2 at this stage of granuloma formation leads to maximal inhibition of granuloma weight. Administration of inhibitors of prostaglandin formation during this stage, may have no benificial effects since they would eliminate the action of an anti-inflammatory endogenous modulator, namely PGE_2.

The most interesting aspect of the actions of indomethacin in the chamber model was the (at that time) unexpected inhibition of HETE formation in the early phase of inflammation. It was then still believed that HETE formation from arachidonate, via the lipoxygenase pathway (Part I, Chapter 1.2c), was insensitive to inhibition by nonsteroidal anti-inflammatory drugs. The lipoxygenase pathway leads to the formation of several hydro-peroxy-fatty acid intermediates, from which HETE or the leukotrienes are subsequently formed. One of the leukotrienes is the local hormone, SRS-A, an important bronchoconstrictor in allergic conditions, while other leukotrienes, especially di- and trihydroxy-fatty acids, are chemotactic for granulocytes. Furthermore, our studies with indomethacin indicated that the formation of products from injected arachidonate could never be fully suppressed, which anticipated our later studies, by suggesting autoxidative formation of metabolites.

The formation of malonaldehyde at the inflamed site, irrespective of the biosynthesis of prostaglandins, during granuloma formation, provides further evidence for the involvement of lipid peroxidation in inflammation (see Appendix, Chapter 4). Our observation that local lipid peroxidation is correlated with the inflammatory strength, has also been observed in patients with different degrees of rheumatoid arthritis (Muus et al, 1979).

The studies on the in vivo metabolism of arachidonate indicated that a redirection of metabolism via the thromboxane pathway, leading to HHT, thromboxane and malonaldehyde formation at the expense of PGE_2, does not occur and is not responsible for the dissociation between locally formed malonaldehyde and PGE_2. The increased levels of malonaldehyde in the liver and plasma of rats with a remote localized inflammation, indicate that the liver is involved in an extra-hepatic inflammation. That the malonaldehyde recovered from the liver is really due to hepatic formation and not to trapping of the aldehyde from the circulation, is demonstrated by the enhanced formation of malonaldehyde on incubation of livers from inflamed rats, irrespective of the inflammagen used (Sharma et al, 1972; see also Appendix, Chapter 4).

In many in vitro experiments, correlations have been demonstrated to exist between increased lipid peroxidation and inhibition of oxidative drug metabolism and *vice versa*, using liver homogenates and microsomal suspensions from various animal species. Possible underlying mechanisms have been discussed in Part I, Chapter 1.2d. We have shown that increased hepatic lipid peroxidation and reduced aminopyrine metabolism occur simultaneously in vivo, during granulomatous inflammation in the rat (see Appendix, Chapters 5 and 6). The reduced aminopyrine metabolism, measured as an increased half-life, was correlated with the inflammatory strenght. The nature of the inhibition of drug metabolism during inflammation has not yet been investigated, but one possibility is that the inhibition is due to destruction of cytochrome P-450 at the heme moiety or at the lipid region. In this respect, it has been reported that during adjuvant arthritis in the rat the amounts of cytochrome P-450 and also of NADPH-cytochrome c reductase were significantly reduced (Matthur et al, 1978). Unfortunately, no time-dependency of the changes have been investigated and thus, it is not possible to establish whether NADPH-cytochrome c reductase is responsible for production of superoxide radicals, when cytochrome P-450 has already been inactivated.

The consequences of reduced drug metabolism may be of importance, if one extrapolates the data with rats to rheumatoid arthritis in man. A major implication of the experimental data would be that the 'safety' of anti-rheumatic drugs depending on hepatic metabolism, in patients with rheumatoid arthritis will be severely overestimated. Toxicity studies are based mainly on observations with healthy animals and volunteers, whereas data obtained

with patients can only be gathered after a long-term controlled study. In other words, a patient suffering from rheumatoid arthritis will generally be more sensitive to toxic drug effects than a normal person, receiving the same dose. Time will show, however, whether it is justifiable to make such extrapolations, as investigations of hepatic drug metabolizing capacity have not yet been carried out on patients with rheumatoid arthritis.

Impairment of hepatic drug metabolizing capacity is not limited to to experimental inflammation (nor possibly to rheumatoid arthritis), as it has also been reported to occur during pregnancy and hepatitis. During the latter conditions, increased serum levels of the acute phase proteins as well as of lipid peroxides have been reported (see Part I, Chapter 1.2d). It is, therefore, tempting to speculate that the same trigger mechanism may play a role in eliciting the acute phase response, hepatic lipid peroxidation and decreased drug metabolism.

During remote localized inflammation, changes occur in hepatic antioxidant levels. Catalase activity and the levels of reduced glutathione and ascorbic acid are markedly depressed, while lipid peroxidation is strongly enhanced (see Appendix, Chapter 6). Possible consequences of lipid peroxidation in the liver are stimulation of lysosomal enzyme release, membrane lysis and degranulation of rough and smooth endoplasmic reticulum with subsequent inhibition of protein synthesis and cellular excretory mechanisms. Decreased hepatic glutathione levels may lead to inhibition of phase II biotransformation of drugs which are normally conjugated to glutathione via hepatic glutathione-S-transferases, for instance, paracetamol (acetaminophen). Unconjugated reactive metabolites, formed during phase I biotransformation (for instance by cytochrome P-450), attack sulphydryl moieties of hepatocytes, leading to hepatocellular damage and ultimately, necrosis.

Should our rat models also be representative of rheumatoid arthritis with respect to hepatic changes, then it is very likely that, during rheumatoid arthritis, some kind of hepatic oxidant stress exists. Indeed, it has been shown that plasma levels of malonaldehyde, the stable lipid peroxidation product, are elevated during rheumatoid arthritis and correlate with disease activity (Muus et al, 1979). As in the rat models, this may indicate altered hepatic function. However, it is not clear whether the lipid peroxidation observed during inflammation is due to the enhanced formation of lipid peroxides or to decreased metabolizing capacity of the

liver with respect to lipid peroxides and xenobiotics. It is generally thought that low 'steady-state' levels of several oxidants (superoxide anions, lipid peroxides, hydrogen peroxide) exist in the normal individual, but the body is able to detoxify them by means of ascorbic acid, vitamin E, glutathione and the enzymes, catalase, superoxide dismutase and glutathione peroxidase (see Chance et al, 1979). Disturbances in the capabilities of these detoxification mechanisms, whatever the underlying cause may be (genetically determined, drug-induced, inherent to states of disease or nutricional insufficiencies, etc.), may lead to increased lipid peroxidation and its sequellae.

It is of particular interest that several reports have described the presence of many biochemical and morphological aberrances in the livers of rheumatoid arthritis patients, varying from the well-known changes in plasma proteins to raised serum levels of alkaline phosphatase, 5'-nucleotidase, bioptic findings of inflammatory cell infiltration and an increase of fibrous tissue around the portal triade (see Lefkovits and Farrow, 1955). Although some observations may be explained by iatrogenic mechanisms, these findings suggest the existence of a reactive hepatitis during rheumatoid arthritis. This may represent one of the feed-back mechanisms involved in the self-curtailing processes of inflammation. Thus, it is a well-known clinical experience that during hepatitis and jaundice, the activity of rheumatoid arthritis is decreased. Several explanations are feasible. One possible explanation maight be that, as a result of liver dysfunction, the catabolism of adrenal corticosteroids by the liver is also impaired, which would lead to an elevated serum level and a longer half-life of these hormones. However, in patients with liver cirrhosis, a condition preceded by chronic hepatitis, plasma levels of 17α-hydroxy-corticosteroids (cortisone, hydrocortisone) are often not elevated, but administered corticosteroids are less rapidly cleared from the blood, when compared to healthy individuals (see Kornreich et al, 1971). This implies that other mechanisms must be involved in the suppression of rheumatoid arthritis than merely the modulation by endogenous anti-inflammatory steroids.

Another, almost similar, explanation is that bile salts and bilirubin displace corticosteroids from their binding sites on plasma transport proteins, since bile duct ligation leads to an improvement of art paw oedema (see Bonta, 1978). A third explanation may be the enhanced synthesis of anti-inflammatory acute phase proteins as a result of hepatitis and other

forms of injury, for instance laparotomy. Pregnancy which is, for instance, characterized by enhanced levels of ceruloplasmin in the blood, also results in an improvement of rheumatoid arthritis.

Ceruloplasmin, the copper transport protein, is a superoxide radical scavenger, is the major serum antioxidant and has anti-inflammatory properties against experimental inflammation. With respect to the scavenging of superoxide radicals, it is much less effective than superoxide dismutase (see Part I, Chapter 2.4). Even at very high dosage levels, we were unable to demostrate any beneficial effects of the local administration of bovine superoxide dismutase in a model of granulomatous inflammation in the rat (see Appendix, Chapter 7). This may be due to the size of the molecule, which prevents superoxide dismutase from reaching its site of action. However, catalase with a molecular weight of about seven times that of superoxide dismutase, was a potent inhibitor of granuloma formation and exudation under the same experimental conditions. This may mean that hydrogen peroxide is a more potent inflammatory mediator than superoxide, but it is also likely that superoxide dismutase is rapidly inactivated by one of its own products, namely hydrogen peroxide. This may also partially explain why the low-molecular weight copper-amino acid chelates, which are relatively stable and also easily cross membranes because of their lipophilicity, are effective anti-inflammatory agents (Sorenson, 1976).

Although we were unable to demonstrate anti-inflammatory effects of hydroxyl radical scavengers in our experiments (see Appendix, Chapter 7), it is still likely that hydroxyl radicals are inflammatory mediators by virtue of their high reactivity to biological molecules, for instance lipids and proteoglycans. This implies that scavengers need to be administered in concentrations high enough to compete with ubiquitous biological molecules as 'substrates' for hydroxyl radicals. Inhibitors of hydroxyl radical formation, rather than scavengers of preformed hydroxyl radicals, would then be expected to display anti-inflammatory properties. In this context, we were able to demonstrate that specific combinations of superoxide dismutase and catalase as well as an iron chelator, were effective as inhibitors of granuloma formation, presumably by preventing the Fenton reaction of iron(II) with hydrogen peroxide, yielding hydroxyl radicals (see Appendix, Chapter 7). A pharmacological implication of these findings is that iron chelators might be promising anti-rheumatic agents in view of their potential inhibiting properties on granuloma formation and synovial

fluid depolymerization.

The observed anti-inflammatory effects of antioxidants (see Appendix, Chapter 7) have a number of pharmacological implications. Some antioxidants are markedly ineffective as inhibitors of prostaglandin biosynthesis and since PGE_2 is an inhibitor of experimentally-induced granuloma formation, this lack of inhibition may be advantageous. In addition, such antioxidants may also lack gastrotoxicity, as the latter seems to be coupled with inhibition of prostaglandin biosynthesis in the stomach. Other antioxidants potentiate the inhibitory actions of indomethacin on carrageenin-induced rat paw edema (Sobański et al, 1976). This means that smaller amounts of indomethacin are required to achieve therapeutically active levels and thus, disadvantageous side effects of nonsteroidal anti-inflammatory drugs may be reduced. Some antioxidants inhibit (albeit not selectively) soybean lipoxygenase. Should mammalian lipoxygenases be sensitive to to inhibition by selected antioxidants, then antioxidants may prevent the formation of chemotactic principles and slow-reacting substances, which are formed via a lipoxygenase mediated pathway.

Finally, it would be interesting to evaluate drugs which act as inducers of the synthesis of acute phase proteins or as inhibitors of repression at the DNA-RNA level. Oestrogens, amongst other agents, may provide a matrix for new drugs to be developed, since they induce enhanced levels of ceruloplasmin and have beneficial effects on inflammatory disorders. They may be used to investigate interactions between lipid peroxidation, drug metabolism and the hepatic acute phase response in animals as well as in man, and to study whether lipid peroxidation products, migrating from the inflamed site, act as triggers of the hepatic adaptive syndrome during inflammation.

PART IV. APPENDIX

1. Cannulated Teflon Chamber Implant in the Rat: A New Model for Continuous Studies on Granulomatous Inflammation

P. C. BRAGT, I. L. BONTA, AND M. J. P. ADOLFS

A granulomatous inflammation model is described. It is a modification of the cannulated sponge method and consists of a subdermally implanted teflon cylinder in the rat. This 'chamber' is cannulated and provided with holes to ensure contact and exchange of materials between the inner chamber and the surrounding tissues. The cannulae are exteriorized at the back of the neck and allow materials to be injected and exudate to be collected at any given time during the development of granulomatous tissue around the chamber. Some parameters of this inflammation, such as exudation, granuloma growth, cellular infiltration in exudate, and the formation of prostaglandins were studied, and a comparison was made with the sponge implant model. The value of the chamber method is illustrated by brief reference to a study on the metabolism of ^{14}C-arachidonic acid during granulomatous inflammation.

Key Words: Arachidonic acid metabolism *in vivo*; Cannulated chamber implant; Granulomatous inflammation; Exudative inflammation; Cells in granulomatous inflammation; Prostaglandins in granulomatous inflammation.

INTRODUCTION

Recently, an inflammatory granuloma model was described (Bonta et al., 1979), consisting of cannulated polyether sponges implanted into the dorsal subdermal tissue of a rat. The merit of this method is that it allows the local injection of materials into the inflamed site at any given time during granuloma development. However, the method has the disadvantage that it does not allow the withdrawal of inflammatory exudate via the cannulae because of the high flow resistance of the sponge material. Another disadvantage, in common with other synthetic sponge implant models, is the fact that compounds (e.g. softeners, stabilizers) added to the sponge material during its manufacture may have an influence on the inflammatory process (Parnham et al., 1977).

Therefore, we have developed a new model that allows materials to be injected and exudate to be withdrawn at any given stage of granuloma development. Our model combines the advantages of both the cannulated sponge implant of Bonta et al. (1979) and the wound chamber described by Lundgren and Lindhe (1973); it

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consists of a cannulated teflon cylinder with a perforated mantle that is implanted into the dorsal subdermal region of a rat. The main constituent, teflon, evokes a minimal tissue reaction by itself (Harrison et al., 1957; Calnan, 1963) and is inert towards almost all chemicals. We compared some parameters of granulomatous inflammation induced by teflon chamber implants with those following sponge implantation. In addition, as the newly developed chamber method enabled us to study the local metabolism of arachidonate in vivo during granulomatous inflammation (Bragt and Bonta, 1979), some data from this study on arachidonate metabolism are presented to illustrate the use of the method. Our results are compared with the results of Chang et al. (1978), who reported arachidonate conversion by granulomatous tissue in vitro.

MATERIALS AND METHODS

Materials

Polytetrafluoroethylene (Teflon, Fluon, Fluoroflex) tube, i.d. 8 mm and wall thickness 1 mm (Eriks; Alkmaar, The Netherlands), was cut into pieces of 30 mm in length. Each piece of teflon was installed in a piercing gauge and 108 holes were made, 1.5 mm in diameter and distributed into 12 rows of 9 holes around the cylinder mantle. After piercing, residual curls of teflon were filed off. One end of the cylinder was closed with a plug of silicone (Silastic Medical Adhesive, Dow Corning; Midland, Texas) and after having inserted two small pieces of Tygon tube (0.7 × 2.3 mm, Rubber; Hilversum, The Netherlands) 20 and 40 mm in length, respectively, through the open end; this end was also sealed with silicone. Two nylon cannulae, about 120 mm in length (0.65 × 1.02 mm, Rubber; Hilversum, The Netherlands), were inserted into the Tygon tubes, after the silicone had been left to harden overnight. This completed the assembling of the unit and the cannulated teflon chamber was ready for implantation into rats.

The materials used permit sterilization of the chamber at 120°C, if this is required. The internal volume of the chamber is 1.5 ml, plus that of the cannulae (0.04 ml each). Thus, after the injection of a compound only 2.7% (by volume) will fail to enter the granuloma. If necessary, this can be overcome by the inclusion of a small volume of air in the injection syringe. The total area of the perforations represents 25.3% of the mantle area.

Sodium carrageenin (Viscarin, Marine Colloids Inc.; Springfield, Mass.) was prepared as a 1% (w/v) gel in sterile pyrogen-free saline, by stirring for several hours at room temperature.

[1-^{14}C] Arachidonic acid (55.5 Ci/mole, The Radiochemical Centre; Amersham, England) was removed from its solvent, benzene, by evaporation under a stream of nitrogen, and redissolved in absolute ethanol to a specific activity of 12.5 μCi/ml.

May-Grünwald (number 1424) and Giemsa (number 9207) solutions were purchased from Merck; Darmstadt, West Germany.

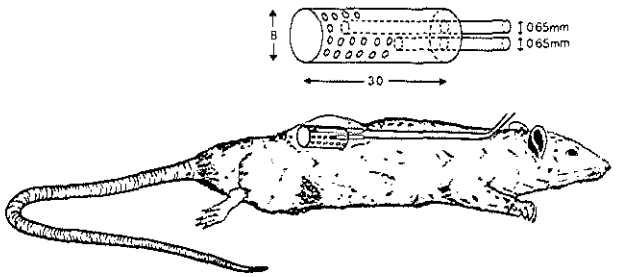

FIGURE 1. Schematic representation of the implanted teflon chamber in the rat, with the cannulae exteriorized at the back of the neck. Sizes of the chamber are given in the upper figure. The cylinder mantle is provided with holes of 1.5 mm in diameter and the total area of the holes is 25.3% of the mantle area. The inner chamber volume is 1.5 ml. The top and bottom of the cylinder have been closed by means of silicone.

Implantation Procedure

Male Wistar rats (TNO; Zeist, The Netherlands), weighing 190–230 g, were maintained on standard laboratory food (Hope Farms; Woerden, The Netherlands) and drinking water, to which they had free access.

The implantation was performed according to the method of Bonta et al. (1979), except for the fact that, instead of a midline incision in the back of the rat, an incision of about 10 mm was made in the flank. The cannulated chamber was then pushed through the incision into a thoraco-sacral position under the dorsal skin and the wound was closed. The situation and the size of the chamber are schematically presented in Figure 1. To illustrate the position of the chamber in the intact animal, a lateral X-ray picture was made without using any contrast fluid. The positive print of this picture is presented as Figure 2. After implantation, 1 ml

FIGURE 2. Lateral X-ray photograph of a rat with an implanted cannulated teflon chamber. In order to visualize the chamber no contrast fluid was injected. The cannulae from the chamber, exteriorized at the back of the neck, are not visible in this photograph.

carrageenin was injected into the chamber via one of the exteriorized cannulae. The cannulae were closed by a plug of tube sealer (Seal-ease, Clay-Adams; Parsippany, N. J.). In order to ensure good contact of the carrageenin (through the perforations) with the tissues surrounding the chamber, the rats were rotated around the thoraco-sacral axis immediately after the injection of the inflammagen. The development of granuloma around the chamber was studied for 7 days following the administration of carrageenin.

Characteristics of the Granulomatous Inflammation

Five groups of 5 rats were used for the study of changes in exudation, cell accumulation in the inflammatory exudate, and postaglandin formation at different stages of granuloma development, i.e.: 6 hr, 1,3,5, and 7 days after the induction of inflammation by carrageenin. The inflammatory response as a result of the implantation of the teflon chamber alone without stimulation by carrageenin, was investigated in two groups of 10 rats on day 3 and day 7 after the implantation. At the appropriate times, rats were killed by chloroform inhalation, bled by an incision in the neck, and the chambers with the surrounding granulomata were removed. The isolated granulomata were cut open and the individual exudates collected in 2 ml heparinized (Thromboliquine, Organon; Oss, The Netherlands) saline by centrifugation of the granulomata over a polypropylene tube (15 min at 800 × g).

The supernatant was used for the determination of the exudate volumes and the bioassay of prostaglandin-like material (PGL) according to the method of Bult et al. (1977), after lipid extraction at pH 3. The pellet, consisting of cells present in the exudate, was resuspended in 2-5 ml heparinized saline depending on the amount of sediment. Twenty microliters suspension was mixed with 20 ml Isoton fluid and the sample was counted for total cells in a Coulter Counter type 3260 B (thresholds: lower 9, upper 100). The total cell number in each exudate was calculated by correcting for the dilutions made. Cell differentiations were performed on smears of sediment by light microscopy, after staining with May-Grünwald-Giemsa. For this differentiation, 350 cells were counted in each smear. The criterion for the discrimination between monocytes and macrophages present in the inflammatory exudate was based on the presence of a vacuolized cytoplasm and a bean-shaped nucleus in the macrophages, while the monocytes did not show vacuoles and had nuclei with irregular shapes. Following the removal of the exudate and separation of the chamber from the granulomata, the latter were weighed, dried at 80°C for 24 hr, and finally reweighed for the determination of the dry granuloma weights. In experiments involving comparison of inflammation in the chamber model with that in the sponge implant model, the latter was used according to the method described earlier (Bonta et al., 1979), except that in the present study the size of the sponges was halved in comparison with the original method.

Arachidonate Metabolism In Vivo by the Granuloma

A group of 4 rats was used for the study of the metabolism of arachidonate by granulomatous tissue *in vivo*. On days 1,4,8, and 12 after the induction of inflam-

mation [1-^{14}C], arachidonic acid (2.5 μCi/rat in 200 μl ethanol) was injected via one of the cannulae while the rats were under ether anaesthesia, the isotope labeled arachidonic acid was left in the rat for a 1-hr incubation in vivo.

Recovery of the Exudates Containing Metabolites of Arachidonate

After the 1-hr incubation, the rats were again subjected to ether anaesthesia and the ends of the cannulae, closed with tube sealer, were cut off. The exudate was withdrawn via one of the cannulae using a 5-ml syringe equipped with a 0.6 × 25 mm needle. To obtain maximal recovery of the exudates containing the radioactive metabolites, 1 ml saline was injected into the other cannula and the perfusate was collected into the first syringe. The perfusates were collected into calibrated, siliconized glass tubes, and identification and quantitation of the arachidonate metabolites were performed by a method combining thin-layer chromatograpy and liquid scintillation counting as described by Vincent and Zijlstra (1978).

Cleaning of the Chamber for Re-use

After use, the cannulae were removed from the Tygon tubes attached to the chamber. The silicone plugs, together with the Tygon tubes, were pulled out, and all parts of the chambers submersed in decontamination fluid to remove residual tissue and radioactivity inside the cylinders and tubes. The decontamination fluid was discarded and frequently refilled until no tissue or radioactivity could be detected either on the inside or the outside of the materials. Finally, the chambers and cannulae were rinsed with water and absolute ethanol, dried under a stream of air, and reassembled ready for re-use.

RESULTS

Properties of the Implanted Chamber

The implantation of chambers into rats is a procedure that can be very easily performed in large groups of animals. Because of the good mechanical and chemical stability, the chambers can be used for long-term studies. Sometimes, the Tygon tubes tend to become less flexible over time, which makes it advisable to replace them after each experiment. As a result of the carrageenin-stimulated inflammatory process, the chambers may be rejected through the skin. This was only observed in rats that had been inflicted with chronic inflammation for more than 20 days.

The cannulation of the chamber allows the injection of materials directly into the inflamed site. As can be seen in Figure 3A the injected fluid spreads over the subdermal layers when no granuloma is present around the chamber. This has the disadvantage that the local concentration at the inflamed site of an injected compound is less than in the presence of a granuloma. Figure 3B shows that, a few minutes after the injection of a contrast fluid, the distribution is limited to the exudate within a 4-day-old granuloma. The main merit of the chamber method for the study of experimental inflammation is the ability to obtain exudate from the

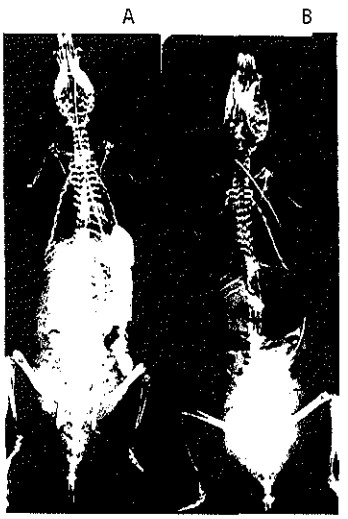

FIGURE 3. Posterior–anterior X-ray photograph of two rats taken within a few seconds after the injection of contrast fluid into the implanted chambers. A: A rat 4 hr after the injection of carrageenin, when no granuloma had yet developed. The contrast fluid is spread over the subdermal space. B: When injected into the chamber of a rat 4 days after the administration of carrageenin, the distribution of the contrast fluid is limited to the intra-granulomatous fluid. Because the rat bent foreward, the major part of the contrast fluid is localized in the rostral part of the chamber. Note that in both pictures only one cannula is filled with contrast fluid.

granuloma at any given moment, without killing the rat, for at least 12 days after the induction of inflammation. When perfusion with saline is carried out as described in the "methods" section, the recovery of exudate is possible in spite of the presence of a spongy granuloma that has grown into the chamber. In this way, about 30% of the radioactivity injected as sodium ^{14}C-arachidonate, could be recovered from an 8-day-old granuloma, either unchanged or as metabolites. Liquid scintillation counting of the homogenates of the isolated granulomata showed that about 35% of the injected radioactivity had been incorporated into the granuloma. The chemical nature of the incorporated radioactivity and the fate of the remaining 35% of the injected radioactivity, was not further investigated. This perfusion technique cannot be applied to the cannulated sponge model (Bonta et al., 1979), because the sponge acts as a filter with an enormous flow resistance. Within the chamber the resistance is relatively low because it is empty, and good mixing of the perfusion fluid with the exudate is ensured by the difference in the inserted-length of the Tygon tubes, to which the nylon cannulae are connected.

Characteristics of the Inflammatory Process with the Chamber Model

The implantation of the teflon material, without the addition of carrageenin, led to an inflammatory response that was macroscopically different from the carrageenin-stimulated response. Rats implanted with teflon alone did not show any retardation in weight gain, and an easily removable granuloma developed. It is remarkable that even on day 7 after implantation, the chamber remained free within the subcutaneous space, being easily movable with the finger. At this stage the inflammatory exudate contained a large number of erythrocytes, together with leucocytes. In contrast, as early as 24 hr after the injection of carrageenin into the newly implanted chamber, a solid granuloma had developed and had grown into the dorsal muscle layer and skin.

Several parameters of the inflammatory process induced by carrageenin are shown in Figure 4B. The development of a granuloma around the chamber was not detectable until day 1 after the administration of carrageenin, whereas a maximal dry granuloma weight of 310 mg was reached on day 5. On days 1–5 the total amount of exudate that could be removed from the granulomata was fairly constant at about 1.5 ml, whereas on day 7 the volume was 0.8 ml. The total number of cells present in the exudate showed a submaximum in the acute inflammatory phase 6 hr after the administration of carrageenin, whereas an almost continuous increase was observed over days 3–7. Differential cell counting revealed that, during the entire period of investigation, the cell population consisted mainly of granulocytes. These granulocytes were almost all neutrophils and practically no eosinophils could be detected. The number of granulocytes was constant from 6 hr to day 3, at about 10×10^6, and then constantly increased to a mean number of 40×10^6 granulocytes/exudate on day 7. The second important population in the exudate was the lymphocytes. Apart from 6 hr, this cell type showed an increase from 1.1×10^6 on day 1 to 3.5×10^6 cells/exudate on day 7. Monocytes and macrophages were present in relatively very small amounts during the whole period of investigation. PGL in the exudate was present in amounts within the picogram (PGE_2 equivalents) range. Figure 5 shows the increase in PGL from the moment of the induction of inflammation to a value of 33 pg equiv. PGE_2 on day 7.

Comparison with the Sponge Model

The courses of the granuloma weights, exudate volumes, and total cells present in the exudate as a result of the implantation of carrageenin-soaked sponges, are depicted in Figure 4A. As in the chamber model, the sponge implantation resulted in the development of granuloma and a maximal dry granuloma weight of 440 mg was reached at the same stage of inflammation, namely on day 5. As can be judged from this granuloma weight, the inflammation is more severe around the sponge implants. The exudate volume (pooled from 2 sponges, implanted in 1 rat) increased from 1.8 ml on day 1 to 2.7 ml on day 7. The total number of cells present in the exudate was maximal on day 1 at 250×10^6 and decreased thereafter to a constant level of 100×10^6 cells/pooled exudate on days 5–7. During the entire period of

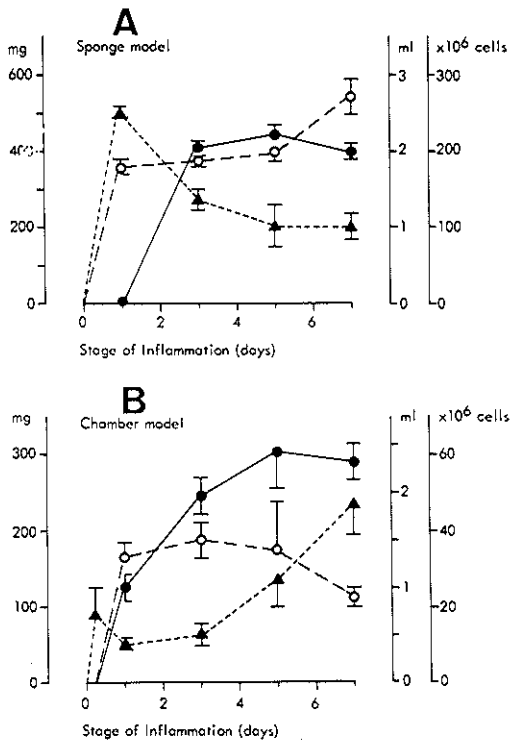

FIGURE 4. Time courses for changes in the exudate volume (open circles, broken line), the dry granuloma weight (closed circles, unbroken line) and the total number of cells present in the exudate (triangles, broken line) following the injection of carrageenin at zero time. A: the model of cannulated sponge implants of Bonta et al. (1979), with two sponges per rat. The dry weights of the separate granulomata were recorded and the exudate from both sponges was pooled for the determination of exudate volume and total cells per exudate. B: the model of cannulated teflon chambers implantation. Each point represents the mean ± s.e.m. of five rats.

investigation, as in the chamber model, the granulocytes formed the major population of the cells in the exudate (>70%). However, lymphocytes were detectable in numbers less than 1% of those of total cells in the sponge exudates. Unlike the chamber model, the PGL in the sponge exudate showed a maximum of 62 ng equiv. PGE_2 on day 1 and subsequent decrease to 8 ng equiv. PGE_2 on day 7 (Fig. 5).

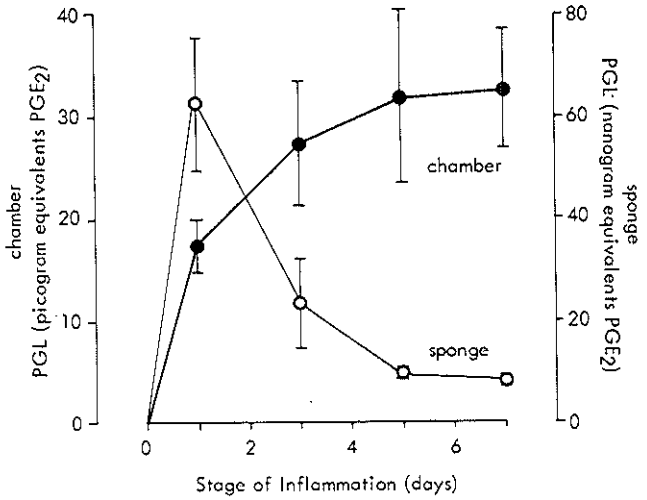

FIGURE 5. Changes in the levels of prostaglandin-like material (PGL) during carrageenin-induced granuloma development in the sponge model (open circles) of Bonta et al. (1979) and in the newly developed chamber model (closed circles). Carrageenin was injected at zero time and PGL was measured by bioassay on the rat stomach and expressed as PGE_2 equivalents. Points represent means ± s.e.m. of five rats.

Prostaglandin Biosynthesis from [1-^{14}C] Arachidonic Acid In Vivo

A concise presentation of the results is shown in Table 1. The major products of the metabolism of arachidonate, injected into the chamber, appeared to be hydroxy fatty acids and PGE_2, whereas 6-keto$PGF_{1\alpha}$ and TXB_2 were hardly formed. We were unable to resolve the fraction of hydroxy fatty acids into 12-L-hydroxy-5,8,10- heptadecatrienoic acid (HHT) and the lipoxygenase product 12-L-hydroxy-5,8,10,14-ei-

TABLE 1 Arachidonate Bioconversion by Granulomatous Tissue In Vivo in Percentages of Total Radioactivity

Inflammation Day	Hydroxy Fatty Acids	PGE_2	$PGF_{2\alpha}$	TXB_2	6-Keto $PGF_{1\alpha}$
1	1.2 ± 0.2	0.5 ± 0.1	0.2 ± 0.04	0.3 ± 0.1	0.1 ± 0.02
4	1.7 ± 0.4	1.1 ± 0.5	0.3 ± 0.1	0.1 ± 0.05	0.1 ± 0.03
8	2.1 ± 0.4	1.0 ± 0.1*	0.4 ± 0.04**	0.2 ± 0.05	0.2 ± 0.03*
12	3.4 ± 1.1*	1.8 ± 0.6**	0.6 ± 0.02**	0.2 ± 0.05	0.3 ± 0.1 *

Mean values of 4 experiments.
*$p < 0.05$, **$p < 0.01$ vs day 1 (Student's t-test).

cosatetraenoic acid (HETE). During the development of granulomatous tissue from days 1-12 of inflammation, the capacity for biosynthesis of all metabolites, except for TXB_2, increased about threefold. A detailed report dealing with the in vivo metabolism of arachidonate during carrageenin-induced inflammation in the rat, using the teflon chamber method, has been published elsewhere (Bragt and Bonta, 1979).

DISCUSSION

The newly developed cannulated teflon chamber for the study of experimental inflammation combines the property of the sponge model of Bonta et al. (1979), namely accurate local injection of materials into the inflamed site, with the advantage of allowing for the withdrawal of inflammatory exudate from the inflamed site, for at least 12 days after the injection of the inflammagen. This means that biochemical changes at the inflamed site can be studied over a desired period of time in one and the same animal. Moreover, this is clearly of economic benefit since smaller numbers of animals need to be used in studies on inflammation. When carrageenin was used for the induction of granuloma growth, the development of the granuloma proceeded in the same fashion in both the sponge and the chamber model. The maximum dry weight occurred at the same time (day 5), but in the sponge model the amount of tissue formed and the exudate volume were higher. Since the total number of cells present in the exudate of the implanted sponges is also high in comparison with the chamber method, this suggests that the inflammatory reaction in response to the injection of carrageenin into the sponges is more severe than when teflon chambers are used. It may well be that this is due to an inflammatory response induced by the sponge itself, whereas teflon evokes only a very moderate tissue reaction in comparison with other materials (see Calnan, 1963). In both models, the granulocytes were the dominant cell type in the exudate. The fact that in the sponge model practically no lymphocytes could be detected, whereas in the chamber model this population was the second most important cell type, may be due to sponge constituents with an inhibitory effect on lymphocyte migration. The effects of a polyether sponge constituent on granuloma formation and on the biosynthesis of prostaglandins has been described recently (Parnham et al., 1977). However, the possibility that one or the other constituent of the synthetic sponge may have an inhibitory effect on lymphocyte infiltration remains conjecture, because there are no data to further support this proposal.

In the sponge model, the appearance of PGL at different stages of inflammation shows a maximum on day 1 with a close resemblance to the plot of the total amount of cells in the exudate. On the other hand, the curve of PGL in the chamber model parallels that of the variation in granuloma weight over time. In relation to the small number of cells present in the exudate, it would seem likely that the major site for the biosynthesis of prostaglandins is localized in the cells constituting the granuloma.

Although teflon has been claimed to produce only a very moderate tissue reaction on implantation (see the Introduction), we observed that the implantation of the

teflon chamber alone evoked appreciable exudation and granuloma growth. However, the injection of carrageenin into the chamber produced a specific inflammatory response that was macroscopically different from and obliterated the effects of mere implantation of teflon. In view of the different tissue responses to carrageenin in the chamber and sponge model, respectively, it may be concluded that the characteristics of the inflammatory response induced by carrageenin are liable to alteration, depending on the material into which the inflammagen has been injected.

The fact that after 1 hr, approximately 65% of the injected radioactivity can be recovered, whether from the exudate or from the granuloma, suggests that the newly developed model may be suitable for general studies on both the local metabolism of drugs and on precursors of local hormones at the inflamed site. This has been demonstrated with the specific example of the injection of the prostaglandin precursor arachidonic acid into the chamber at different stages of granulomatous inflammation. Thus, the study of the in vivo metabolism of arachidonate at the site of inflammation shows that hydroxy fatty acids and PGE_2 are the main products formed (Bragt and Bonta, 1979). These results are in marked contrast with the results of Chang et al. (1978), who reported TXB_2 and 6-ketoPGF$_{1\alpha}$ as the major metabolites formed by granulomata in vitro. The use of the chamber model for studies in vivo, may avoid the introduction of interfering factors during in vitro handling and thus speculative extrapolations from in vitro to in vivo conditions are unnecessary.

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REFERENCES

Bonta IL, Adolfs MJP, Parnham MJ (1979) Cannulated sponge implants for the study of time dependent pharmacological influences on inflammatory granulomata. *J Pharmacol Meths* 2: 1–10.

Bragt PC, Bonta IL (1979) In vivo metabolism of [1-^{14}C] arachidonic acid during different phases of granuloma development in the rat. *Biochem Pharmac* 28: 1581–1586.

Bult H, Parnham MJ, Bonta IL (1977) Bioassay by cascade superfusion using a highly sensitive laminar flow technique. *J Pharm Pharmac* 29: 369–370.

Calnan J (1963) The use of inert plastic material in reconstructive surgery. I. A biological test for tissue acceptance. II. Tissue reactions to commonly used materials. *Br J Plast Surg* 16: 1–22.

Chang WC, Murota SI, Tsurufuji S (1978) Inhibition of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ formation by anti-inflammatory drugs in carrageenin-induced granuloma. *Biochem Pharmac* 27: 109–111.

Harrison JH, Swanson DS, Lincoln AF (1957) A comparison of the tissue reactions to plastic materials. *Arch Surg* 74: 139–144.

Lundgren D, Lindhe J (1973) Exudation, inflammatory cell migration and granulation tissue formation in performed cavities. A methodological study. *Scand J Plast Reconstr Surg* 7: 1–9.

Parnham MJ, Bonta IL, Adolfs MJP, Bragt PC (1977) A polyether sponge constituent which is antioxidant, anti-inflammatory and increases prostaglandin concentrations at the inflamed site. *Agents and Actions* 7: 539–544.

Vincent JE, Zijlstra FJ (1978) Nicotinic acid inhibits thromboxane synthesis in platelets. *Prostaglandins* 15: 629–635.

Biochemical Pharmacology, Vol. 28, pp. 1581–1586.

2. *IN VIVO* METABOLISM OF [1-^{14}C]ARACHIDONIC ACID DURING DIFFERENT PHASES OF GRANULOMA DEVELOPMENT IN THE RAT

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Abstract—The metabolism of [1-^{14}C]arachidonic acid was studied *in vivo* during the development of carrageenin-induced granuloma in the rat. For this purpose a double-cannulated teflon cylinder was implanted into the dorsal subdermal tissue of rats, and the cannulae were exteriorised through the skin at the scruff of the neck. The "chamber" allowed the injection of substances and collection of exudate at different stages of granuloma development. When [1-^{14}C]arachidonate was injected into the chamber one hour before recovery of the exudate at periods up to 12 days after initiating the inflammation, hydroxy fatty acids and prostaglandin E_2 appeared to be the main products formed. Only very small amounts of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ were detectable. These *in vivo* results are in marked contradiction to observations of other workers with granuloma tissue *in vitro*. The present findings are discussed in relation to the postulated negative-feedback function of E-type prostaglandins and a possible role of hydroxy fatty acids in inflammation.

The main precursor of endogenous prostaglandins (PG's) is arachidonic acid (AA), which occurs abundantly in cell membrane lipids of virtually all mammalian tissues [1]. PG's are released upon a variety of stimuli [2] and increased amounts of PG's, particularly those of the E-type, are detectable at inflamed sites [3]. PG's exert pro-inflammatory actions, such as vasodilation [4], increased vessel permeability [5] and potentiation of similar actions of other inflammatory mediators [6]. It is now widely accepted that PGE's contribute significantly to the development of acute inflammatory conditions [7]. However, with respect to chronic inflammation the function of PG's is not clear [8]. Dietary depletion of AA in rats, resulting in a shortage of PG's at the inflamed site, leads to an enhancement of the granulomatous component of chronic inflammation [9] and this enhancement is associated with an increased synthesis of collagen [10]. While this indirectly suggested that PG's exert a suppressive action on granulomatous inflammation, direct evidence was obtained more recently. Thus it was shown that local administration of PGE_1 inhibits the formation of inflammatory granulomata in rats, provided that the administration is carried out a stage of granuloma development when tissue growth is already in progress [11]. A similar inhibitory action on granuloma formation was also demonstrated with PGE_2 [12].

Prostacyclin (PGI_2), a recently discovered product of arachidonate bioconversion, has been found to be even more potent than PGE_2 in producing vasodilation [13, 14]. However, PGI_2 fails to inhibit granuloma formation, when injected locally in the chronic phase of inflammation at a dose which is equivalent to the inhibitory dose of PGE_2 [12].

Studies involving *in vitro* incubations of AA with a fraction of the homogenate of rat granuloma tissue, identified thromboxane B_2 (TXB_2) and 6-keto$PGF_{1\alpha}$ (a metabolite of PGI_2) as the major products formed [15, 16]. Furthermore, at different stages during the development of granuloma tissue, the capacity of the particular tissue fraction used to convert AA into TXB_2 increased, whereas its capacity to form 6-keto$PGF_{1\alpha}$ decreased [17].

The present study was undertaken to investigate the metabolism of AA *in vivo* at different stages of granuloma development in the rat, using a modification of the model involving implantation of sponges with indwelling cannulae [18]. This modification allowed withdrawal of exudate from the intact rat at any desired moment during the development of inflammatory granuloma.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (specific radioactivity 56.2 Ci/mole) was purchased from the Radiochemical Centre, Amersham, England; Carrageenin (Viscarin) from Marine Colloids Inc. Springfield, U.S.A.; arachidonic acid, prostaglandin A_2, E_2 and $F_{2\alpha}$ from Sigma, St. Louis, U.S.A.; 12L-hydroxy-5,8,-10,14-eicosatetraenoic acid (HETE) and 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) were gifts from Unilever, Research, Vlaardingen, The Netherlands. 6-Keto$PGE_{1\alpha}$ was a gift from Wellcome Research, Beckenham, England and PGD_2 and TXB_2 from Upjohn, Kalamazoo, U.S.A. Phosphomolybdic acid and 60 F_{254}-silicagel thin layer chromatography (t.l.c.) plates were purchased from E. Merck, Darmstadt, Germany and dioxane scintillation fluid from Packard, Brussels, Belgium. Teflon tube: 8 × 10 mm, was purchased from Eriks, Alkmaar. The Netherlands, silicone adhesive (Silastic) from Dow Corning Co., Midland, U.S.A. and nylon tube: 0.62 × 1.02 mm, from Rubber, Hilversum, The Netherlands. All other chemicals used

were of the highest degree of purity commercially available.

Animal treatment. 4 Male Wistar rats (TNO, Zeist, The Netherlands), weighing 245–260 g, were each implanted with a Teflon cylinder (length 30 mm, inner diameter 8 mm) provided with 108 holes of 1.5 mm diameter and two longitudinally indwelling nylon cannulae of 0.65 mm in diameter. The ends of the cylinder were sealed with silicone kit and the whole then implanted subcutaneously in the lumbar region, the cannulae being exteriorised at the back of the neck.

Inflammation was induced by injection through a cannula of 1 ml of a 1% (w/v) gel of carrageenin in sterile pyrogen-free saline into the implanted cylinder. The cannulae were then closed by means of tube-sealer. The injected carrageenin had access to the surrounding tissue via the holes in the implanted chamber, thus inducing granulomatous inflammation. The granuloma developed initially on the outside of the cylinder, subsequently growing in through the holes. Macroscopically, the whole granuloma had a similar appearance to that induced by carrageenin-soaked sponges [18]. A detailed description of the methodology will be published elsewhere.

Rats had free access to water and standard laboratory food (Hope Farms, Woerden, The Netherlands).

Injection and collection of radioactive material. Arachidonic acid (10 μCi) was dissolved in 800 μl ethanol after having evaporated the stock solvent, benzene, under a stream of nitrogen. Of this solution 200 μl were injected through the cannula into the teflon cylinder in each of the 4 rats on days 1, 4, 8 and 12 of the inflammatory process. After 1 hour of *in vivo* incubation with the arachidonate, exudate was collected by infusing 1 ml saline into one cannula, while fluid was drawn out of the other one into a 5-ml syringe. During handling, rats were under slight ether anaesthesia. Exudate was transferred into a calibrated glass tube and its volume recorded.

Extraction and thin layer chromatography. Extraction of lipid material was carried out twice, by mixing the exudate with 4 ml ethylacetate after acidification to a pH of 3 with 2 M HCl. Phase separation was facilitated by centrifugation at 1000 *g* for 5 min. The separate extracts of each exudate were pooled, the volumes recorded and subsequently the solvent was evaporated *in vacuo* at room temperature. Samples of 10 and 100 μl exudate and extract, respectively, were taken up into 10 ml scintillation fluid and recovery was estimated by counting radioactivity in a Packard Tricarb Model 3375 liquid scintillation counter with external standardization for quench correction. T.l.c. plates were activated at 100° for 10 min and after cooling 5 μg samples of arachidonic acid, PGA_2, D_2, E_2, $F_{2\alpha}$ and 6-keto$PGF_{1\alpha}$ were spotted at one point as internal standards. The extract residue was dissolved in 50 μl chloroform and spotted on the t.l.c. plate at the same point as the reference compounds.

T.l.c. plates were initially developed in a solvent system consisting of chloroform:methanol:acetic acid:water (90:8:1:0.8) [19] and subsequently in the organic phase of a mixture of ethylacetate:*iso*-octane:acetic acid:water (11:5:2:10) [20]. This two-dimensional chromatography procedure produced excellent separation of the metabolites under study.

Radioactive spots were visualized by scanning the t.l.c. plates in a Berthold LB 2723 thin layer scanner equipped with a dot scan unit for β-radiation. After scanning, plates were sprayed with a freshly prepared 7.5% (w/v) solution of phosphomolybdic acid in absolute ethanol followed by heating for 10 min at 100°. Blue coloured spots, and finally the uncoloured remainder, were scratched off the plates into scintillation vials, mixed with 10 ml scintillation fluid and radioactivity was estimated by liquid scintillation counting.

Calculation of the radioactivity of metabolites. Conversion of c.p.m. into d.p.m. was performed by correction for quenching via an external standard. Amounts of metabolites of arachidonic acid were expressed as percentages of total plate radioactivity. Activity representing PGE_2 was estimated by calculating the sum of radioactivities of the original spots of PGE_2 and PGA_2, because handling solutions containing PGE_2 may lead to dehydration and hence PGA_2 formation [21]. In addition, enzymatic conversion of PGE_1 into PGA_1 by a placental dehydrase has been reported [22]. In our experiments radioactivity representing PGA_2 never exceeded that for PGE_2.

RESULTS

The implantation of a porous Teflon chamber with two indwelling cannulae appeared to be a convenient model for studies of the metabolism of [1-^{14}C]arachidonic acid *in vivo* in carrageenin-induced inflammation. It enables the biochemical changes occurring within the inflamed locus to be investigated at any time during granuloma development in the same animal. Recovery of exudate is a simple atraumatic procedure, although on day 12 of inflammation accumulation of cell debris occurred within the chamber. Injection of 1 ml saline into one cannula to reduce viscosity and removal of the perfusate through the other one overcame the problem of obstruction. The injected [1-^{14}C]arachidonate was more than 99.9 per cent pure as revealed by prior thin layer chromatography and subsequent scanning of radioactive dots.

T.l.c. of the extract of the inflammatory exudate indicated that arachidonate was metabolised into hydroxy fatty acids, prostaglandins and thromboxane B_2 (Fig. 1). Maximally, 1.5 per cent of total radioactivity remained at the origin after development in both solvent systems. The amount of unconverted arachidonate, which represents the main spot in the chromatogram, showed a decrease during the development of inflammation. In contrast, the total amounts of products of cyclo-oxygenase activity, i.e. prostaglandins, thromboxane B_2 and hydroxy fatty acids, increased three-fold from day 1 to day 12 (Fig. 2). During this period radioactivity representing other lipids, running at the solvent front, increased from 8.2 per cent on day 1 to 21.8 per cent of total radioactivity on day 12 (Table 1). Elution of this fraction, followed by rechromatography, indicated that the fraction consisted mainly of triglycerides (unpublished results), presumably originating from the membranes of accumulated cells.

Our results further indicate that the major products formed by arachidonate cyclo-oxygenase activity were hydroxy fatty acids and PGE_2 (Fig. 1). The major part of the hydroxy fatty acids, produced during metabolism

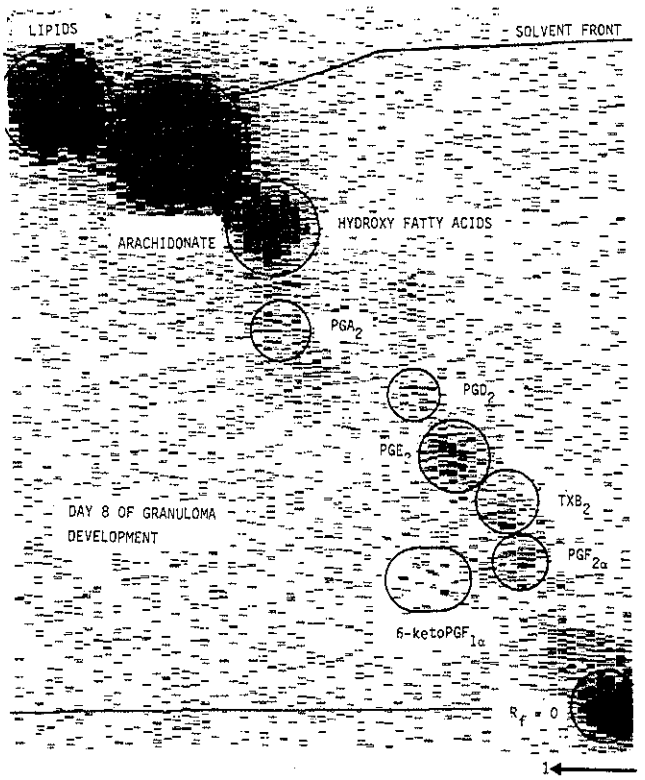

Fig. 1. Distribution of radioactivity on a t.l.c. plate after two-dimensional chromatography of the lipid material from an inflammatory exudate. Radioactive spots, accentuated by circles, were visualised by dot-scanning and the intensity of a dot is proportional to its radioactivity. $R_f = 0$ indicates the origin.

Table 1. Frequency of radioactive materials originating from locally injected arachidonate after 1 hour *in vivo* incubation

Radioactive spot	Per cent of total radioactivity*			
	Day 1	Day 4	Day 8	Day 12
PGE_2	0.23 ± 0.04	0.88 ± 0.42	0.76 ± 0.14‡	1.40 ± 0.59§
PGF_2	0.18 ± 0.04	0.27 ± 0.10	0.38 ± 0.04‡	0.58 ± 0.20§
PGD_2	0.11 ± 0.02	0.23 ± 0.07	0.24 ± 0.01**	0.35 ± 0.12§
PGA_2	0.23 ± 0.05	0.24 ± 0.03	0.29 ± 0.03	0.40 ± 0.06§
Unidentified lipids	8.21 ± 4.14	9.19 ± 2.67	19.73 ± 3.68§	21.80 ± 4.42§
Origin	0.57 ± 0.14	0.76 ± 0.15	1.11 ± 0.20§	1.35 ± 0.25§§
Ratio+ $\frac{TXB_2}{PGE_2 + PGA_2 + PGF_2}$	0.41 ± 0.13	0.11 ± 0.01§	0.17 ± 0.04	0.09 ± 0.03§

*Values represent means ± S. E. M. of 4 rats at different stages of granuloma development. The total formation of PGE_2 (not included in this table) was calculated from the sum of the individual values of PGE_2 and PGA_2 shown in the table.

+Ratios of TX/PGs were calculated for individual rats and the ratios shown represent means ± S. E. M. of 4 rats.

Metabolites of arachidonate included in the figures are not presented in this table. Significance was tested versus day 1 (one-tailed Student's *t* test. § = $P < 0.05$, ‡ = $P < 0.01$, §§ = $P < 0.025$ and ** = $P < 0.001$).

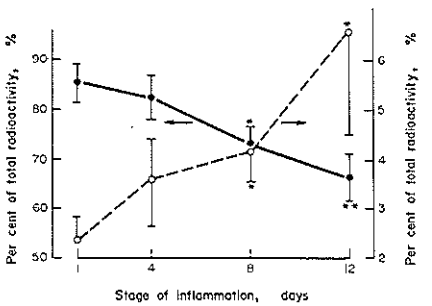

Fig. 2. Decrease in the amount of unconverted arachidonate (closed circles, unbroken lines) and increase in the total amount of prostaglandins, hydroxy fatty acids and thromboxane (open circles, broken line), during the development of granuloma in the rat. Arrows indicate the respective axes for the two curves. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus day 1 values (one-tailed Student's t test. * = $P < 0.05$, ** = $P < 0.01$).

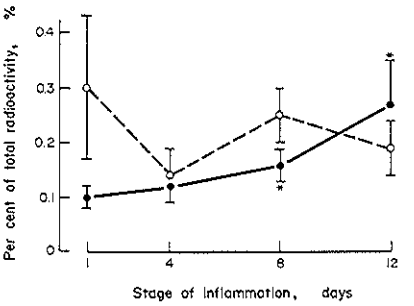

Fig. 4. Bioconversion of the injected arachidonate to 6-keto-prostaglandin $F_{1\alpha}$ (closed circles, unbroken line) and thromboxane B_2 (open circles, broken line) at different stages of granulomatous inflammation in the rat. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus values from day 1 of granuloma development (one-tailed Student's t test. * = $P < 0.05$).

of arachidonate *in vivo*, may consist of 12L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) as deduced from the R_F value of the unlabelled standard. However, this cannot be concluded with certainty because the closely related substance, 5L-hydroxy-6,8,12,14-eicosatetraenoic acid [23], may occupy more or less the same position in the chromatogram. Moreover, the relatively small resolution capacity of the dot-scanner did not allow specification of the exact chemical nature of the fraction of hydroxy fatty acids. As our interest was primarily the formation of PGE_2, 6-keto$PGF_{1\alpha}$ and TXB_2, we did not make an attempt to separate the fraction into HETE and HHT.

The capacity of the inflamed locus to convert arachidonate into both hydroxy fatty acids and PGE_2 showed an approximately threefold increase during granuloma development, reflecting an enhancement of cyclo-oxygenase activity in the later stages of inflammation (Fig. 3). At all time periods investigated, hydroxy fatty acids were formed in greater amounts than PGE_2. However, $PGF_{2\alpha}$ and PGD_2 were minor metabolites, which also increased approximately threefold between days 1 and 12 of the inflammatory process (Table 1). Another interesting observation was the very small capacity of the tissue for the biosynthesis of TXB_2 and 6-keto$PGF_{1\alpha}$ from arachidonate *in vivo*. From day 4 to day 12 both metabolites were synthesized in amounts even smaller than those of PGD_2 and $PGF_{2\alpha}$. The capacity for synthesis of 6-keto$PGF_{1\alpha}$ increased during granuloma development by two- to threefold (Fig. 4). TXB_2 formation did not increase in later stages when compared with day 1, but actually showed a tendency to decrease during the development of inflammation. From day 1 to day 8 TXB_2 formation exceeded that of 6-keto$PGF_{1\alpha}$, whereas on day 12 6-keto$PGF_{1\alpha}$ synthesis dominated.

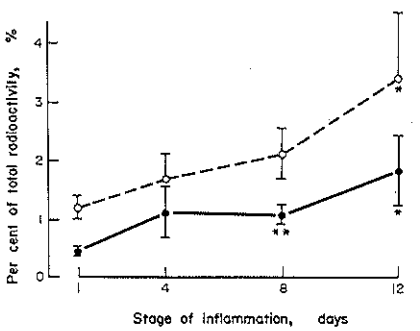

Fig. 3. Increase in hydroxy fatty acids (open circles, broken line) and prostaglandin E_2 (closed circles, unbroken lines) as a result of the *in vivo* metabolism of arachidonate by a developing granuloma in the rat. Each point represents the mean ± S.E.M. of 4 experiments. Significance was tested versus values from day 1 of inflammation (one-tailed Student's t test, * = $P < 0.05$, ** = $P < 0.005$).

The ratio $TXB_2/(PGE_2 + PGF_{2\alpha})$ showed a progressive decrease from day 1 to day 12 (Table 1). As the ethanol vehicle might possibly have influenced conversion of arachidonic acid or the ratios between the different metabolites, we carried out a separate experiment, using two rats, in which we investigated the metabolism of arachidonate, injected in a saline vehicle, on day 1 of inflammation. However, when suspending the arachidonate in saline it was found that a large percentage of the fatty acid adhered to the test tube and did not enter suspension. Nevertheless, when the small amount of arachidonate, which was suspended in saline, was injected into the cylinder, TXB_2 and 6-keto$PGF_{1\alpha}$ were again minor metabolites as compared to hydroxy fatty acids and PGE_2 while hydroxy fatty acid levels were somewhat less than those obtained using arachidonate dissolved in ethanol (data not shown). When acetone was used as a vehicle the results were similar to those obtained with the ethanol vehicle (data not shown).

DISCUSSION

The presence of prostaglandin-like activity in the exudate of a carrageenin-induced granuloma in the rat was first demonstrated by Willis in 1969 [3], using bioassay. Ever since, the role of prostaglandins as mediators of the early inflammatory response has been widely investigated [24–26]. However, it was discovered recently that PGE_1 can display an anti-inflammatory effect during the later stages of chronic inflammation [9, 11]. Possible mechanisms underlying this phenomenon have been reviewed [8].

It has also been suggested that labile intermediates in prostaglandin biosynthesis may also play an important role in inflammation [27], and granuloma tissue has been shown to convert arachidonate into TXB_2 [15] and 6-keto$PGF_{1\alpha}$ [16] *in vitro* as major metabolites.

The sodium salt and methylester of prostacyclin (PGI_2) both increased local vascular permeability when injected into an 8 day old granuloma in the rat. 6-Keto$PGF_{1\alpha}$ and TXB_2 were inactive in this respect [28]. PGE_1 and PGE_2 also increased vascular permeability, when administered locally on day 7, but failed to exhibit any effect when injected on day 1 [29].

Depriving rats of the essential fatty acid precursors for prostaglandin and thromboxane biosynthesis leads to a reduction in exudate accumulation but an increase in granuloma formation, when examined on day 8 of granuloma development, suggesting an inhibitory effect of fatty acid derivatives on the granulomatous component of inflammation [9]. Indeed, local injection of 1 μg PGE_1/day on days 4–7 of inflammation resulted in a decreased dry granuloma weight, whereas injection on day 1 enhanced granuloma formation [11]. Administration of PGE_2 showed similar effects on granuloma formation, but PGI_2 failed to reduce the weight of an 8 day old granuloma, when injected locally at 1 μg twice a day on days 4–7 of granuloma development [12]. The results reported in the present paper indicate that PGE_2 is a major metabolite formed from locally injected arachidonate by granulomatous tissue *in vivo*. During development of the granuloma an increased cyclo-oxygenase activity, measured as the sum of prostaglandins, thromboxane and hydroxy fatty acids formed from a fixed amount of AA in 1 hr, seems to parallel tissue growth. Capacity for biosynthesis of PGE_2 is enhanced in the later stage of granuloma development as compared to the initial phase, emphasising the possibility that PGE plays an important role in the chronic phase of inflammation. However, it should be noted that other authors have observed a decrease in the concentration of PGE in exudate during later phases of carrageenin-induced granuloma [30].

Our results further show the very small capacity of the granuloma tissue to convert arachidonate into TXB_2 and 6-keto$PGF_{1\alpha}$ *in vivo*. This is in strong disagreement with the observations of Chang *et al.* [31] obtained from *in vitro* experiments, showing that PGE_2 and $PGF_{2\alpha}$ were hardly formed but rather two other products, later established as being TXB_2 [15] and 6-keto$PGF_{1\alpha}$ [16], were synthetised. However, these results were obtained by the use of the 600 *g* supernatant of the homogenized granuloma tissue as an enzyme source, while membrane fractions of the homogenate were not investigated.

Moreover, in our inflammatory model, consisting of a s.c. implanted Teflon cylinder containing carrageenin, the metabolism of injected AA is not restricted to the granulomatous tissue itself. The inflammatory exudate may contribute to the metabolism of AA by supplying enzymes originating from cell breakdown. Phagocytozing polymorphonuclear leukocytes [32, 33], macrophages [34, 35], fibroblasts [36, 37], and platelets [38] accumulating in the inflamed area [39], have been shown to convert AA into prostaglandins, thromboxanes and hydroxy fatty acids.

Our results reveal the small tendency for the bioconversion of injected AA into TXB_2. This is consistent with results from the clinical experiments of Trang and his colleagues [40], who measured TXB_2 and PG levels in the synovial fluid of patients with rheumatoid arthritis. PGE_2 amply exceeded TXB_2 levels and the ratio $TXB_2/(PGF_{2\alpha} + PGE_2)$ was an average of 0.1 ± 0.05 in 5 patients. This ratio is in good agreement with the ratio in the chronic phase of experimental inflammation observed in our study (Table 1). Only at day 1 was this ratio relatively high, and this may indicate that thromboxanes play a major role in the early inflammatory stage.

The observation that hydroxy fatty acids are the major products formed from AA *in vivo*, suggests a possible role for these compounds in inflammation. It has been shown that the hydroxy fatty acids HETE and HHT exert a selective chemotactic effect on human polymorphonuclear (PMN) leukocytes but not on monocytes, and these compounds enhance random cell migration [41, 42]. In addition, increased concentrations of HETE have been reported to occur in the epidermis during psoriasis, an inflammatory skin disorder [43].

The hydroxy fatty acid HHT is formed from arachidonate by a cyclo-oxygenase, whereas HETE is formed by lipoxygenase activity, which is insensitive to inhibition by nonsteroidal anti-inflammatory drugs [38]. Both HHT and HETE can be identified and quantified by chromatography in combination with mass spectrometry, using deuterated standards [38]. Despite the availability of authentic standard compounds, we did not attempt to further specify whether HHT or HETE were present in the inflammatory exudate, because the resolution capacity of the dot-scanner was insufficient. Furthermore we had no access to mass spectrometry.

Nevertheless, our observation that hydroxy fatty acids, irrespective of their exact chemical nature, are the main metabolites formed during arachidonate bioconversion *in vivo*, combined with the chemotactic properties of these molecules, tempts speculation that HHT and HETE play an important role in inflammation.

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REFERENCES

1. G. J. Nelson, in *Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism* p. 336. Wiley Interscience, New York (1972).

2. B. Samuelsson, E. Granström, H. Green, M. Hamberg and S. Hammarström, *A. Rev. Biochem.* **44**, 669 (1975).
3. A. L. Willis, *J. Pharm. Pharmac.* **21**, 126 (1969).
4. P. Crunkhorn and A. L. Willis, *Br. J. Pharmac.* **36**, 216 (1969).
5. S. Arora, P. K. Lahiri and R. K. Sanyal, *Int. Archs Allergy appl. Immun.* **39**, 186 (1970).
6. J. R. Vane, *J. Allerg. Clin. Immunol.* **58**, 691 (1976).
7. R. J. Flower, *Naunyn-Schmiedeberg's Arch. Pharmac.* **297**, S 77 (1977).
8. I. L. Bonta and M. J. Parnham, *Biochem. Pharmac.* **27**, 1611 (1978).
9. I. L. Bonta, M. J. Parnham and M. J. P. Adolfs, *Prostaglandins* **14**, 295 (1977).
10. M. J. Parnham, S. Shoshan, I. L. Bonta and S. Neiman-Wollner, *Prostaglandins* **14**, 709 (1977).
11. I. L. Bonta and M. J. Parnham, *Br. J. Pharmac.* **62**, 417P (1977).
12. M. J. Parnham, I. L. Bonta and M. J. P. Adolfs, *Agents and Actions* Suppl., in press.
13. M. J. Peck and T. J. Williams, *Br. J. Pharmac.* **62**, 464P (1978).
14. G. A. Higgs, S. Moncada and J. R. Vane, *J. Physiol., Lond.* **275**, 30P (1978).
15. W. C. Chang, S. Murota and S. Tsurufuji, *Prostaglandins* **13**, 17 (1976).
16. W. C. Chang, S. Murota, M. Matsuo and S. Tsurufuji, *Biochem. biophys. Res. Commun.* **72**, 1259 (1976).
17. W. C. Chang, S. Murota and S. Tsurufuji, *Biochem. Pharmac.* **27**, 109 (1978).
18. I. L. Bonta, M. J. P. Adolfs and M. J. Parnham, *J. Pharmac. Meths* **2**, 1 (1979).
19. D. H. Nugteren and E. Hazelhof, *Biochim. biophys. Acta* **326**, 448 (1973).
20. F. Cottee, R. J. Flower, S. Moncada, J. A. Salmon and J. R. Vane, *Prostaglandins* **14**, 413 (1977).
21. W. P. Schneider, J. E. Pike and F. P. Kupiecki, *Biochim. biophys. Acta* **125**, 611 (1966).
22. N. A. Alam, P. Clary and P. T. Russel, *Prostaglandins* **4**, 363 (1973).
23. P. Borgeat, M. Hamberg and B. Samuelsson, *J. biol. Chem.* **251**, 7816 (1976).
24. J. W. Hinman, *A. Rev. Biochem.* **41**, 161 (1972).
25. R. B. Zurier, *Archs int. Med.* **133**, 101 (1974).
26. J. R. Vane, in *Advances in Prostaglandin and Thromboxane Research* (Eds B. Samuelsson and R. Paoletti), Vol. 2, p. 791. Raven Press, New York (1976).
27. F. A. Kuehl Jr., J. L. Humes, R. W. Egan, E. A. Ham, G. C. Beveridge and C. G. van Arman, *Nature, Lond.* **265**, 170 (1977).
28. S. Murota, I. Morita, S. Tsurufuji, H. Sato and K. Sugio, *Prostaglandins* **15**, 297 (1978).
29. W. C. Chang and S. Tsurufuji, *Eur. J. Pharmac.* **36**, 7 (1976).
30. W. C. Chang, S. Murota and S. Tsurufuji, *Jap. J. Pharmac.* **25**, 219 (1975).
31. W. C. Chang, S. Murota and S. Tsurufuji, *Biochem. Pharmac.* **25**, 2045 (1976).
32. G. A. Higgs, E. McCall and L. J. F. Poulten, *Br. J. Pharmac.* **53**, 539 (1975).
33. G. A. Higgs, S. Bunting, S. Moncada and J. R. Vane, *Prostaglandins* **12**, 749 (1976).
34. I. L. Humes, R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl Jr. and P. Davies, *Nature, Lond.* **269**, 149 (1977).
35. M. Glatt, H. Kälm, K. Wagner and K. Brune, *Agents and Actions* **7**, 321 (1977).
36. D. R. Robinson, M. B. McGuire and L. Levine, *Ann. N.Y. Acad. Sci.* **256**, 318 (1975).
37. D. S. Newcombe and Y. Ishikawa, *Prostaglandins* **12**, 849 (1976).
38. M. Hamberg, J. Svensson and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3824 (1974).
39. J. E. Vincent, I. L. Bonta and F. J. Zijlstra, *Agents and Actions* **8**, 291 (1978).
40. L. E. Trang, E. Granström and O. Lövgren, *Scand. J. Rheumat.* **6**, 151 (1977).
41. S. R. Turner, J. A. Tainer and W. S. Lynn, *Nature, Lond.* **257**, 680 (1975).
42. E. J. Goetzl and R. R. Gorman, *J. Immun.* **120**, 526 (1978).
43. S. Hammarström, M. Hamberg, B. Samuelsson, E. A. Duell, M. Stawiski and J. J. Voorhees, *Proc. natn. Acad. Sci. U.S.A.* **72**, 5130 (1975).

3. Indomethacin inhibits the in vivo formation of the lipoxygenase product HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid) during granulomatous inflammation in the rat

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During both acute and chronic phases of carrageenan-induced granulomatous inflammation in the rat, arachidonic acid is mainly converted in vivo into poly-unsaturated hydroxy fatty acids and PGE_2 (Bragt & Bonta 1979). The hydroxy fatty acids HETE and HHT (12-hydroxy-5,8,10-heptadecatrienoic acid) are chemotactic to polymorphonuclear (PMN) leucocytes (Turner et al 1975; Goetzl & Gorman 1978). The formation of HHT can be blocked by non-steroidal anti-inflammatory drugs (NSAIDs) and thromboxane synthetase inhibitors (Diczfalusy et al 1977), whereas the lipoxygenase-dependent formation of HETE is not blocked by the NSAIDs, but rather stimulated, at least in human platelets (Hamberg et al 1974). In addition, aspirin, phenylbutazone and indomethacin, when given orally 1 h before sponge implantation in the rat, significantly inhibit the migration of PMN leucocytes into the 5 h sponge exudate (Ford-Hutchinson et al 1975). More recently, it was demonstrated that low doses of indomethacin and aspirin stimulate the migration of PMN leucocytes into the inflamed area 24 h after the implantation of sponges, but high doses inhibit. Moreover, the migration was also inhibited by the compound BW755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline), an inhibitor of both lipoxygenase and cyclo-oxygenase (Higgs et al 1979). Higgs (1979) subsequently suggested that high doses of aspirin and indomethacin might inhibit lipoxygenase activity.

We have investigated the effects of indomethacin on the in vivo metabolism of arachidonate, 24 h and 8 days after the implantation of cannulated Teflon chambers and the induction of granuloma growth around the chamber by the injection of carrageenan.

Cannulated Teflon chambers were implanted, subdermally, into the backs of male Wistar rats (190–220 g), divided into two groups of 5 animals, as previously described (Bragt et al 1979). Inflammation was induced immediately afterwards by the injection through each cannula of 1 ml 1% (w/v) carrageenan in 0·9% NaCl (saline). One group of rats received a daily dose of indomethacin (200 μg) directly into the chamber, whereas the other group received the vehicle. Four hours after these injections, on days 1 and 8 of granuloma development, sodium [1-^{14}C]arachidonate was injected into the chambers. The in vivo incubation procedure, the recovery of the inflammatory exudate by perfusion and the identification and quantification of the arachidonate metabolites formed, were carried out as previously described (Bragt et al 1979). One sample of the labelled arachidonate was aerobically incubated for 1 h at 37 °C and stirred at 500 rev min^{-1}, as a control for the autoxidative formation of prostaglandin-like products.

Effects of indomethacin on arachidonate metabolism. The results of the present set of experiments are summarized in Table 1. The local injection of indomethacin, immediately after the induction of inflammation, resulted, after 24 h, in a significant decrease in the formation of PGE_2, $PGF_{2\alpha}$, TXB_2 and HHT. While the synthesis of 6-keto$PGF_{1\alpha}$, the metabolite of prostacyclin, was unaltered by indomethacin, the synthesis of the lipoxygenase product HETE was markedly inhibited, to a level approaching the amount of HETE-like material formed during autoxidation.

When the local administration was continued daily up until day 8 of granuloma development, only the formation of PGE_2 was significantly inhibited by indomethacin. The formation of all other metabolites, including HETE, was not significantly reduced at this stage. Results not included in Table 1 show that, whereas on day 1 there was an increased conversion of the total injected arachidonate after indomethacin treatment (24·8 ± 2·2% in control and 16·0 ± 1·0% in drug treated rats, $P < 0.01$, one-tailed Student's *t*-test), arachidonate conversion was unchanged by the drug on day 8. Furthermore, the amount of arachidonate incorporated into the phospholipid ($R_F = 0$) fraction on day 1 was significantly enhanced by treatment of the rats with indomethacin (14·1 ± 1·4% in control animals against a mean ± s.e.m. of 18·2 ± 0·7% of total radioactivity in indomethacin-treated rats ($P < 0.025$). No difference in this parameter was observable on day 8 of inflammation. Similar changes were observed in the glyceride fraction (data not shown).

Except for the hydroxy fatty acids, HHT and HETE, the capacity for biosynthesis of the arachidonate metabolites was more pronounced on day 8 than on day 1 (Table 1). On day 8, the animals were killed and the granulomata were dissected out and weighed. There was almost no reduction in the mean wet granuloma weight following indomethacin treatment (2·3 ± 0·3 g against 2·4 ± 0·1 g in control rats, $P > 0.4$), although the formation of PGE_2 was still significantly inhibited at this stage of inflammation (Table 1). Thus, granuloma growth and the formation of PGE_2 can be influenced independently of each other, as recently reported by Bonta et al (1979).

Our data clearly show that the daily administration of indomethacin (200 μg, locally), inhibits the biosynthesis

* Correspondence.

Table 1. Effects of daily administration of indomethacin (200 μg, locally) on the in vivo metabolism of sodium [1-^{14}C]arachidonate on days 1 and 8 of carrageenan-induced granulomatous inflammation in the rat.

	Product formed as a percentage of total plate radioactivity				
	Day 1		Day 8		
Metabolite	Vehicle	Drug	Vehicle	Drug	Autoxidation
PGE_2	0·88 ± 0·04	0·50 ± 0·09**	5·30 ± 2·40	0·72 ± 0·10*	0·14
$PGF_{2\alpha}$	0·28 ± 0·04	0·20 ± 0·02*	0·73 ± 0·31	0·24 ± 0·02	0
6-keto$PGF_{1\alpha}$	0·34 ± 0·07	0·29 ± 0·09	0·83 ± 0·63	0·10 ± 0·03	0
TXB_2	0·34 ± 0·03	0·25 ± 0·03*	0·85 ± 0·31	0·42 ± 0·14	0
HHT	1·86 ± 0·25	1·16 ± 0·10*	0·68 ± 0·11	0·72 ± 0·12	0·35
HETE	1·27 ± 0·04	0·71 ± 0·06**	0·63 ± 0·17	0·50 ± 0·10	0·71

Values represent means ± s.e.m. of 5 rats. Significance of the differences between indomethacin and vehicle treated rats was tested by the one-tailed Student's *t*-test (* $P < 0·05$ and ** $P < 0·01$). The values ranked in the last column represent the formation of products during the autoxidation of arachidonate with identical R_F-values as the metabolites formed in vivo.

of most arachidonate metabolites during the acute phase of inflammation, whereas in the chronic phase the inhibition is less pronounced. This finding is consistent with the results of other investigators that indomethacin is a more effective inhibitor of granuloma growth during the acute, rather than the chronic phase (Fukuhara & Tsurufuji 1969). Furthermore, our experiments confirm the observation that the pool of cyclo-oxygenase involved in the ultimate synthesis of prostacyclin is relatively insensitive towards inhibition by low doses of NSAID in vivo (Basista et al 1978).

Although the inhibition of HETE formation on day 1 might be the result of a direct inhibition of lipoxygenase(s) by indomethacin, two other explanations are also possible, as indomethacin has not been reported to be a lipoxygenase inhibitor in vitro (Hamberg et al 1974). Indomethacin inhibits the migration of leucocytes (Ford-Hutchinson et al 1975) and this inhibition may lead to a reduced amount of arachidonate converting enzymes at the inflamed site and a reduced formation of metabolites. The insensitivity of the formation of arachidonate metabolites on day 8 towards inhibition by indomethacin, may be due to the relative dominance of other cell types in this phase of inflammation.

It is of particular interest that at least a part of the injected arachidonate is incorporatedin to phospholipids and indomethacin increases the amount of arachidonate incorporated into this fraction on day 1. Kaplan et al (1978) have shown that indomethacin, in very low concentrations, inhibits the phospholipase A_2 of rabbit PMN leucocytes. If the major part of the arachidonate administered in our inflammation model is indeed incorporated into membranes and subsequently released by a phospholipase, it is not inconceivable that the inhibition by indomethacin of the formation of HETE and other products during the acute stage of inflammation is due to the inhibition of phospholipase A_2, resulting in a decreased availability of arachidonic acid for cyclo-oxygenase and lipoxygenase.

We thank Drs M. J. Parnham and J. E. Vincent for helpful discussions and we are also indebted to Dr M. J. Parnham for corrections of the text.

September 20, 1979

Note added in proof:

It has recently been shown that aspirin-like drugs inhibit the formation of HETE by human platelet microsomes, owing to the inhibition a HPETE peroxidase (Siegel, M. I., McConnell, R. T., Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. U.S.A. 76: 3774–3778).

REFERENCES

Basista, M., Dobranowski, J., Gryglewski, R. J. (1978) Pharmacol. Res. Commun. 10: 759–763

Bonta, I. L., Adolfs, M. J. P., Parnham, M. J. (1979) Agents Actions Suppl. 6 in the press

Bragt, P. C., Adolfs, M. J. P., Bonta, I. L. (1980) J. Pharmacol. Methods in the press

Bragt, P. C., Bonta, I. L. (1979) Biochem. Pharmacol. 28: 1581–1586

Diczfalusy, U., Falardeau, P., Hammarstrøm, S. (1977) FEBS Lett. 84: 271–274

Ford-Hutchinson, A. W., Smith, M. J. H., Elliott, P. N. C., Bolam, J. P., Walker, J. R., Lobo, A. A., Badcock, J. K., Colledge, A. J., Billimoria, F. J. (1975) J. Pharm. Pharmacol. 27: 106–112

Fukuhara, M., Tsurufuji, S. (1969) Biochem. Pharmacol. 18: 475–484

Goetzl, E. J., Gorman, R. R. (1978) J. Immunol. 120: 526–531

Hamberg, M., Svensson, J., Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71: 3824–3838

Higgs, G. A. (1979) Agents Actions Suppl. 6 in the press

Higgs, G. A., Flower, R. J., Vane, J. R. (1979) Biochem. Pharmacol. 28: 1959–1961

Kaplan, L., Weiss, J., Elsbach, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75: 2955–2958

Turner, S. R., Tainer, J. A., Lynn, W. S. (1975) Nature 257: 680–681

Prostaglandins and Medicine 2: 51-61, 1979

4. DISSOCIATION BETWEEN PROSTAGLANDIN AND MALONDIALDEHYDE FORMATION IN EXUDATE AND INCREASED LEVELS OF MALONDIALDEHYDE IN PLASMA AND LIVER DURING GRANULOMATOUS INFLAMMATION IN THE RAT.

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ABSTRACT

During kaolin-induced granuloma pouch inflammation in the rat the concentration of malondialdehyde (MDA) in the exudate, measured by the 2-thiobarbiturate method, steadily increased and this increase was correlated with the increase in granuloma weight. There was a complete lack of correlation between the concentrations of MDA and prostaglandin (PG)-like material in the inflammatory exudate. Whereas the concentration of PGs reached a maximum 1 day after the induction of inflammation and returned to control levels on day 4, the MDA concentration continuously increased from the initiation of inflammation up to day 16. During granuloma development the concentration of MDA in the plasma reached a maximum on day 2 in parallel with the concentration in the liver, then both plasma and liver MDA decreased to levels observed in control rats. Between day 2 and 8 of the inflammatory process homogenates of livers from inflamed rats showed an increased formation of MDA on incubation, the amount formed being maximal on day 4.
The results of the present study are discussed in view of a possible involvement of lipid peroxidation in inflammation.

INTRODUCTION

Mammalian tissues contain large amounts of polyunsaturated fatty acids (PUFAs) in their cell membranes. PUFAs are highly susceptible to oxidation by oxygen, and the process in which fatty acids become rancid on exposure to atmospheric oxygen, is called 'autoxidation'. It has now been amply shown that lipid peroxidation can occur in living organisms, presumably having the same underlying free radical mechanism and kinetics as the autoxidation process.
Malondialdehyde (MDA) is a product of both cyclo-oxygenase-dependent (1) and independent (2) peroxidation of certain PUFAs. The enzymatic bioconversion of arachidonic acid (20:4, ω6) into prostaglandins at the inflamed site and its relevance for the acute as well as the chronic phase of inflammation has already been the subject of numerous investigations (3,4). However, there is evidence that lipid peroxidation and the

parallel formation of MDA is not limited to the site of inflammation. Thus, it has been shown that incubation of liver homogenates obtained from mice and rats with carrageenin-induced paw edema, results in enhanced lipid peroxide formation, when compared with livers from normal animals (5,6). However, with adjuvant arthritic rats, liver homogenates showed a decreased tendency to produce lipid peroxides on incubation (6). The liver is a source of the enzymes superoxide dismutase (S.O.D.), glutathione peroxidase, and of the acute phase protein ceruloplasmin. SOD scavenges the superoxide free radical ($O_2^{\cdot}$), which can, by itself, induce inflammation (7), and has been shown to inhibit microsomal lipid peroxidation in vitro (8). In addition, SOD inhibits the depolymerization of synovial fluid caused by superoxide (9) and is antiinflammatory (10). Glutathione peroxidase is very effective in reducing organic hydroperoxides (11) and the copper enzyme ceruloplasmin, which is present in increased concentrations in the serum of patients with rheumatoid arthritis (12), has antioxidant properties (13). Other antioxidants such as N,N-diphenyl-*p*-phenylenediamine, propyl gallate, and d-α-tocopherol, also have antiinflammatory activity in inflammation models (14-16).
In the present study lipid peroxidation was studied at different stages of kaolin-induced granulomatous inflammation in the rat, by determining thiobarbituric acid reactive substances (MDA) and prostaglandins in the exudate, furthermore MDA levels in plasma and in homogenates of the liver before and after incubation.

MATERIALS AND METHODS

Animals and induction of inflammation

Male Wistar rats (TNO Zeist, The Netherlands), weighing about 250 g were divided at random into groups of 5 animals and kept on standard laboratory food (Hope Farms, Woerden, The Netherlands) and water, to which they had free access. Under light ether anaesthesia, the backs of the rats were shaved and 6 ml air was injected s.c. With the needle remaining in situ, inflammatory granuloma was initiated by the injection into the preformed airsac of 4 ml of a 10% (w/v) kaolin suspension in sterile, pyrogen-free saline.

Tissue removal and processing

At different stages of granulomatous inflammation rats were lightly anaesthesized under ether, and blood was obtained from each rat by cardiac puncture, using a heparinized needle, and collected in a polystyrene tube. Immediately afterwards, the liver was removed, rinsed with ice-cold saline, and homogenized in liquid nitrogen, using a mortar and pestle. The frozen liver powser from each rat was stored under nitrogen at -25°C, until required.
The blood samples were centrifuged for 20 min at 1,500 x g and the clear plasma fraction was used for the determination of MDA levels.
Finally, the granuloma pouch was removed and the exudate collected by cutting the pouch on one side and rinsing, with 2 ml saline into siliconized glass tubes containing 100 µg indomethacin added in 10 µl ethanol to prevent prostaglandin biosynthesis and hence artefactual MDA formation during handling.

The exudate volumes and the dry (20h, 100°C) granuloma weights were recorded. "Exudate" was collected from control rats by injecting kaolin, followed by immediate sucking up of the fluid. Further treatment was carried out as for the inflamed rats.
Fractions of the frozen liver powder (0.5g weighed at 0°C) were suspended in 4.5 ml of an ice-cold 150 mM KCl solution (5), and aliquots of 1 ml were removed for measurement of intrinsic liver MDA levels. The remainder was incubated for 3h at 37°C in 25 ml vials in a Lab-Line Model 3569 metabolic shaker (oscillations: 120 min^{-1}, stroke length: 5 cm). Reactions were terminated by adding an equal volume of 15% (w/v) trichloroacetic acid and after centrifugation (800xg, 15 min) the supernatants were used for the determination of MDA.

Assay methods

Protein was determined according to Lowry et al. (17), using 10 µl diluted exudate with bovine serum albumin as a standard. Protein levels were calculated for the undiluted exudate.
After addition of 10 µl [5,6-^{3}H] PGE_1 (approx 25,000 dpm) ethanol, 1 ml diluted exudate was acidified with 1 M HCl to pH 3, and lipids were extracted by mixing with 2 volumes chloroform. Phase separation was facilitated by brief centrifugation. The organic phase was then collected and the solvent evaporated in vacuo at room temperature. The residue was dissolved in 100 µl saline and from this solution 10 µl was used for the determination of recovery by liquid scintillation counting in a Packard Tricarb Model 3375. The remaining 90 µl was used for bioassay of prostaglandins. Prostaglandin-like material (PGL) was bioassayed against authentic PGE_2 on a rat stomach strip superfused with aerated Krebs buffer, containing a mixture of antagonists (18), at a rate of 1.3 ml/min at 37°C. PGL in exudates was expressed as nanogram equivalents PGE_2 per ml exudate, after correction for recovery.
MDA was determined according to the thiobarbituric acid (TBA) method of Smith (19), using 1 ml aliquots of plasma, diluted exudates, and liver homogenates from inflamed and control rats. The TBA reagent was prepared according the method of Stuart (20). Hydrolysed samples of tetramethoxypropane served as a standard for the MDA determination, the molar extinction coefficient being $(1.33 \pm 0.02) \times 10^5$ l/mole/cm (mean ± sem of 14 experiments). The amounts of MDA present in plasma and in the inflammatory exudates were expressed as nmoles MDA/ml, whereas MDA levels in the liver homogenates, were expressed as nmoles/g wet liver. Net MDA production during incubation was calculated by subtraction of the MDA values, found in the unincubated samples, from those formed in the corresponding incubated samples.
As bilirubin or biliverdin might have interfered with our assay for MDA (21), a qualitative test was performed with representative samples from plasma and liver to investigate this possibility. Extraction of the chromophor from the acid aqueous phase into butanol, followed by re-extraction into an alkaline aqueous phase (21), revealed that no interference had occurred, as no shift in colour from pink to blue could be observed.

Materials

The following agents were purchased: narcose ether from Hoechst, Frankfurt, Germany; kaolin from Baker Chemicals, Deventer, The Netherlands; tetramethoxypropane from Merck-Schuchardt, Hohenbrunn, Germany, 2-thiobarbituric acid from Merck, Darmstadt, Germany; heparin (Thromboliquine) from Organon, Oss, The Netherlands; PGE_2 from Sigma, St. Louis, U.S.A.; [5,6-^{3}H] PGE_1 from The Radiochemical Centre, Amersham, England, and dioxane scintillation fluid from Packard, Brussels, Belgium. Indomethacin, and other antagonists used for bioassay of prostaglandins, were obtained from the pharmacist, Dijkzigt Academic Hospital, Rotterdam, The Netherlands.

RESULTS

The acute inflammatory response was characterized by marked exudation and accumulation of exudate protein within the granuloma pouch, the total amount of protein reaching its maximum on day 1 of inflammation (Fig.1).
The granuloma weight, a component of the more chronic phase of inflammation, steadily increased from the time of kaolin injection reaching a maximum on day 8 (Fig.1). The granuloma removed 6 hours after injection of kaolin, were soft and had a jelly-like appearance, whereas on day 16 the granulomata were relatively hard and had a more or less spherical shape.
The concentration of PGL in the exudate increased rapidly from the time of kaolin administration, reaching a maximum on day 1 of inflammation (Fig.2). In contrast, the levels of MDA in the exudate began to increase 6 hours after kaolin and did not reach a maximum within the period of investigation (Fig.2). Thus, alterations in MDA concentration in the exudate did not show a correlation with alterations in the concentration of PGL (Spearman rank test $p > 0.05$). However, the MDA concentration in the exudate showed a significant correlation (Spearman rank test, $p < 0.01$) with the granuloma weight in 2 separate experiments (Fig.3). When plasma fractions from inflamed and control rats were examined for their content of MDA, increased levels were observed in animals with a developing granuloma pouch. The MDA concentration in the plasma obtained from rats with 1, 2 and 4 days inflammation was significantly increased in comparison with control animals ($p < 0.01$, Student's t-test), achieving maximal levels on day 2 of inflammation (Fig.4, lower part). From 4 to 16 days the plasma MDA level decreased to the value observed at the moment of the induction of inflammation.

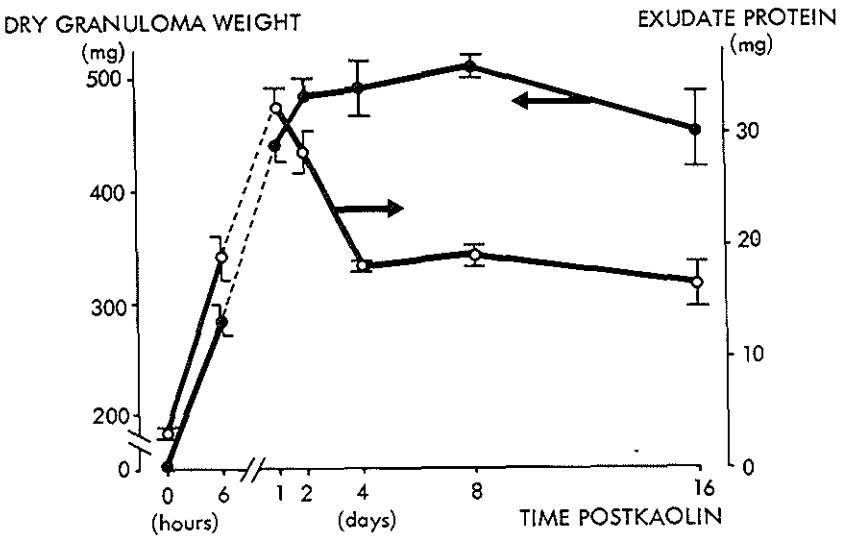

Figure 1. Development of the granuloma, expressed as the increase in dry granuloma weight (closed circles), and the accumulation of protein (open circles) in the inflammatory exudate during the development of the kaolin granuloma pouch. Inflammation was induced at zero time by the injection of 4 ml kaolin (10% w/v in saline) into a preformed subdermal airsac. Arrows indicate the respective axes. Each point represents the mean $\pm$ s.e.m. of 5 animals.

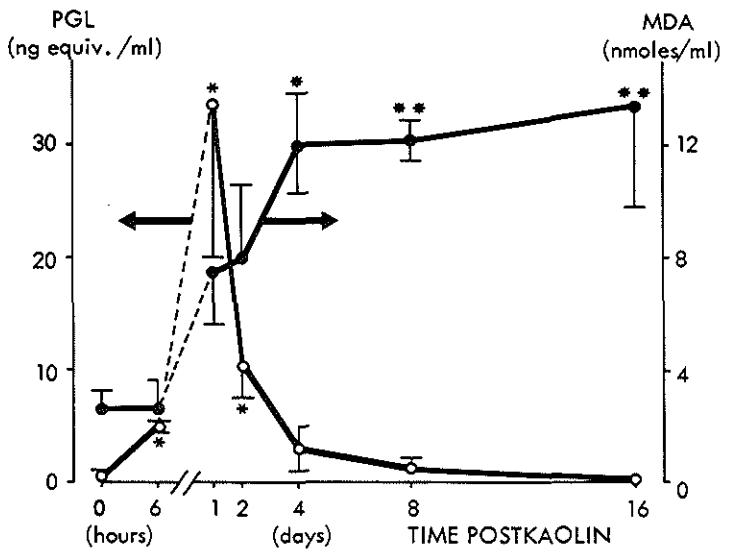

Figure 2. Time dependency of the concentrations of prostaglandin-like material (PGL, open circles) and malondialdehyde (MDA, closed circles) in the inflammatory exudate. PGL, measured by bio-assay on a rat fundus strip, is expressed as nanogram equivalents PGE_2 per ml. Arrows indicate the respective axes. Each point represents the mean $\pm$ s.e.m. of 5 animals. Significance was tested versus values at zero time. (one-tailed Student's t-test, * = $p<0.01$, ** = $p<0.001$).

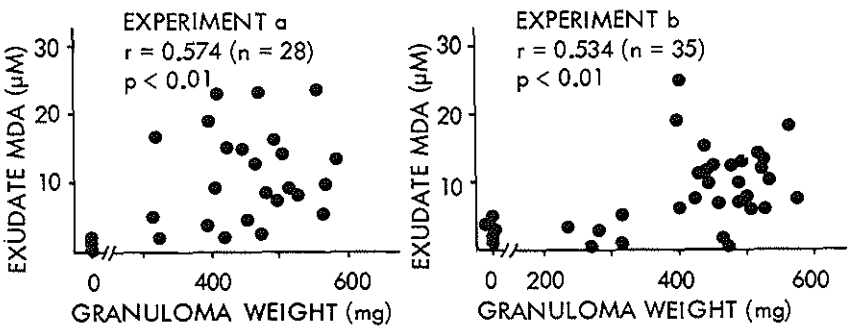

Figure 3. Correlation of the malondialdehyde (MDA) concentration in the pouch exudate with the dry granuloma weight in two separate experiments. Correlation coefficients and degrees of significance were calculated with the Spearman rank test.

Increased intrinsic MDA levels were observed in the liver homogenates from rats with 1 and 2 days old granuloma pouches (Fig.4, upper part). The maximal intrinsic MDA concentration in the liver coincided with the maximum concentration in the plasma, but, although the plasma concentration was still significantly elevated on day 4, the MDA concentration in the liver had already decreased to values detectable in uninflamed rats (Fig.4, upper part).

When aerobic incubation of liver homogenates from inflamed and control rats was carried out at 37° C for 3h, formation of MDA was significantly increased in the livers from inflamed rats, when compared with controls, 2, 4 and 8 days after the initiation of inflammation (Fig.4, upper part).

DISCUSSION

The results in this paper indicate that remote local inflammation in the rat leads to elevated lipid peroxide levels in the plasma and in the liver. Interestingly, the extent of lipid peroxidation at the inflamed site is correlated with the severity of the inflammatory process (i.e. granuloma weight).
Our data also clearly indicate that the observed formation of MDA in the pouch exudate cannot have solely been the result of the action of PG synthetase. In granulomatous inflammation in the rat, induced by carrageenin, locally formed metabolites of arachidonate consist mainly of PGE_2 (22). As the carrageenin-induced granulomatous inflammation is akin to the granuloma pouch model, used in the present study, it appears justified to compare the levels of PGE_2 and MDA in two models. Thus, on a molar base, local levels of MDA exceed PG levels by more than a hundred-fold, assuming that the bioconversion of endogenous arachidonate is identical in both models. In addition, the time courses of PGL and MDA formation

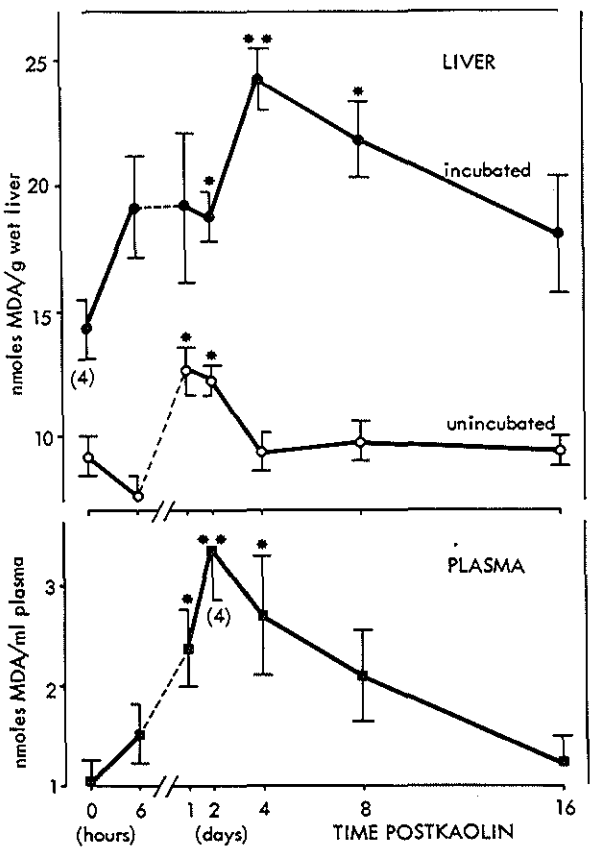

Figure 4. Changes in the malondialdehyde concentrations in plasma (squares) and in incubated (closed circles) and unincubated (open circles) homogenates of livers from rats at different stages of kaolin-induced granuloma pouch development. Homogenates were made 10% w/v in 150 mM KCl. Incubations were performed for 3h at 37°C in a metabolic shaker. Unincubated samples were withdrawn at zero time. Net MDA production on incubation was calculated by subtracting the MDA values in the corresponding unincubated samples. Unless otherwise indicated (in brackets) each point represents the mean ± s.e.m. of 5 animals. Significance was tested versus the moment of the induction of inflammation (one-tailed Student's t-test, * $p<0.01$, ** $p<0.001$).

are totally dissimilar (Fig.2). In fact, at any time when PG levels have already decreased to values observed in control rats, the concentration of MDA in the exudate is still increasing.
During the phagocytosis of particles by polymorphonuclear leucocytes and macrophages, the respiration of these cells is dramatically increased, and large quantities of the superoxide anion (O_2^-) are produced by the membrane-bound NAD(P)H oxidase system (23). The inner part of the phagocytosing cell is protected against this radical by the enzyme superoxide dismutase (SOD), which converts superoxide into oxygen and hydrogenperoxide. However, part of the superoxide is released into the extracellular space, thereby inducing peroxidation of tissue cell membranes with concomitant MDA formation, either by itself (24), or via conversion into reactive species like the hydroxyl radical (25) and singlet oxygen (26). As this generation of oxygen metabolites during phagocytosis is completely independent of PG biosynthesis, it may very well explain the absolute dissimilarity between the local formation of MDA and PGs.
Besides an increase in local MDA levels, we observe increased MDA levels in the plasma and liver from rats with a granuloma pouch. There may be two major explanations for this phenomenon.
One possible reason for the increase in the concentration of plasma and hepatic MDA is a decreased elimination of lipid peroxides by protecting enzymes, of which glutathione peroxidase is the most important one (11). The activity of this enzyme depends on the availability of reduced glutathione. As it has been reported that, during inflammation in rats, levels of hepatic glutathione and plasma thiols are reduced (27,28), it is not inconceivable that the peroxide metabolizing system is also affected. The increased hepatic synthesis of the acute phase reactant ceruloplasmin, a protein with strong antioxidant properties (13), can then be explained as a physiologically operating feedback mechanism to restore the peroxide metabolizing capacity.
Another valid explanation may be that, during inflammation, increased synthesis of lipid peroxides occurs in the liver. It has been shown that 'uncoupling' of the cytochrome P-450 system leads to the production of superoxide radicals on the one hand and a decreased metabolism of drugs on the other one (29). Interestingly, a reduction of the in-vivo capacity of drug metabolism has been reported to occur during inflammation in the rat (30), whereas lipid peroxidation interferes with hepatic drug metabolism in vitro (31).
This tempts to speculate that a relation exists between the increased hepatic synthesis of the antioxidant acute phase protein ceruloplasmin, the increased lipoperoxide concentration in the plasma and liver, and the decreased hepatic drug metabolism during the inflammatory process. However, experimental evidence for such a correlation has still to be delivered.
Finally, it is worth mentioning, that the phenomenon of increased plasma MDA levels is apparently not restricted to the presently investigated animal model of inflammation, but occurs also under clinical inflammatory conditions, i.e. in patients with rheumatoid arthritis (32).

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REFERENCES

1. Hamberg M., Samuelsson B. Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. J.Biol.Chem. 242: 5344, 1967.

2. Pryor WA, Stanley JP. A suggested mechanism for the production of malondialdehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J.Org.Chem. 40: 3615, 1975.

3. Vane JR. Prostaglandins as mediators of inflammation. p.791 in Advances in Prostaglandin and Thromboxane Research. Vol.2 (B. Samuelsson, R. Paoletti eds) Raven Press, New York, 1976.

4. Bonta IL, Parnham MJ. Prostaglandins and chronic inflammation. Biochem. Pharmac. 27: 1611, 1978.

5. Sharma SC, Mukhtar H, Sharma SK, Krishna Murti CR. Lipid peroxide formation in experimental inflammation. Biochem.Pharmac. 21: 1210, 1972.

6. Robak J. Adjuvant-induced and carrageenin-induced inflammation and lipic peroxidation in rat spleen, liver and lungs. Biochem.Pharmac. 27: 531, 1978.

7. Ohmori H, Komoriya K., Azuma A, Hashimoto Y, Kurozumi S. Xanthine oxidase-induced foot edema in rats: involvement of oxygen radicals. Biochem.Pharmac. 27: 1397. 1978.

8. Zimmermann R, Flohé L, Weser U, Hartmann HJ. Inhibition of lipid peroxidation in isolated inner membrane of rat liver mitochondria by superoxide dismutase. FEBS Letters 29: 117, 1973.

9. McCord JM. Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase. Science 185: 529, 1974.

10. Lund-Olesen K, Menander KB. Orgotein - New anti-inflammatory metalloprotein drug - Preliminary evaluation of clinical efficacy and safety in degenerative joint disease. Curr.Ther.Res. 16: 706, 1974.

11. Flohé L. Glutathion peroxidase: Enzymology and biological aspects (in German). Klin.Wochenschr. 49: 669, 1971.

12. Niedermeier W. Concentration and chemical state of copper in synovial fluid and blood serum of patients with rheumatoid arthritis. Ann. Rheum.Dis. 24: 544, 1965.

13. Al-Timimi DJ, Dormandy TL. The inhibition of lipid autoxidation by human caeruloplasmin. Biochem.J. 168: 288, 1977.

14. Levy L, Kerley TL. The use of DPPD (N,N'-diphenyl-p-phenylenediamine) as an anti-inflammatory agent. Life Sci. 14: 1917, 1974.

15. McDonald-Gibson WJ, Saeed SA, Schneider C. The local antinociceptive and topical anti-inflammatory effects of propyl gallate in rodents. Br.J.Pharmac. 58: 573, 1976.

16. Stuyvesant VW, Jolley WB. Anti-inflammatory activity of d-α-tocopherol (vitamin E) and linoleic acid. Nature 216: 585, 1967.

17.Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J.Biol.Chem. 193: 265, 1951.

18.Bult H, Bonta IL. Prostaglandin endoperoxides, serotonin and the superfused rabbit aorta: Possible pitfalls in the bio-assay of R.C.S. Agents and Actions 6: 712, 1976.

19.Smith JB, Ingerman CM, Silver MJ. Malondialdehyde formation as an indicator of prostaglandin production by human platelets. J.Lab.Clin. Med. 88: 167, 1976.

20.Stuart MJ, Murphy S, Oski FA. A simple nonradioisotope technique for the determination of platelet life-span. New Engl.J.Med. 292: 1310, 1975.

21.Gutteridge JMC, Tickner TR. The thiobarbituric acid-reactivity of bile pigments. Biochem.Med. 19: 127, 1978.

22.Bragt PC, Bonta IL. In vivo metabolism of [1-^{14}C] arachidonic acid during different phases of granuloma development in the rat. Submitted for publication.

23.Roos D. Oxidative killing of microorganisms by phagocytic cells. Trends Biochem.Sci. 2: 61, 1977.

24.Pederson TC, Aust SD. The role of superoxide and singlet oxygen in lipid peroxidation promoted by xanthin oxidase. Biochem.Biophys. Res.Commun. 53: 1071, 1973.

25.Haber F, Weiss J. The catalytic decomposition of hydrogen peroxide by iron salts. Proc.R.Soc.London Ser A 147: 332, 1934.

26.Kellogg EW, Fridovich I. Superoxide, hydrogenperoxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J.Biol. Chem. 250: 8812, 1975.

27.Marozzi A, Malone MH. Analgesic and anti-inflammatory evaluation of thymotic acid and certain homologs. J.Pharmac.Sci. 57: 589, 1968.

28.Baumgartner WA, Beck FWJ, Lorber A, Pearson CM, Whitehouse MW. Adjuvant disease in rats: Biochemical criteria for distinguishing several phases of inflammation in rats. Proc.Soc.exp.Biol.Med. 145: 625, 1974.

29.Kuthan H, Tsuji H, Graf H, Ullrich V, Werringloer J, Estabrook RW. Generation of superoxide anion as a source of hydrogen peroxide in a reconstituted monooxygenase system. FEBS Letters 91: 343, 1978.

30.Beck FJ, Whitehouse MW. Impaired drug metabolism in rats associated with acute inflammation: A possible assay for anti-injury agents. Proc.Soc.exp.Biol.Med. 145: 135, 1974.

31.Wills ED. Lipid peroxide formation in microsomes. Relationship of hydroxylation to lipid peroxide formation. Biochem.J. 113: 333, 1969.

32.Muus P., Bonta IL, Den Oudsten SA. Plasma levels of malondialdehyde, a product of cyclo-oxygenase dependent and independent lipidperoxydation in rheumatoid arthritis: A correlation with disease activity. Submitted for publication.

Br.J.Pharmac. (1980), 68, 123P-124P.

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5. Increased lipid peroxidation and decreased hepatic aminopyrine metabolism during carrageenin-induced granulomatous inflammation in the rat

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Hepatic drug metabolism is decreased during adjuvant arthritis in the rat (Beck & Whitehouse, 1973). We observed increased levels of the stable lipid peroxidation product malondialdehyde (MDA) in plasma and liver of rats with kaolin granuloma pouch inflammation (Bragt, Schenkelaars & Bonta, 1979). We have now investigated both hepatic aminopyrine demethylation and lipid peroxidation during carrageenin-induced granuloma in the rat. Wistar rats weighing 200–250 g were used. Lipid peroxidation was estimated by determining MDA in plasma and livers from rats with carrageenin-induced (10 mg) granulomata growing around subcutaneously implanted teflon chambers (Bragt, Bonta & Adolfs, 1979). Drug metabolism was measured *in vivo* by the expiration of $^{14}CO_2$ after the i.p. injection (120 mg/kg, 0.4 μCi/kg) of [^{14}C]-aminopyrine (Radiochemical Centre, Amersham), as described by Lauterburg & Bircher (1976), in rats which had either polyether sponge implants, carrageenin-soaked (10 mg/sponge) sponge implants (Bonta, Adolfs & Parnham, 1979), or which were sham-operated.

The injection of carrageenin resulted in a significant rise in the MDA concentrations in plasma and liver after 6 h (340% and 64%, respectively, $P < 0.01$ vs day 0, Mann-Whitney U test) a change which was still present after 7 days (700% and 54% respectively, $P < 0.01$). The increase in liver MDA was associated with a decrease in hepatic reduced glutathione (GSH) content (~40%) throughout the entire period of investigation (data not shown). The plasma MDA concentration showed a correlation with the severity of inflammation expressed as wet granuloma weight ($r_s = 0.53$, $n = 25$, $P < 0.01$ Spearman rank test). Five days after operation (i.e. peak of granuloma formation), the half-life ($T_{\frac{1}{2}}$) of aminopyrine was longer in rats with a stronger inflammatory response (Table 1); the $T_{\frac{1}{2}}$ correlated significantly with the amount of granuloma formed ($r_s = 0.76$, $n = 18$, $P < 0.01$). The extended $T_{\frac{1}{2}}$ during inflammation might be due to the reduced hepatic levels of GSH, since the binding of demethylated aminopyrine metabolites would also be reduced. Consequently, these metabolites would compete with the aminopyrine for the active site of cytochrome P-450. We, thus, injected GSH (400 mg/kg i.v.) into rats implanted with carrageenin-soaked sponges, 45 min prior to the administration of aminopyrine. The $T_{\frac{1}{2}}$ was not altered (Table 1). Therefore, GSH depletion *per se* may not be responsible for the decreased drug metabolism during inflammation.

As the two sets of experiments indicate that increased lipid peroxidation and a reduction in drug metabolism occur in parallel during carrageenin-induced granuloma, it is not inconceivable that the two phenomena are interrelated.

References

BECK, F.J. & WHITEHOUSE, M.W. (1973). Effect of adjuvant disease in rats on cyclophosphamide and isophosphamide metabolism. *Biochem. Pharmac.*, **22**, 2453-2468.

BONTA, I.L., ADOLFS, M.J.P. & PARNHAM, M.J. (1979). Cannulated sponge implants in rats for the study of time-dependent pharmacological influences on inflammatory granulomata. *J. Pharmac. Meths.*, **2**, 1-11.

BRAGT, P.C., BONTA, I.L. & ADOLFS, M.J.P. (1979). Cannulated teflon chamber implant in the rat: a new model for continuous studies on granulomatous inflammation. *J. Pharmac. Meths.*, (in press).

Table 1 Extension of the *in vivo* half-life ($T_{1/2}$) of [^{14}C]-aminopyrine in rats 5 days after subdermal sponge implantation

	Granuloma formed (*wet weight g*)	*Aminopyrine* $T_{1/2}$ (*min*)
Sham-operation	0	62 ± 4
Sponge implant without carrageenin	1.45 ± 0.03	71 ± 3*
Carrageenin-soaked sponge implant	5.06 ± 0.47	103 ± 26**
Carrageenin-soaked sponge implant + GSH	n.d.	106 ± 9**

Values represent the means ± s.e. mean of 6 rats. $^{14}CO_2$ was trapped in ethanolamine and collected every 15 minutes. $T_{1/2}$ values were calculated from trapped radioactivity during the elimination phase of aminopyrine, starting 30 min after drug injection and continuing for up to 150 minutes. *: $P < 0.05$, **: $P < 0.01$, one-tailed Mann Whitney U test vs sham-operated rats. n.d. = not determined.

BRAGT, P.C., SCHENKELAARS, E.J.P.M. & BONTA, I.L. (1979). Dissociation between prostaglandin and malondialdehyde formation in exudate and increased levels of malondialdehyde in plasma and liver during granulomatous inflammation in the rat. *Prostaglandins and Medicine*, **2**, 51-61.

LAUTERBURG, B.H. & BIRCHER, J. (1976). Expiration measurement of maximal aminopyrine demethylation *in vivo*: effect of phenobarbital, partial hepatectomy, portacaval shunt and bile duct ligation in the rat. *J. Pharmac. exp. Ther.*, **196**, 501-509.

Agents & Actions Supplement 7 (1980).

6. Depletion of Hepatic Antioxidants During Granulomatous Inflammation in the Rat and Local Anti-Inflammatory Effects of Free Radical Scavengers

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Abstract

Changes in hepatic oxidative events have been studied during extrahepatic granulomatous in the rat, together with the local anti-inflammatory effects of scavengers of reactive oxygen species. During remote localized inflammation in the rat, we observed increased hepatic lipid peroxidation and reduced hepatic levels of protecting substances such as ascorbic acid, catalase and reduced glutathione, the specific substrate for the peroxide-metabolizing enzyme, glutathione peroxidase. The levels of superoxide dismutase did not alter significantly during the period of investigation (7 days). The half-life of aminopyrine was longer in rats with a stronger inflammatory response. In addition, catalase and the anti-oxidants, α-tocopherol and propyl gallate, inhibited granuloma development on local injection during the acute phase of inflammation. Superoxide dismutase alone was devoid of anti-inflammatory effects, but markedly enhanced the effect of catalase. Scavengers of hydroxyl radicals and of singlet oxygen failed to display anti-inflammatory activity. The results indicate that peroxides may act as mediators of inflammation and that increased lipid peroxidation is not limited to the site of inflammation.

Introduction

Apart from local changes which occur at a site of inflammation, systemic changes are also seen. These include increased body temperature, increased pituitary-adrenal function and increased hepatic synthesis of the acute phase reactants (APR), reflected by elevated levels of these substances in the blood [1]. The function of the APR is generally thought to be a protective one. For example, the copper storage protein, ceruloplasmin, which is a superoxide radical ($\cdot O_2^-$) scavenger, has anti-inflammatory properties in a rat model of inflammation [19]. In this respect, it resembles the copper and zinc containing enzyme, superoxide dismutase (SOD), which is present in the [2] cytoplasma and catalyses the reaction: $\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ [3]. The protective function of the superoxide scavengers during inflammation is probably due to their capacity to eliminate a potentially damaging radical, which is released during the phagocytosis by polymorphonuclear leukocytes. It has been shown that, during the synthesis of the APR, the liver 'neglects' the synthesis of other substances, as a result of the adaptive syndrome [4], and it is possible that the synthesis of protective substances (e.g. scavengers of reactive intermediates) may also be reduced. We, therefore, investigated the changes in hepatic SOD, catalase, reduced glutathione (GSH) and ascorbic acid during granulomatous inflammation in the rat and the effects of administration of some of these protective agents into the inflamed site.

Materials and methods

Male Wistar rats (about 200 g) were implanted with polyether sponges or with teflon chambers, in the dorsal subdermal region [5, 6]. Both the sponges and the chambers were provided with indwelling cannulae, which were exteriorized at the back of the neck. Carrageenin (10 mg) was used to induce the formation of granulomata around the implanted materials. At different stages of granulomatous inflammation, the livers were removed from the chamber-implanted rats, homogenized and assayed for lipid peroxidation as previously described [7]. SOD and catalase activities and the hepatic concentrations of GSH and ascorbic acid were determined by established assay methods [8–11].

Scavengers of superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen ($^1O_2$) and anti-oxidants were administered into the cannulae of sponge-implanted rats, either in a single dose immediately after implantation or daily, on days 3–6 of granulomatous inflammation. The vehicles were either saline or arachis oil (200 μ), depending on the lipophilicity of the drugs used. Control rats received vehicle only. The effects of the administration of the drugs on the inflammation were

estimated from the exudate volumes and the dry weights of the granulomata on day 7 after the implantation of the sponges.

The in vivo demethylation of ^{14}C-labelled aminopyrine (120 mg/kg, 0.4 μCi/kg, i.p.) was investigated after the induction of inflammation with differing severity, namely in sham-operated rats and in rats either implanted with sponges alone, or with carrageenin-soaked sponges. The metabolism studies were performed on day 5 after the operation (the time of maximal granuloma weight) and the aminopyrine half-life was estimated from the expiration of $^{14}CO_2$, essentially as described by LAUTERBURG and BIRCHER [12].

Results

Some of these results have been communicated to the British Pharmacological Society [13]. As a result of a remote localized inflammation, the hepatic concentration of GSH was significantly reduced ($p < 0.025$, one-sided Mann–Whitney U test versus control) from 6 hours after the induction of inflammation up until day 7 (Fig. 1A). On day 3, the hepatic concentration of ascorbate was also significantly reduced ($p < 0.025$, Fig. 1D)

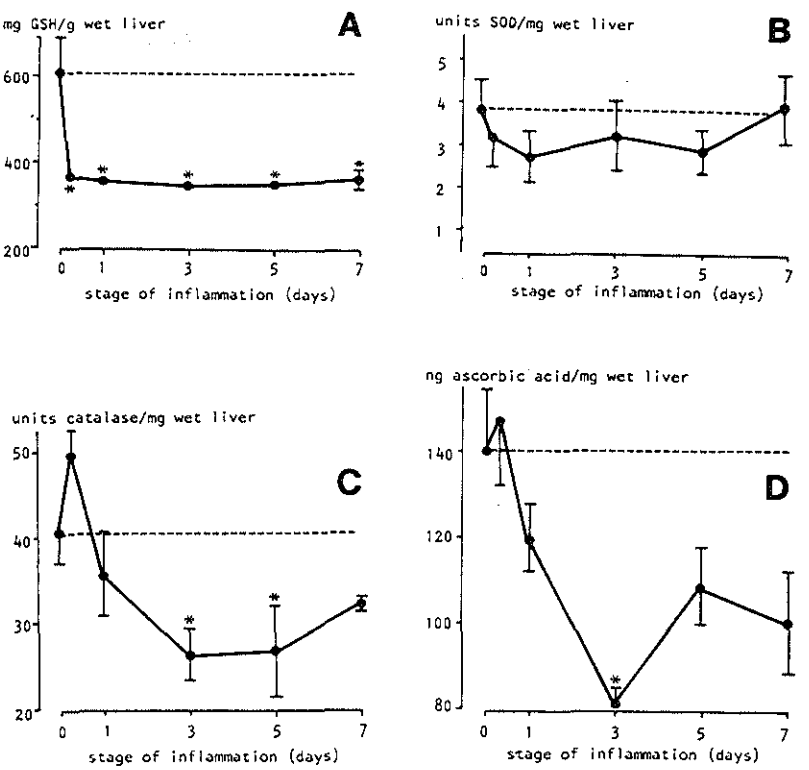

Figure 1

Time-dependent changes in the hepatic levels of A: reduced glutathione (GSH), B: superoxide dismutase (SOD), C: catalase and D: ascorbic acid, during a remote localized inflammation in the rat. Inflammation was induced at $t = 0$ and the corresponding control values are indicated with dotted lines. Each point represents the mean ± S.E.M. of at least 5 rats. With GSH, the S.E.M. values are smaller than the size of the dots. Asterisks indicate that the values differ significantly from the corresponding controls ($p < 0.025$, one-tailed Mann–Whitney U test).

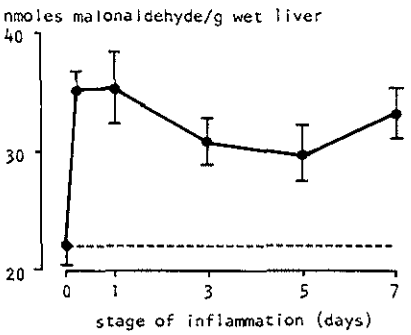

Figure 2
Time-dependent changes in hepatic lipid peroxidation following the induction of inflammation at zero time. Lipid peroxidation was estimated by the determination of malonaldehyde by the 2-thiobarbituric acid method. Values indicate means ± S.E.M. The dotted line represents the control values. Points, other than $t = 0$, differ significantly from control ($p < 0.01$, one-tailed Mann–Whitney U test).

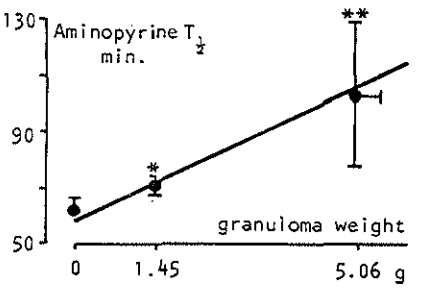

Figure 3
Aminopyrine half-life plotted against the granuloma weight in sham-operated rats, rats implanted with sponges alone and rats implanted with carrageenin-soaked sponges. Each point represents the mean aminopyrine half-life with the corresponding mean granuloma weight (± S.E.M.) in a group of 6 rats. * and ** indicate significant differences in the aminopyrine half-lives against sham-operated rats at the 5 and the 1% level, respectively (one-tailed Mann–Whitney U test).

and was still low on day 7 of inflammation. After a small initial rise, there was a decreased hepatic catalase activity, which was significantly lower than control on days 3–5 of inflammation ($p < 0.025$). From day 5 to day 7, the catalase activity tended to increase slightly towards control levels (Fig. 1C). Whereas there was no significant change ($p > 0.05$) in the activity of hepatic SOD (Fig. 1B), there was a dramatic increase in the concentration of the lipid peroxidation product malonaldehyde (Fig. 2). From 6 hours after the induction of granulomatous inflammation up until day 7, hepatic lipid peroxidation was significantly increased ($p < 0.01$) in inflamed rats in comparison with healthy animals. Simultaneously, there was also a drastic change in the hepatic capacity for metabolizing drugs. Thus, the aminopyrine half-life ($T_{\frac{1}{2}}$) was longer in rats with a more severe inflammation, the $T_{\frac{1}{2}}$ showing a significant positive correlation ($r_s = 0.76$, $n = 18$ rats with a $p < 0.01$, Spearman rank test) with the wet granuloma weight. Figure 3 shows the linearity of the mean half-lives plotted against the mean granuloma weights in three groups of rats.

SOD (363–12,088 U/sponge) neither inhibited granuloma growth nor exudation following local administration at the time of sponge implantation. In contrast, catalase showed a dose-dependent anti-inflammatory effect. A dose of 46,667 U per sponge inhibited both granuloma growth and exudation (Table 1). The addition of 363 U SOD, which was inactive in itself, markedly potentiated the inhibitory effect of catalase on the granuloma weight (Table 1). This effect was due to an enzymic activity, since denaturation prior to injection abolished the inhibition. The hydroxyl radical scavengers, mannitol, benzoate and thiourea, failed to inhibit the parameters of inflammation to any significant extent. In fact, thiourea enhanced the granuloma growth (Table 1). In addition, the singlet oxygen scavengers DABCO and β-carotene were inactive in inhibiting granuloma growth and exudation following administration immediately after the induction of inflammation. However, on administration into a preformed granuloma (i.e. daily on days 3–6), both compounds tended to enhance exudation and granuloma weights (Table 1). GSH (30 mg/sponge) was inactive under both dose regimes (data not shown). The anti-

Table 1
Effects of scavengers of reactive oxygen species on granulomatous inflammation in the rat.

Scavenger and dose[a]	Intermediate scavenged	Time of administration[b]	Inhibition granuloma (% change vs. control)[c]	Inhibition exudation (% change vs. control)[c]
SOD (363–12,088 U)	$\cdot O_2^-$	day 0	−12–6	16–−5
CAT (313–46,667 U)	H_2O_2	day 0	−14–35†	2–27 †
CAT (6260 U) + SOD (363 U)	·OH?	day 0	42†	17
Denatured CAT + SOD		day 0	−11	−25
Mannitol (40 mg)	·OH	day 0	−33	0
Sodium benzoate (26 mg)	·OH	day 0	14	9
Thiourea (20 mg)	·OH	day 0	−70†	−13
DABCO (3.5 mg)	$^1O_2$	day 0	3	36
		days 3–6	−12	−8
β-Carotene (125 μg)	$^1O_2$	day 0	12	10
		days 3–6	−41†	−1
α-Tocopherol (19 mg)	$^1O_2$/LOO·	day 0	20	27†
		days 3–6	−27†	12
Propyl gallate (1 mg)	LOO·	day 0	32†	−18
		days 3–6	21†	34

[a] The doses were administered into each sponge (2 sponges per rat) in rats of about 200 g.
[b] The drugs were given either in a single dose on day 0, or daily on days 3–6.
[c] The inhibition of granuloma weight and exudation is expressed as percent change versus controls. Significance was tested by the one-tailed Mann–Whitney U test versus controls (†$p < 0.05$). Negative signs indicate stimulation of granuloma or exudation.
SOD: superoxide dismutase, CAT: catalase, DABCO: 1,4-diazabicyclo{2,2,2}octane, $\cdot O_2^-$: superoxide anion, ·OH: hydroxyl radical, $^1O_2$: singlet oxygen, LOO·: lipid-peroxy radical.

oxidant and singlet oxygen scavenger, α-tocopherol, inhibited granuloma growth and exudation following administration on day 0, whereas it significantly enhanced the granuloma weight following injection on days 3–6 (Table 1). The anti-oxidant propyl gallate inhibited granuloma weight independently of the time of administration (Table 1).

Discussion

The involvement of the liver in a remote localized inflammation is not only obvious from the production of the acute phase proteins, but also from an increased lipid peroxidation and a reduced hepatic availability of reducing substances (GSH, ascorbate) and protecting enzymes (catalase), as shown in the present study. Since in vitro studies revealed that lipid peroxidation and microsomal drug metabolism are competitors for reducing equivalents, it is not surprising that, during inflammation, there is also a reduced hepatic drug metabolism. The reduced hepatic drug metabolism was clearly demonstrated by the elongation of the aminopyrine half-life in rats with different degrees of granulomatous inflammation. These results are in close agreement with the observations of BECK and WHITEHOUSE [14], who showed an increased sleeping-time following the administration of hexobarbital and trichloroethanol and a decreased hepatic metabolism of aminopyrine and cyclophosphamide in vitro, in rats with acute inflammation. As far as present knowledge is concerned, it is not clear what actually causes the hepatic changes. It has, for example, been postulated that some kind of humoral factor from the inflamed site induces an adaptive syndrome in the liver, after its transport via the blood [15].

As it seemed conceivable that the liver, via catalase, SOD and ceruloplasmin, may protect the organism against oxidative damage by reactive intermediates ($\cdot O_2^-$, $^1O_2$, H_2O_2, lipid peroxides) generated at the inflamed site, we locally injected some scavengers of these

intermediates into the inflamed site to investigate their possible anti-inflammatory effects. Using this route of administration, 'false-positive' anti-inflammatory drugs are excluded, by avoiding counter-irritation effects. From the fact that catalase exerted a dose-dependent inhibition of the granuloma formation it may be concluded that hydrogen peroxide plays a role as an inflammatory mediator. It is not likely that superoxide also plays such a role, since SOD was not inhibitory even at extremely high dosage levels. The addition of specific amounts of SOD to catalase prior to injection, resulted in a potentiated inhibition of the granuloma weight. As combinations of SOD and catalase may prevent the formation of hydroxyl radicals in vitro [16], we tested some established HO· scavengers. None of the compounds tested could significantly reduce the amount of granuloma formed, thus, it is unlikely that the hydroxyl radical is an important pro-inflammatory species in the acute phase of granulomatous inflammation. The same can be said of singlet oxygen in view of the results with the $^1O_2$ scavengers. However, $^1O_2$ may be involved in the breakdown of collagen or in the inhibition of macrophage proliferation in the chronic phase of inflammation, since the injection of specific scavengers at this stage, tended to enhance the granuloma weight. This observation is in agreement with the results of other investigators [17], who showed that singlet oxygen causes the cleavage of non-helical regions of the collagen molecule in vitro, by an oxidative attack on susceptible amino acids. When applied to a pre-existing granuloma, α-tocopherol behaves like the other $^1O_2$ scavengers tested. However, on administration immediately after the induction of inflammation, it inhibits both granuloma formation and exudation. In addition, the anti-oxidant propyl gallate inhibits the granuloma growth irrespective of the time of administration. In view of this and of the fact that α-tocopherol is a very poor inhibitor of the enzyme fatty acid cyclo-oxygenase, which produces prostaglandins and thromboxanes from specific polyunsaturated fatty acids [18], it is likely that lipid peroxides (LOOH) or lipid-peroxy radicals (LOO·) are potential mediators of inflammation.

Therefore, efforts should be made to develop and to test drugs, which inhibit the formation of lipid peroxides, via the lipoxygenase pathway or via the free radical mediated pathway.

Acknowledgments

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References

[1] M.W. Whitehouse, *Some Biochemical Complexities of Inflammatory Disease Affecting Drug Action*, Agents and Actions *Suppl. 2*, 135–147 (1977).

[2] W. Huber, T.L. Schulte, S. Carson, R.E. Goldhamer and E.E. Vogin, *Some Chemical and Pharmacologic Properties of a Novel Anti-Inflammatory Protein*, Tox. Appl. Pharmacol. *12*, 308 (1968).

[3] I. Fridovich, *Superoxide Dismutases*, Adv. Enzymol. *41*, 35–97 (1974).

[4] G.G. Shutler, J.M. Langstaff, J.C. Jamieson and D.N. Burton, *The Effects of Experimental Inflammation on Adaptive Synthesis of Rat Liver Fatty Acid Synthetase*, Arch. Biochem. Biophys. *183*, 710–717 (1977).

[5] I.L. Bonta, M.J.P. Adolfs and M.J. Parnham, *Cannulated Sponge Implants for the Time Dependent Pharmacological Influences on Inflammatory Granulomata*, J. Pharmacol. Meth. *2*, 1–10 (1979).

[6] P.C. Bragt, I.L. Bonta and M.J.P. Adolfs, *Cannulated Teflon Chamber Implant in the Rat: a New Model for Continuous Studies on Granulomatous Inflammation*, J. Pharmacol. Meth. 3, 51-61 (1980)

[7] P.C. Bragt, E.P.M. Schenkelaars and I.L. Bonta, *Dissociation Between Prostaglandin and Malondialdehyde Formation in Exudate and Increased Levels of Malondialdehyde in Plasma and Liver During Granulomatous Inflammation in the Rat*, Prostaglandins Med. *2*, 51–61 (1979).

[8] C. Beauchamp and I. Fridovich, *Superoxide Dismutase: Improved Assays and an Assay Applicable to Acrylamide Gels*, Anal. Biochem. *44*, 276–287 (1971).

[9] H. AEBI, *Catalase*, in: H.U. BERGMEYER, *Methods of Enzymatic Analysis*, vol. 2 (Verlag Chemie Weinheim/Academic Press Inc., New York and London, 1974).

[10] V.H. COHN and J. LYLE, *A Fluorometric Assay for Glutathione*, Anal. Biochem. *14*, 434–440 (1966).

[11] E.C. ATTWOOD, E.D. ROBEY, J. ROSS, F. BRADLEY and J.J. KRAMER, *Determination of Platelet and Leukocyte Vitamin C and the Levels Found in Normal Subjects*, Clin. Chim. Acta *54*, 95–105 (1974).

[12] B.H. LAUTERBURG and J. BIRCHER, *Expiratory Measurement of Maximal Aminopyrine Demethylation in vivo: Effects of Phenobarbital, Partial Hepatectomy, Portacaval Shunt and Bile Duct Ligation in the Rat*, J. Pharmacol. Exp. Ther. *196*, 501–509 (1976).

[13] M.J.P. ADOLFS, I.L. BONTA and P.C. BRAGT, *Increased Lipid Peroxidation and Decreased Hepatic Aminopyrine Demethylation During Carageenin-Induced Granulomatous Inflammation in the Rat*, Br. J. Pharmacol. 68, 123P–124P (1980).

[14] F.J. BECK and M.W. WHITEHOUSE, *Impaired Drug Metabolism in Rats Associated with Acute Inflammation: a Possible Assay for Anti-Injury Agents*, Proc. Soc. Exp. Biol. Med. *145*, 135–140 (1974).

[15] I.L. BONTA, *Endogenous Modulators of the Inflammatory Response*, in: J.R. VANE and S.H. FERREIRA, *Inflammation, Handbook of Experimental Pharmacology*, vol. 50/I (Springer Verlag, Berlin and Heidelberg, 1978).

[16] K.L. FONG, P.B. MCCAY and J.L. POYER, *Evidence that Peroxidation of Lysosomal Membranes is Initiated by Hydroxyl Free Radicals Produced During Flavin Enzyme Activity*, J. Biol. Chem. *248*, 7792–7797 (1973).

[17] K. VENKATASUBRAMANIAN and K.T. JOSEPH, *Action of Singlet Oxygen on Collagen*, Ind. J. Biochem. Biophys. *14*, 217–220 (1977).

[18] K.H. GIBSON, *Prostaglandins, Thromboxanes, PGX: Biosynthetic Products from Arachidonic Acid*, Chem. Soc. Rev. *6*, 489–510 (1977).

[19] C.W. DENKO, *Protective Role of Ceruloplasmin in Inflammation*, Agents and Actions *9*, 333–336 (1979).

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7. ANTIINFLAMMATORY EFFECTS OF FREE RADICAL SCAVENGERS AND ANTIOXIDANTS
Further Support for Proinflammatory Roles of Endogenous Hydrogen Peroxide and Lipid Peroxides[1]

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Abstract—Scavengers of reactive oxygen species were tested by local administration during granulomatous inflammation in the rat, induced by the subdermal implantation of carrageenin-soaked sponges. Drugs were administered either in a single dose immediately after sponge implantation, or in daily doses on days 3–6 of inflammation. The effects of the injected drugs were assessed using the day 7 granuloma. When given at the moment of sponge implantation, catalase showed antiinflammatory effects, whereas superoxide dismutase did not. However, the addition of superoxide dismutase to catalase, prior to injection, markedly potentiated the inhibition of granuloma formation by catalase alone. Negative results obtained with scavengers of hydroxyl radicals and singlet molecular oxygen suggest that protection of superoxide dismutase by catalase from inactivation by hydrogen peroxide, is a likely explanation for the observed potentiation. When administered at the moment of the induction of inflammation, α-tocopherol and propyl gallate, both antioxidants, also inhibited granuloma formation. All drugs tested were either ineffective or even enhanced granuloma weight following administration into a preformed granuloma. An inhibitor of both pathways of arachidonate metabolism, phenidone, inhibited granuloma formation irrespective of the moment of administration. The results presented in this paper suggest proinflammatory roles for hydrogen peroxide and lipid peroxides and a possible involvement of hydroxyl radicals and singlet oxygen in the breakdown of collagen.

INTRODUCTION

During inflammation, a massive invasion of granulocytes and/or macrophages occurs at the inflammatory site. Both cell types are capable of

[1]Some of the data presented in this paper were communicated at the International Meeting on Inflammation, Verona, Italy, September 1979.

producing the cytotoxic superoxide anion ($\cdot O_2^-$) during the phagocytosis of bacteria or particles (1, 2). That the intracellularly generated superoxide can escape into the extracellular space, thereby stimulating the pro-inflammatory response, is demonstrated by the observation that superoxide dismutase (SOD), a copper- and zinc-containing enzyme which converts superoxide into hydrogen peroxide and oxygen, has antiinflammatory properties (3, 4). In vitro experiments have shown that superoxide can react with other molecules to produce the extremely reactive hydroxyl radical ($\cdot OH$), which is considered to be the main initiator of lipid peroxidation with deleterious consequences for the organism (5). Recently, evidence has been provided that hydroxyl radicals are produced as a result of the zymosan-stimulated oxygen burst in monocytes and granulocytes (6, 7). The exact mechanism of the generation of hydroxyl radicals in biological systems is still speculative, but likely sources are the iron(II)-catalyzed decomposition of hydrogen peroxide (8) and lipid hydroperoxides (9). This is confirmed by the observation that the iron chelator, diethylenetriamine pentaacetic acid, inhibits the formation of hydroxyl radicals in a system containing iron, hydrogen peroxide and superoxide anions as essential components (10).

A possible role of hydroxyl radicals in the amplification of an inflammatory process might be anticipated from their ability to cause depolymerization of hyaluronic acid and bovine synovial fluid (11), labilization of lysosomal membranes (5), lipid peroxidation with subsequent generation of chemotactic principles from lipids (12), and enhancement of the release of anaphylactic mediators from challenged sensitized guinea pig lungs (13).

In the present study, we investigated the possible antiinflammatory effects of catalase, superoxide dismutase, and combinations of both, scavengers of hydroxyl radicals and singlet oxygen and of the iron chelator bathophenanthroline sulfonate. Furthermore, we tested the antioxidants propyl gallate and α-tocopherol and an inhibitor of both the lipoxygenase and cyclooxygenase-dependent oxidative metabolism of arachidonic acid, phenidone (14). The effects of the administered drugs on the intensity of inflammation were determined from their effects on granuloma weight, exudation and, for some drugs, on the amounts of prostaglandin-like material (PGL) in the exudate.

MATERIALS AND METHODS

Animals and Treatment. Inflammation was induced in male Wistar rats (TNO, Zeist, The Netherlands) by the implantation of two carrageenin-soaked and cannulated polyether sponges ($20 \times 10 \times 5$ mm^3), according to the method of Bonta et al. (15). The rats weighed 175–200 g and were maintained on standard laboratory food (Hope Farms; Woerden, The Nether-

lands) and tap water, to which they had free access. For the study of drug effects, the rats were randomly divided into groups of 5, and the drugs to be tested or the corresponding vehicles were injected through the cannulae into both sponges, either as a single dose directly after implantation, or daily on days 3–6 of inflammation. On day 7 of inflammation, the rats were reweighed, sacrificed by carbon dioxide inhalation, and bled by carotid incision. The effects of the test drugs on the intensity of inflammation were assessed from the volume of accumulated exudate, the dry weights of the granulomata formed, and corrected for the differences in body weights. Furthermore, for some drugs, the amounts of prostaglandin-like material in the exudates were estimated by bioassay on a rat stomach fundus strip in the presence of a mixture of antagonists in the superfusion fluid (16).

Preparation of Sponges. In order to prevent interference from an antioxidant material (17) present in the polyether during testing of the drugs, the sponges were boiled in absolute ethanol for 30 min, dried, impregnated with 0.5 ml of a 2% (w/v) carrageenin solution, and finally dried again before implantation.

Drugs. Superoxide dismutase (Palosein®, 2900 units/mg) was a gift from Diagnostic Data Inc., Mountain View, California and another batch was purchased (bovine superoxide dismutase, 2700–2900 units/mg) from Sigma, St. Louis, Missouri. Also from Sigma were: thymol-free bovine liver catalase (10,000 units/mg), the iron chelator, bathophenanthroline sulfonate, and the lipoxygenase and cyclooxygenase inhibitor, 1-phenyl-3-pyrazolidone (phenidone). The hydroxyl radical scavenger, D-mannitol, and the antioxidants, α-tocopherol and propyl gallate, were purchased from ICN, Cleveland, Ohio. The hydroxyl radical scavengers, sodium benzoate and thiourea, were obtained from E. Merck, Darmstadt, West Germany. The singlet oxygen quenchers, DABCO [1,4-diazabicyclo(2,2,2)octane] and trans-β-carotene, were from Fluka, Buchs, Switzerland, and Aldrich, Milwaukee, Wisconsin, respectively. Reduced glutathione (GSH) was purchased from Boehringer, Mannheim, West Germany.

The lipophilic substances were dissolved in arachis oil and the water soluble drugs in saline in such concentrations that the volume of the solutions administered to the animals was always 200μl.

Statistics. Statistical analysis was performed using the one-tailed Mann-Whitney U-test. Differences between drug- and vehicle-treated groups of animals were considered to be significant at the 5% level. Linear regression and correlation analysis were performed with the method of the least squares and the Spearman rank test, respectively.

RESULTS

Table 1 shows that the administration of catalase during the acute phase of inflammation results in a dose-dependent inhibition of both granuloma formation and exudation. The inhibition of exudation, however, was only statistically significant at the highest dose tested, i.e., 46,667 units/sponge (4.7 mg protein). The lowest dose of catalase significantly inhibited the accumulation of prostaglandins in the exudate, without displaying and effect on granuloma formation and exudation. An opposite phenomenon was observed with the combined administration of 6260 units/sponge catalase and 2417 units/sponge SOD namely, a significant increase in prostaglandins in association with a lack of effect on granuloma formation and exudation.

Table 1. Effects of Local Injection of Catalase, with and without Different Additions of Superoxide Dismutase (SOD), on Some Parameters of Granulomatous Inflammation in the Rat, when Given Immediately after Induction of Inflammation by Implantation of Carrageenin-Soaked Sponges[a]

Drug combination tested		Dry granuloma weight (g)		Exudate volume (ml)		PGL (ng equiv PGE_2)[b]	
SOD (unit/sponge)	Catalase	Drug	Vehicle	Drug	Vehicle	Drug	Vehicle
0	313[c]	0.37 ± 0.05[c]	0.34 ± 0.02	1.34 ± 0.25	1.36 ± 0.05	29.4 ± 11.6*	40.5 ± 14.7
0	6,260	0.33 ± 0.03*	0.40 ± 0.03	1.50 ± 0.14	1.74 ± 0.15	—	—
0	23,334	0.33 ± 0.04**	0.45 ± 0.04	1.66 ± 0.14	1.94 ± 0.21	—	—
0	46,667	0.31 ± 0.02**	0.47 ± 0.05	1.22 ± 0.07**	1.68 ± 0.21	—	—
363	313[c]	0.32 ± 0.03*	0.40 ± 0.03	1.23 ± 0.21	1.74 ± 0.15	—	—
363	6,260[d]	0.30 ± 0.02**	0.47 ± 0.05	1.40 ± 0.17	1.68 ± 0.21	—	—
363	23,334	0.35 ± 0.03*	0.47 ± 0.05	1.66 ± 0.07	1.68 ± 0.21	—	—
2,417	6,260	0.31 ± 0.03	0.35 ± 0.04	1.54 ± 0.05	1.76 ± 0.10	48.5 ± 6.0*	30.5 ± 5.3
2,417	23,334	0.41 ± 0.01	0.47 ± 0.05	1.30 ± 0.15	1.68 ± 0.21	—	—
12,088	23,334	0.37 ± 0.05*	0.47 ± 0.05	1.52 ± 0.23	1.68 ± 0.21	—	—

[a] The parameters were determined on day 7 of granulomatous inflammation.
[b] PGL = prostaglandin-like material in the inflammatory exudate, detected by bioassay on a rat stomach strip.
[c] Following daily administration on days 3-6 of inflammation, this drug combination was ineffective.
[d] Denaturation, prior to injection, abolished the inhibitory properties on the granuloma formation.
* $P < 0.05$, ** $P < 0.01$, one-tailed Mann-Whitney U test versus control.
Values represent means ± *SEM* of 5 rats, unless indicated otherwise.

For the drug treatment on the day of implantation, correlation analysis was performed with the parameters of inflammation determined on day 7. The Spearman rank correlation coefficients were determined for granuloma weight and exudate volume against prostaglandin-like activity in the exudate, respectively. There was no correlation between granuloma weight and PGL ($r_s = 0.38$, $P > 0.05$) or between exudate volume and PGL ($r_s = 0.14$, $P > 0.05$) for eight different drug treatments. The granuloma weight and the exudate volume were significantly correlated ($r_s = 0.67$, $P < 0.05$).

Following administration on day 0, SOD alone was devoid of any effect on the parameters of inflammation, even at the highest dose tested, i.e., 12,088 units/sponge (4.2 mg protein). In contrast, the addition of low doses of SOD to catalase, prior to injection, resulted in a marked potentiation of the inhibition of granuloma formation, but this combination was ineffective in inhibiting exudation. Figures 1 and 2 show the dose–effect

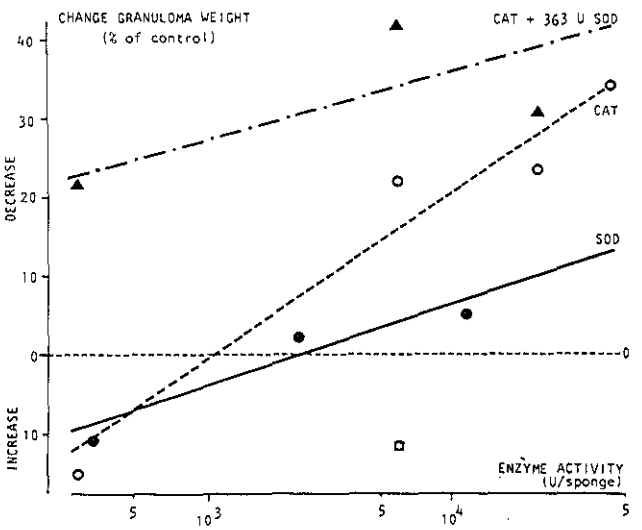

Fig. 1. Percent changes in the dry granuloma weight in response to the local injection of different doses of catalase (CAT, --○--), superoxide dismutase (SOD, —●—), and combinations of catalase with 363 units superoxide dismutase (CAT + 363 units SOD, -·-·▲-·-·-) into sponges, at the moment of sponge implantation. Denaturation prior to injection abolished the inhibitory effect on the granuloma weight of the most effective combination of catalase and superoxide dismutase (□). Each point represents the mean percent change for a group of 5 rats versus its corresponding, vehicle-treated, control group of 5 rats.

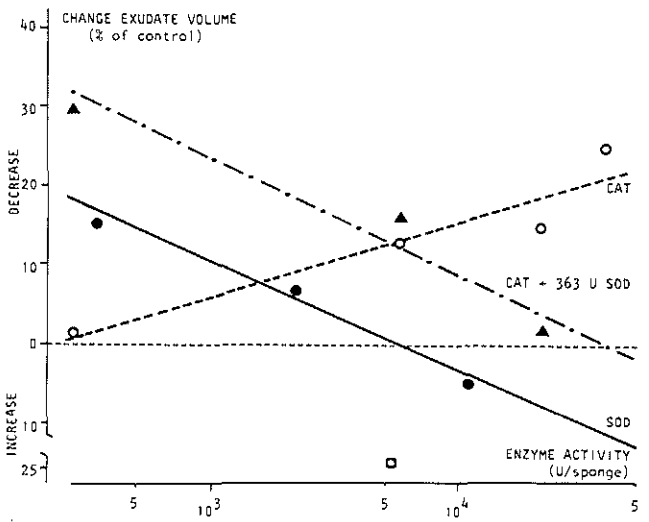

Fig. 2. Percent changes in the exudate volume in response to the local injection of different doses of catalase (CAT, --○--), superoxide dismutase (SOD, —●—), and combinations of catalase and superoxide dismutase (CAT + 363 units SOD, -·--·▲·--·-). In order to test the requirement for enzymatic activity, the combination of 363 units SOD with 6260 units CAT was denatured. Denaturation led to a reversal of the effect of the combination (□). See the legend of Figure 1 for further details.

relationships for the percent changes in granuloma dry weight and exudate volume, respectively, as a function of different doses of catalase, SOD, and combinations of both. The combined administration of 363 units SOD and 6260 units catalase per sponge was the most effective treatment for the inhibition of granuloma formation (Figure 1). However, this inhibition was completely abolished when the enzymes were heat-denaturated before injection, showing that the inhibition of granuloma formation was due to an enzymic action. When given daily to a preexisting granuloma (days 3–6), catalase (313 units) and SOD (363 units), alone or in combination, were ineffective in inhibiting granuloma formation and exudation (data not shown), but the combination significantly enhanced the granuloma weight.

As the results with some of the combinations of SOD and catalase suggested the involvement of hydroxyl radicals in the acute phase of inflammation, we tested some established hydroxyl radical scavengers, with

Table 2. Effects of Local Administration of Free Radical Scavengers and Antioxidants on Some Parameters of Granulomatous Inflammation in the Rat, when Given at Different Stages of Granuloma Development in Response to Implantation of Carrageenin-Soaked Sponges

Scavenger tested[a]	Dose (mg/sponge)	Dry granuloma weight (g)		Exudate volume (ml)		PGL (ng equiv PGE_2)	
		Drug	Vehicle	Drug	Vehicle	Drug	Vehicle
Mannitol	0.4	0.32 ± 0.03	0.34 ± 0.02	1.68 ± 0.21	1.36 ± 0.05	21.0 ± 6.0	40.5 ± 14.7
	4	0.38 ± 0.04	0.45 ± 0.04	1.38 ± 0.09*	1.94 ± 0.21	—	—
	40	0.20 ± 0.03	0.14 ± 0.01	0.84 ± 0.07	0.77 ± 0.10	5.5 ± 1.5	6.1 ± 3.6
Sodium benzoate	25.5	0.38 ± 0.03	0.45 ± 0.04	1.76 ± 0.20	1.94 ± 0.21	—	—
Thiourea[b]	20	0.35 ± 0.07*	0.21 ± 0.02	0.85 ± 0.29	0.73 ± 0.10	—	—
BPS[c]	10	0.15 ± 0.02*	0.21 ± 0.02	0.55 ± 0.06	0.73 ± 0.10	—	—
DABCO							
Day 0	3.5	0.36 ± 0.02	0.39 ± 0.04	1.14 ± 0.19*	1.78 ± 0.10	62.7 ± 13.7	104.8 ± 35.4
Days 3–6	3.5	0.29 ± 0.03	0.26 ± 0.02	1.62 ± 0.14	1.50 ± 0.09	—	—
Carotene							
Day 0	0.125	0.33 ± 0.03	0.39 ± 0.04	1.60 ± 0.21	1.78 ± 0.10	52.5 ± 10.1	104.8 ± 35.4
Days 3–6	0.125	0.36 ± 0.04*	0.26 ± 0.02	1.52 ± 0.21	1.50 ± 0.09	—	—
Tocopherol							
Day 0	18.75	0.30 ± 0.03*	0.39 ± 0.04	1.30 ± 0.13*	1.78 ± 0.10	49.8 ± 15.1	104.8 ± 35.4
Days 3–6	75	0.33 ± 0.02*	0.26 ± 0.02	1.32 ± 0.16	1.50 ± 0.09	—	—
Pr. gallate							
Day 0	1	0.35 ± 0.03**	0.48 ± 0.03	1.16 ± 0.08	0.98 ± 0.02	—	110.5 ± 22.0
Days 3–6	1	0.37 ± 0.04	0.45 ± 0.03	1.24 ± 0.20	1.87 ± 0.37	—	—
Phenidone							
Day 0	0.835	0.27 ± 0.04**	0.48 ± 0.03	0.92 ± 0.10	0.98 ± 0.02	56.3 ± 12.1*	110.5 ± 22.0
Days 3–6	0.835	0.30 ± 0.02*	0.45 ± 0.03	1.82 ± 0.26	1.87 ± 0.26	—	—
GSH							
Day 0	30	0.33 ± 0.03	0.35 ± 0.04	1.84 ± 0.09	1.76 ± 0.10	56.9 ± 9.7*	30.5 ± 5.3
Days 3–6	30	0.31 ± 0.02	0.30 ± 0.03	2.06 ± 0.19	2.34 ± 0.25	128.1 ± 48.8	80.0 ± 7.5

[a]Scavengers were administered either in a single dose, immediately after the induction of inflammation, or daily, on days 3–6 of inflammation. Abbreviations: BPS = bathophenanthroline sulfonate, DABCO = 1,4-diazabicyco(2,2,2)octane, pr. gallate = propyl gallate, phenidone = 1-phenyl-3-pyrazolidone, GSH = reduced glutathione.

*$P < 0.05$, **$P < 0.01$, one-tailed Mann-Whitney U test versus control.

See also legend of Table 1.

[b]4 Rats in the drug-treated group, of which 2 died within 24 h.

[c]4 Rats in the drug-treated group.

completely different chemical structures, as possible inhibitors of the development of granuloma. Neither mannitol, benzoate, nor thiourea showed an inhibition of the dry granuloma weight (Table 2). The median dose of mannitol significantly reduced exudation. Moreover, thiourea, besides being extremely toxic, appeared to be an irritant since it significantly stimulated the granuloma weight. Interestingly, the administration of an iron chelator, with the intention of preventing the formation of hydroxyl radicals via a Fenton-like reaction, resulted in a significantly decreased granuloma weight (Table 2). The singlet oxygen scavengers, DABCO and β-carotene, did not inhibit granuloma weight, following administration during the acute phase of inflammation, but rather tended to enhance the amount of granuloma formed, when given on days 3–6. Whereas only DABCO strongly inhibited exudation, both DABCO and carotene reduced the accumulation of prostaglandins in the exudate, albeit not significantly (Table 2).

The antioxidant, propyl gallate, reduced the amount of granuloma formed, following injection shortly after sponge implantation. The antioxidant and singlet oxygen scavenger, α-tocopherol, behaved like propyl gallate when given at the moment of induction of inflammation, and also inhibited exudation, whereas following administration to a preexisting granuloma it behaved like the other singlet oxygen scavengers as it enhanced the amount of granuloma formed. GSH, a nonspecific scavenger and the essential electron donor in the glutathione peroxidase-catalyzed reduction of peroxides, was ineffective with respect to granuloma formation and exudation, but significantly enhanced the amount of prostaglandins in the exudate on administration during the acute phase of inflammation. Finally, the inhibitor of enzyme-catalyzed oxidative metabolism of arachidonic acid, phenidone, was the only drug which inhibited the granuloma formation, irrespective of the stage of administration. It also significantly inhibited prostaglandin formation, when injected on sponge implantation (Table 2).

DISCUSSION

The present study demonstrates that SOD, even at high doses (i.e., 12,088· units/sponge), does not display antiinflammatory properties in the inflammation model used. Thus, we failed to confirm the results of Huber and coworkers (18), who showed that SOD (orgotein) is an effective antiinflammatory agent in several species, including the rat, mouse, guinea pig, dog, horse, and man. However, McCord and coworkers (19) showed that native bovine superoxide dismutase was ineffective, after intravenous injection, against carrageenin-induced paw edema and the

passive Arthus reaction in the rat. As the plasma half-life of native SOD was about 6 min and as the coupling of SOD to Ficoll resulted in a half-life of about 24 h which was associated with antiinflammatory effects in both models, it was concluded that renal clearance limits the antiinflammatory effects of native SOD (19). The local injection of SOD directly into the inflamed site, which was performed in our studies and which is not prone to interference by renal clearance because its high molecular weight prevents SOD from crossing membranes, appears to be ineffective. However, on administration in the same way, catalase showed a dose-dependent antiinflammatory effect in our model, although McCord claimed that the plasma half-life of catalase is even shorter than that of SOD.

From these data and from the fact that combined administration of catalase and SOD was more effective than the administration of catalase alone, it is likely that other mechanisms than merely the interference of renal clearance should be considered. The fact that catalase inhibits granuloma development and exudation on administration at the moment of induction of inflammation suggests that hydrogen peroxide is a proinflammatory species in the acute phase of inflammation. In fact, hydrogen peroxide is produced during the SOD-catalyzed dismutation of superoxide radicals. In view of the marked potentiation by SOD of the inhibition of granuloma formation by catalase, it is likely that catalase protects SOD from inactivation by hydrogen peroxide (20).

An alternative explanation for the potentiation is that specific combinations of SOD and catalase prevent the generation of hydroxyl radicals, which, at least in vitro, have been shown to be produced in solutions containing iron, hydrogen peroxide, and superoxide anions (10). Scavengers of hydroxyl radicals, however, failed to mimic the effects of combinations of SOD and catalase in our experiments, thus making an involvement of hydroxyl radicals unlikely. Surprisingly, the iron chelator, BPS, which is effective in the prevention of the hydroxyl radical-induced degradation of hyaluronic acid (21), was also effective in the inhibition of granuloma formation in our model. Probably, inhibiting hydroxyl radical formation is much more efficient than scavenging the radical before it reacts with biologically important molecules.

An involvement of singlet oxygen in the acute phase of inflammation is unlikely in light of the ineffectiveness of the quenchers DABCO and β-carotene as inhibitors of granuloma formation. Only DABCO inhibited exudation. The fact that the administration of the scavengers into a preexisting granuloma (daily administration on days 3–6) tend to enhance the granuloma weight, raises the possibility that the scavengers directly stimulated fibroblast functions or that singlet molecular oxygen is involved in the breakdown of collagen. The latter is likely, since it has recently been shown that reactive oxygen species may evoke the degradation of collagen in vitro (22, 23).

The antioxidants α-tocopherol and propyl gallate inhibited granuloma formation on administration during the acute phase of inflammation. Moreover, α-tocopherol also inhibited the exudation. On administration during the chronic phase of inflammation, propyl gallate was ineffective whereas α-tocopherol enhanced the granuloma weight, thus behaving in a manner related to its properties as a singlet oxygen scavenger, rather than to its antioxidant properties. The lipoxygenase and cyclooxygenase inhibitor phenidone was effective in inhibiting granuloma formation, irrespective of the time of administration. Therefore, it is likely that lipid peroxides, besides prostaglandins, are involved in inflammation as mediators, because α-tocopherol, as an inhibitor of granuloma in the acute phase, is a very weak inhibitor of cyclooxygenase (24) and phenidone is equally potent in inhibiting lipoxygenase and cyclooxygenase (14). The involvement of lipid peroxides is also emphasized when one considers the fact that the levels of prostaglandin-like material did not correlate with the granuloma weight under any conditions of drug administration. In addition, we have recently shown that the exudate levels of malonaldehyde, a compound which is formed both during the biosynthesis of prostaglandins and during radical-induced lipid peroxidation, were significantly correlated with the granuloma weight but not with prostaglandin-like material (25).

In conclusion, while bearing in mind the limitation that we are completely ignorant as to the pharmacokinetics of the majority of the scavengers used, it is evident that hydrogen peroxide plays an important role in the acute phase of inflammation, and it is likely that lipid peroxides are also involved. Knowledge of the interaction of these substances with the humoral and cellular events occurring during the inflammatory response may provide new perspectives for antiinflammatory drug design.

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REFERENCES

1. Babior, B. M., R. Kipnes and J. Cornutte. 1973. Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* 52:741.
2. Ōyanagui, Y. 1976. Inhibition of superoxide anion production in macrophages by antiinflammatory drugs. *Biochem. Pharmacol.* 25:1473.
3. Huber, W., T. L. Schulte, S. Carson, R. E. Goldhamer and E. E. Vogin. 1968. Some chemical and pharmacologic properties of a novel antiinflammatory protein. *Toxicol. Appl. Pharmacol.* 12:308.
4. Ōyanagui, Y. 1976. Participation of superoxide anions at the prostaglandin phase of carrageenan foot-edema. *Biochem. Pharmacol.* 25:1465.

5. Fong, K.-L., P. B. McCay, J. L. Poyer, B. B. Keele and H. Misra. 1973. Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced drug flavin enzyme activity. *J. Biol. Chem.* **248**:7792.
6. Weiss, S. J., G. W. King and A. F. Logublio. 1977. Evidence for hydroxyl radical generation by human monocytes. *J. Clin. Invest.* **60**:370.
7. Weiss, S. J., P. K. Rustagi and A. F. Lobuglio. 1978. Human granulocyte generation of hydroxyl radical. *J. Exp. Med.* **147**:316.
8. Walling, C. 1975. Fentons reagent revisited. *Acc. Chem. Res.* **8**:125.
9. Kharash, M. S., W. Nudenberg and F. S. Arimoto. 1951. The formation of free alkoxy (RO·) radicals in solution. *Science* **113**:392.
10. Halliwell, B. 1978. Superoxide-dependent formation of hydroxyl radicals in the presence of iron chelates. *FEBS Lett.* **92**:321.
11. McCord, J. M. 1974. Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase. *Science* **185**:529.
12. Turner, S. R., J. A. Cambell and W. S. Lynn. 1975. Polymorphonuclear leukocytes chemotaxis toward oxidized lipid components of cell membranes. *J. Exp. Med.* **141**:1437.
13. Adcock, J. J., L. G. Garland, S. Moncada and J. A. Salmon. 1973. Enhancement of anaphylactic mediator release from guinea pig perfused lungs by fatty acid hydroperoxides. *Prostaglandins* **16**:163.
14. Blackwell, G. J. and R. J. Flower. 1978. 1-Phenyl-3-pyrazolidone: An inhibitor of arachidonate oxidation in lung and platelets. *Br. J. Pharmacol.* **63**:360P.
15. Bonta, I. L., M. J. P. Adolfs and M. J. Parnham. 1979. Cannulated sponge implants for the study of time-dependent pharmacological influence on inflammatory granulomata. *J. Pharmacol. Methods* **2**:1.
16. Bult, H. and I. L. Bonta. 1976. Prostaglandin endoperoxides, serotonin and the superfused rabbit aorta: Possible pitfalls in the bio-assay of R.C.S. *Agents Actions* **6**:712.
17. Parnham, M. J., I. L. Bonta, M. J. P. Adolfs and P. C. Bragt. 1977. A polyether sponge constituent which is antioxidant, anti-inflammatory and increases prostaglandin concentrations at the inflamed site. *Agents Actions* **7**:539.
18. Huber, W., K. B. Menander-Huber, M. G. P. Saifer and P. H.-C. Dang. 1977. Studies on the clinical and laboratory pharmacology of drug formulations of bovine Cu-Zn superoxide dismutase (orgotein). *In* Perspectives in Inflammation: Future Trends and Developments. D.A. Willoughby, J.P. Giroud, and G.P. Velo, editors. (MTP Press Ltd., Lancaster, England. 527.
19. McCord, J. M., S. H. Stokes and K. Wong. 1979. Superoxide radical as a phagocyte-produced mediator of inflammation. *In* Advances in Inflammation Research, Vol. 1. G. Weissman, editor. Raven Press, New York. 273.
20. Hodgson, E. K. and I. Fridovich. 1975. Interaction of bovine erythrocyte SOD with hydrogen peroxide: Inactivation of the enzyme. *Biochemistry* **14**:5294.
21. Halliwell, B. 1978. Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. *FEBS Lett.* **96**:238.
22. Venkatasubramanian, K. and K. T. Joseph. 1977. Action of singlet oxygen on collagen. *Ind. J. Biochem. Biophys.* **14**:217.
23. Greenwald, R. A. and W. W. Moi. 1979. Inhibition of collagen gelation by action of the superoxide radical. *Arthritis Rheum.* **22**:251.
24. Panganamala, R. V., J. S. Miller, E. T. Gwebu, H. M. Sharma and D. G. Cornwell. 1977. Differential inhibitory effects on vitamin E and other antioxidants on prostaglandin synthetase, platelet aggregation and lipoxydase. *Prostaglandins* **14**:261.
25. Bragt, P. C., E. J. P. M. Schenkelaars and I. L. Bonta. 1979. Dissociation between prostaglandin and malondialdehyde formation in exudate and increased levels of malondialdehyde in plasma and liver during granulomatous inflammation in the rat. *Prostaglandins Med.* **2**:51.

REFERENCES

Adams, D.O. (1976). The granulomatous inflammatory response. A review. Am. J. Pathol. 84, 164-191.

Al-Timimi, D.J. & T.L. Dormandy. (1977). The inhibition of lipid autoxidation by human ceruloplasmin. Biochem. J. 168, 283-288.

Amphlett, C.B., G.E. Adams & B.D. Michael. (1968). Pulse radiolysis studies of deaerated aquous salicylate solutions. Adv. Chem. Ser. 81, 231-250.

Anbar, M. (1964). Possible role of copper ions in radiobiological damage. Nature 200, 376.

Anbar, M. & P. Neta. (1967). A compilation of specific bimolecular rate constants for the reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solution. Int. J. Appl. Rad. Isotopes 18, 493-523.

Askawa, T. & S. Matsushita. (1979). Thiobarbituric acid test for detection of lipid peroxides. Lipids 14, 401-406.

Babior, B.M., R.S. Kipnes & J.T. Curnutte. (1973). Biological defense mechanisms: The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52, 741-744.

Baehner, R.L., L.A. Boxer, J.M. Allen & J. Davis. (1977). Auto-oxidation as a basis for altered function by polymorphonuclear leukocytes. Blood 50, 327-335.

Bailey, K.R. & A.L. Sheffner. (1967). The reduction of experimentally induced inflammation by sulphydryl compounds. Biochem. Pharmacol. 16, 1175-1182.

Baird, M.B., H.R. Massie & M.J. Piekielniak. (1977). Formation of lipid peroxides in isolated rat liver microsomes by singlet molecular oxygen. Chem. Biol. Interact. 16, 145-153.

Barber, A.A. & F. Bernheim. (1967). Lipid peroxidation: Its measurement, occurrence and significance in animal tissues. Adv. Gerontol. Res. 2, 355-403.

Bidlack, W.R. & A.L. Tappel. (1973). Fluorescent products of phospholipids during lipid peroxidation. Lipids 8, 203-208.

Blackwell, G.J., W.G. Duncombe, R.J. Flower, M.F. Parsons & J.R. Vane. (1977). The distribution and metabolism of arachidonic acid in rabbit platelets and its modification by drugs. Br. J. Pharmacol. 59, 353-366.

Bonta, I.L. (1978). In: Inflammation. Handbook of Experimental Pharmacology, Vol. 50/I (JR Vane & SH Ferreira, eds), Springer Verlag, Berlin, Heidelberg, New York. pp. 523-567.

Bonta, I.L. & M.J. Parnham. (1978). Prostaglandins and chronic inflammation. Biochem. Pharmacol. 27, 1611-1623.

Bonta, I.L., M.J. Parnham, J.E. Vincent & P.C. Bragt. (1980). Anti-rheumatic drugs: Present deadlock and new vistas. Progr. Med. Chem. 17, 185-273.

Borgeat, P. & B. Samuelsson. (1979). Arachidonic acid metabolism in polymorphonuclear leukocytes: Unstable intermediate formation of dihydroxy acids. Proc. Nat. Acad. Sci. USA 76, 3213-3217.

Boxer, L.A., R.E. Harris & R.L. Baehner. (1979). Regulation of membrane peroxidation in health and disease. Pediat. Suppl. 713-718.

Boxer, L.A., J.M. Oliver & S.P. Spielberg. (1979). Protection of granulocytes by vitamin E in glutathione synthetase deficiency. New Engl. J. Med. 301, 901-905.

Bragt, P.C., J.I. Bansberg & I.L. Bonta. (1980). Depletion of hepatic antioxidants during granulomatous inflammation in the rat and local anti-inflammatory effects of free radical scavengers. Agents Actions Suppl. 7, in press.

Brigelius, R., R. Spöttl, W. Bors, E. Lengfelder, M. Saran & U. Weser. (1974). Superoxide dismutase activity of low molecular weight Cu^{2+} studied by pulse radiolysis. FEBS Lett. 105, 195-196.

Canonico, P.G., W. Rill & E. Ayala. (1977). Effects of inflammation on peroxisomal enzyme activities, catalase synthesis and lipid metabolism. Lab. Invest. 37, 479-468.

Carlson, T.H., S.H. Wentland, B.D. Leonard, M.A. Ruder & E.B. Reeve. (1978). Effects of prostaglandin E_2, analogs, fatty acids and indomethacin on fibrinogen level. Am. J. Physiol. 235, H223-H230.

Chance, B., H.Sies & A. Boveris. (1979). Hydroperoxide metabolism in mammalian species. Physiol. Rev. 59, 527-605.

Chang, W.C., S.I. Murota & S. Tsurufuji. (1978). Inhibition of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ formation by anti-inflammatory drugs in carrageenin-induced granuloma. Biochem. Pharmac. 27, 108-111.

Chio, K.S. & A.L. Tappel. (1969). Synthesis and characterization of the fluorescent products derived from malonaldehyde and amino acids. Biochem. 8, 2821-2827.

Chio, K.S., U. Reiss, B. Fletcher & A.L. Tappel. (1969). Peroxidation of subcellular organelles: Formation of lipofuscin-like fluorescent pigments. Science 166, 1535-1536.

Cook, H.W. & W.E.M. Lands. (1976). Mechanism for suppression of cellular biosynthesis of prostaglandins. Nature 260, 630-632.

Coon, M.J. (1978). Oxygen activation in the metabolism of lipids, drugs and carcinogens. Nutr. Rev. 36, 319-328.

Cooper, E.H. & A. Milford Ward. (1979). Acute phase proteins as aids to monitoring disease. Invest. Cell. Pathol. 2, 293-301.

Cranfield, L.M., J.L. Gollan, A.G. White & T.L. Dormandy. (1979). Serum antioxidant activity in normal and abnormal subjects. Ann. Clin. Biochem. 16, 299-306.

Curnutte, J.T., D.M. Whitten & B.M. Babior. (1974). Defective superoxide production by granulocytes from patients with chronic granulomatous disease. New Engl. J. Med. 290, 593-597.

Danon, A. & G. Assouline. (1978). Inhibiiton of prostaglandin biosynthesis by corticosteroids requires DNA and protein synthesis. Nature 273, 552-554.

Denko, C.W. (1979). Protective role of ceruloplasmin in inflammation. Agents Actions 9, 333-336.

Donovan, D.H. & D.B. Menzel. (1978). Mechanisms of lipid peroxidation: Iron

catalyzed decompodition of fatty acid hydroperoxides as the basis of hydrocarbon evolution in vivo. Experientia 34, 775-776.

Dorfman, L.M. & G.E. Adams. (1973). Reactivity of the hydroxyl radical in aquous solutions. NSRDS-NBS, nr 46. US Dept. Commerce, Natl. Bur. Stand.

Duboulez, P. & J. Dumas. (1954). On the metabolism of lipid peroxides. IV. Presence of lipid peroxides in the skin during inflammation (in French). Bull. Stê. Chim. Biol. 36, 983-991.

Egan, R.W., P.H. Gale, G.C. Beveridge & G.B. Phillips. (1978). Radical scavenging as the mechanism for stimulation of prostaglandin cyclo-oxygenase and depression of inflammation by lipoic acid and sodium iodide. Prostaglandins 16, 861-869.

Egan, R.W., J. Paxton & F.A. Kuehl Jr. (1976). Mechanism for irreversible self-deactivation of prostaglandin synthetase. J. Biol. Chem. 251, 7329-7335.

Feldberg, W. & C.H. Kellaway. (1938). Liberation of histamine and formation of lysocithin-like substances by cobra venom. J. Physiol. 94, 187-226.

Ferreira, S.H. (1972). Prostaglandins, aspirin-like drugs and analgesia. Nature 240, 200-203.

Fielden, E.M., P.B. Roberts, R.C. Bray, D.J. Lowe, G.N. Mantner, G. Rotilio & L. Calabres. Mechanism of action of superoxide dismutase from pulse-radiolysis and electron paramagnetic-resonance. Evidence that only half active sites function in catalysis. Biochem. J. 139, 49-60.

Flohé, L. (1971). Glutathione peroxidase. Enzymology and biological aspects (in German). Klin. Wochenschr. 49, 669-683.

Flower, R.J. (1974). Drugs which inhibit prostaglandin biosynthesis. Pharmacol. Rev. 26, 33-67.

Foote, C.S. (1976). In: Free Radicals in Biology. Vol. 2 (WA Pryor, ed). Acad. Press, New York. pp. 85-133.

Fridovich, I. (1974). Superoxide dismutases. Adv. Enzymol. 41, 35-97.

Fujihara, E., V.A. Sandeman & M.W. Whitehouse. (1979). Pathobiodynamics: Reduction in hepatic and intestinal ligandin (glutathione-S-transferase) levels in rats with severe acute and chronic inflammation. Biochem. Med. 22, 175-191.

Fujihara, E. & M.W. Whitehouse. (1977). Pathobiodynamics: Reduced ascorbate excretion by rats with severe inflammation. Proc. Soc. Exp. Biol. Med. 155, 361-364.

Fujita, T. (1977). Effect of ascorbic acid and iron on lipid content in plasma and liver of rats. Japan. J. Pharmacol. 27, 914-916.

Gibson, K.H. (1977). Prostaglandins, thromboxanes, PGX: Biosynthetic products from arachidonic acid. Chem. Soc. Rev. 6, 489-510.

Gitlin, J.D., J.I. Gitlin & D. Gitlin. (1977). Localization of C-reactive protein in synovium of patients with rheumatoid arthritis. Arthr. Rheum. 20, 1491-1499.

Goldstein, B.D., O.J. Balchum, H.B. Demopoulos & P.S. Duke. (1968). Electron paramagnetic resonance spectroscopy: Free radical signals associated with ozonization of linoleic acid. Arch. Environ. Hlth. 17, 46-49.

Goldstein, I.M., H.B. Kaplan, H.S. Edelsson & G. Weissmann. (1979). Ceruloplasmin. A scavenger of superoxide radical anions. J. Biol. Chem. 254, 4040-4045.

Gravela, E., E. Albano, M.U. Dianzani, G. Poli & T.F. Slater. (1979). Effects of carbon tetrachloride on isolated rat hepatocytes. Inhibition of protein and lipoprotein secretion. Biochem. J. 178, 509-512.

Gray, J.I. (1978). Measurement of lipid oxidation: A review. J. Am. Oil Chem. Soc. 55, 539-546.

Green, A.F., L.G. Garland & H.F. Hodson. (1979). In: Anti-inflammatory Drugs. Handbook of Experimental Pharmacology. Vol. 50/II (JR Vane & SH Ferreira, eds.). Springer Verlag, Berlin, Heidelberg, New York. pp. 415-466.

Green, M.R., H.A.O. Mill, M.J. Okolow-Zubowska & A.W. Segal. (1979). The production of hydroxyl and superoxide radicals by stimulated human neutrophils. Measurements by EPR spectroscopy. FEBS Lett. 100, 23-26.

Gryglewski, R.J., S. Bunting, S. Moncada, R.J. Flower & J.R. Vane. (1976). Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. Prostaglandins 12, 685-713.

Gutteridge, J.M.C. (1978). Caeruloplasmin: A plasma protein, enzyme and antioxidant. Ann. Clin. Biochem. 15, 293-296.

Ham, E.A., K.J. Cirillo, M. Zanetti, T.Y. Shen & F.A. Kuehl. (1972). In: Prostaglandins in Cellular Biology (PW Ramwell & BB Harris, eds.). Plenum Press, New York. pp. 415-466.

Hamberg, M. & B. Samuelsson. (1967). Oxygenation of unsaturated fatty acids by the vesicular gland of sheep. J. Biol. Chem. 242, 5344-5354.

Hemler, M.E. & W.E.M. Lands. (1977). Biosynthesis of prostaglandins. Lipids 12, 591-595.

Hemler, M.E., H.W. Cook & W.E.M. Lands. (1979). Prostaglandin biosynthesis can be triggered by lipid peroxides. Arch. Biochem. Biophys. 193, 340-345.

Hidaka, H. & T. Asano. (1977). Stimulation of human platelet guanylate cyclase by unsaturated fatty acid peroxides. Proc. Nat. Acad. Sci. USA 74, 3657-3661.

Higgs, G.A., R.J. Flower & J.R. Vane. (1979). A new approach to anti-inflammatory drugs. Biochem. Pharmacol. 28, 1959-1961.

Higgs, G.A. & J.A. Salmon. (1979). Cyclo-oxygenase products in carrageenin-induced inflammation. Prostaglandins 17, 737-746.

Hochstein, P. & L. Ernster. (1963). ADP-activated lipid peroxidation coupled to TPNH (NADPH) oxidase system of microsomes. Biochem. Biophys. Res. Commun. 12, 388-394.

Hocman, C. (1979). Biochemistry of ageing. Int. J. Biochem. 10, 867-876.

Högberg, J., S.Orrenius & R.E. Larson. (1975). Lipid peroxidation in isolated hepatocytes. Eur. J. Biochem. 50, 595-602.

Hokama, Y., K. Yamada, S.N. Moikeha & E.T. Nishimura. (1969). The metabolic interrelationship and physicochemical analysis of C-reactive protein and hepatic catalase. Canc. Res. 29, 542-548.

Hokama, Y., K. Yamada, C. Scheller & E.T. Nishimura. (1970). Relationship of Cx-reactive protein response to induction of liver and kidney catalase following hematin administration into rabbits. Canc. Res. 30, 1319-1326.

Huber, W., K.B. Menander-Huber, M.G.P. Saifer & P.H.C. Dang. (1977). In: Future Trends in Inflammation. Vol. 3 (DA Willoughby, JP Giroud & GP Velo, eds.). MTP Press, Lancaster, England. pp. 527-544.

Ishihara, M. (1978). Studies on lipoperoxide of normal pregnant women and of patients with toxemia of pregnancy. Clin. Chim. Acta 84, 1-9.

Judah, J.D., A.E.M. McLean & E.K. McLean. (1979). Biochemical mechanisms of liver injury. Am. J. Med. 49, 609-616.

Kamataki, T. & H. Kitagawa. (1973). Effects of lipid peroxidation on activities of drug-metabolizing enzymes in liver microsomes of rats. Biochem. Pharmacol. 22, 3199-3207.

Kaplan, H.A. & J.C. Jamieson. (1977). The effect of inflammation on rat liver β-galactosidase and β-N-acetylglucosaminidase. Life Sci. 21, 1311-1316.

Kaplan, L., J. Weiss & P. Elsbach. (1978). Low concentrations of indomethacin inhibit phospholipase A_2 of rabbit polymorphonuclear leukocytes. Proc. Nat. Acad. Sci. USA 75, 2955-2958.

Kellaway, C.H. & E.R. Trethewie. (1940). The liberation of a slow-reacting smooth muscle-stimulating substance in anaphylaxis. Quart. J. Exp. Physiol. 30, 121-145.

Kellogg III, E.W. & I. Fridovich. (1975). Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by a xanthine oxidase system. J. Biol. Chem. 250, 8812-8817.

Koj, A. (1974). In: Structure and Function of Plasma Proteins. Vol. 1 (AC Allison, ed.). Plenum Press, London, New York. pp. 73-131.

Koka, P. & P.S. Song. (1978). Protection of chlorophyll a by carotenoid from photodynamic decomposition. Photochem. Photobiol. 28, 509-515.

Koppenol, W.H. & J. Butler. (1977). Mechanism of reactions involving singlet oxygen and the superoxide anion. FEBS Lett. 83, 1-6.

Kornreich, H., N.N. Malouf & V. Hanson. (1971). Acute hepatic dysfunction in juvenile rheumatoid arthritis. J. Pediat. 79, 27-35.

Kuehl Jr, F.A., J.L. Humes, R.W. Egan, E.A. Ham & C.G. Van Arman. (1977). Role of prostaglandin endoperoxide PGG_2 in inflammatory processes. Nature 265, 170-173.

Kurzrok, R. & C. Lieb. (1930). Biochemical studies of human semen. II. The action of semen on the human uterus. Proc. Soc. Exp. Biol. Med. 28, 268-272.

Lai, C.S. & L.H. Piette. (1978). Spin-trapping studies of hydroxyl radical production in lipid peroxidation. Arch. Biochem. Biophys. 190, 27-38.

Lands, W.E.M. (1979). The biosynthesis and metabolism of prostaglandins. Ann. Rev. Physiol. 41, 633-652.

Lefkovits, A.M. & I.J. Farrow. (1955). The liver in rheumatoid arthritis. Ann. Rheum. Dis. 14, 162-169.

Levin, W., A.Y.H. Lu, M. Jacobson, R. Kuntzman, J.L. Poyer & P.B. McCay.

(1973). Lipid peroxidation and the degradation of cytochrome P-450 heme. Arch. Biochem. Biophys. 158, 842-852.

Levy, L. & T.L. Kerley. (1974). The use of DPPD (N,N'-diphenyl-*p*-phenylene diamine) as an anti-inflammatory agent. Life Sci. 14, 1917-1925.

Lightner, D.A. & Y.T. Park. (1978). Singlet oxygen ractivity of bilirubin and related tetrepyrroles. Experientia 34, 555-557.

Lin, W.S. & D.A. Armstrong. (1977). Glutathione mediation of papain inactivation by hydrogen peroxide and hydroxyl radicals. Radiat. Res. 69, 434-441.

Loury, M. (1972). Possible mechanisms of autoxidative rancidity. Lipids 7, 671-675.

Lu, A.Y.H. (1976). Liver microsomal drug metabolizing enzyme system: Functional components and their properties. Fed. Proc. 35, 2460-2463.

Marnett, L.J., M.J. Bienkowski, M. Raban & M.A. Tuttle. (1979). Studies on the hydrolysis of ^{14}C-labelled tetraethoxypropane to malondialdehyde. Anal. Biochem. 99, 458-463.

Marnett, L.J. & C.L. Wilcos. (1977). Stimulation of prostaglandin biosynthesis by lipoic acid. Biochim. Biophys. Acta 487, 222-230.

Marozzi, F.J. & M.H. Malone (1968). Analgesic and anti-inflammatory evaluation of thymotic acid and certain homologues. J. Pharm. Sci. 57, 989-994.

Matthur, P.P., R.D. Smyth, C.M. Witmer & G.S. Carr. (1978). Effects of a nonsteroidal anti-inflammatory agent and phenobarbital on hepatic microsomal mono-oxygenases in adjuvant disease in the rat. Biochem. Pharmacol. 27, 1771-1774.

May, H.E. & P.B. McCay. (1968). Reduced triphosphoridine nucleotide oxidase-catalyzed alterations of membrane phospholipids. I. Nature of the lipid alterations. J.Biol. Chem. 243, 2288-2295.

Mazur, A., S. Baez & E. Shorr. (1955). The mechanism of iron release from ferritin as related to its biological properties. J. Biol. Chem. 213, 147-160.

McCallister, J.A., R.E. Harris & R.L. Baehner. (1980). Alteration of microtubule function in glutathione peroxidase deficient polymorhonuclear leukocyte. J. Reticuloendothel. Soc. 27, 59-66.

McCord, J.M. (1974). Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase (SOD). Science 185, 529-531.

McCord, J.M., S.H. Stokes & K. Wong. (1979). In: Advances in Inflammation Research. Vol. 1 (G Weissmann, B Samuelsson & R Paoletti, eds.). Raven Press, New York. pp. 273-280.

McDonald-Gibson, W.J., S.A. Saeed & C. Schneider. (1976). The local anti-nociceptive and topical anti-inflammatory effects of propyl gallate in rodents. Br. J. Pharmacol. 58, 573-581.

Mead, J.F. (1976). In: Free Radicals in Biology. Vol. 1 (WA Pryor, ed.). Acad. Press, New York. pp. 51-68.

Mead, J.F. & A.J. Fulco. (1976). The Unsaturated and Polyunsaturated Fatty Acids in Health and Disease. CC Thomas Publ., Springfield. pp. 153-174.

Menander-Huber, K.B. & W. Huber. (1977). In: Superoxide and Superoxide Dis-

mutases (AM Michelson, JM McCord & I Fridovich, eds.). Acad. Press, London. pp. 537-549.

Miles, P.R., J.R. Wright, L. Bowman & H.D. Colby. (1980). Inhibition of hepatic microsomal lipid peroxidation without drug metabolism. Biochem. Pharmacol. 29, 565-570.

Mittal, C.K. & F. Murad. (1977). Activation of guanylate cyclase by superoxide dismutase and hydroxyl radical: A phusiological regulator of guanosine 3',5'-monophosphate formation. Proc. Nat. Acad. Sci. USA 74, 4360-4364.

Morris, H.R., G.W. Taylor, P.J. Piper & J.R. Tippins. (1980). Structure of slow-reacting substance of anaphylaxis from guinea pig lungs. Nature 285, 104-106.

Murphy, R.C., S. Hammarström & B. Samuelsson. (1979). Leukotriene C: A slow-reacting substance from murine mastocytoma cells. Proc. Nat. Acad. Sci. USA 76, 4275-4279.

Muus, P., I.L. Bonta & S.A. den Oudsten. (1979). Plasma levels of malondialdehyde a product of cyclo-oxygenase dependent and independent lipid peroxidation in rheumatoid arthritis. Prostaglandins Med. 2, 63-64.

Needleman, P. (1978). Experimental criteria for evaluating prostaglandin biosynthesis and intrinsic function. Biochem. Pharmacol. 27, 1515-1518.

Nickander, R., F.G. McMahon & A.S. Ridolfo. (1979). Nonsteroidal anti-inflammatory drugs. Ann. Rev. Pharmacol. Toxicol. 19, 469-490.

O'Brien, P.J. (1978). Hydroperoxides and superoxides in microsomal oxidations. Pharmac. Ther. 2, 517-536.

Okuma, M. & H. Uchino. (1977). Platelet lipoxygenase deficiency. New Engl. J. Med. 297, 1351-1352.

Packer, J.E., T.F. Slater & R.L. Willson. (1979). Direct observation of a free radical interaction between vitamin E and vitamin C. Nature 278, 737-738.

Paine, A.J. (1978). Excited states of oxygen in biology. Their possible involvement in cytochrome P-450 -inked oxidations as well as in the induction of the P-450 system by many divers compounds. Biochem. Pharmacol. 27, 1805-1813.

Panganamala, R.V., J.S. Miller, E.T. Gwebu, H.M. Sharma & D.G. Cornwell. (1977). Differential inhibitory effects of vitamin E and other antioxidants on prostaglandin synthetase, platelet aggregation and lipoxydase. Prostaglandins 14, 261-271.

Pederson, T.C., J.A. Buege & S.D. Aust. (1973). Microsomal electron transport. Role of NADPH-cytochrome c reductase in liver microsomal lipid peroxidation. J. Biol. Chem. 248, 7134-7141.

Pessayre, D., P. Mazel, V. Descatoire, E. Rogier, G. Feldmann & J.P. Benhamou. (1979). Inhibition of hepatic drug-metabolizing enzymes by arachidonic acid. Xenobiotics 9, 301-310.

Piette, L.H., G. Bulow & I. Yamazaki. (1964). EPR studies of the chlorpromazine free radical formed during enzymatic oxidation by peroxidase-hydrogen peroxide. Biochem. Biophys. Acta 88, 120-129.

Pitt, E. & D.A. Lewis. (1979). Stimulatory action of steroidal anti-inflamma-

tory drugs of plasma alpha$_1$-antitrypsin levels in the rat. Br. J. Pharmacol. 66, 454P-455P.

Placer, Z., J. Erazimová, R. Poledne & M. Vidláková. (1971). Cumulation of liver lipids after administration of iron. Experientia 27, 1274-1275.

Placer, Z., A. Veselková & R. Rath. (1965). Kinetics of malondialdehyde in the organism (in German). Experientia 21, 19-20.

Porter, N.A., R.A. Wolff, E.M. Yarbro & H.Weenen. (1979). The autoxidation of arachidonic acid: Formation of the proposed SRS-A intermediate. Biochem. Biophys. Res; Commun. 89, 1058-1064.

Pryor, W.A. (1973). Free radical reactions and their importance in biochemical systems. Fed. Proc. 32, 1862-1869.

Pryor, W.A. (1978). The formation of free radicals and the consequences of their reactions in vivo. Photochem. Photobiol. 28, 787-801.

Pryor, W.A. & J.P. Stanley. (1975). A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J. Org. Chem. 40, 3615-3617.

Pryor, W.A., J.P. Stanley & E. Blair. (1976). Autoxidation of polyunsaturated fatty acids. II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. Lipids 11, 370-379.

Puig-Parellada, P. & J.M. Planas. (1978). Synovial fluid degradation induced by free radicals. In vitro action of several free radical scavengers and anti-inflammatory drugs. Biochem. Pharmacol. 27, 535-537.

Qyanagui, Y. (1976). Inhibition of superoxide anion production in macrophages by anti-inflammatory drugs. Biochem. Pharmacol. 25, 1473-1480.

Rabek, J.F. & B. Ranby. (1978). The role of singlet oxygen in the photooxidation of polymers. Photochem. Photobiol. 28, 557-570.

Recknagel, R.O. & A.K. Ghoshal. (1966). New data on the question of lipoperoxidation in carbon tetrachloride poisoning. Exp. Molec. Pathol. 5, 108-117.

Richardson, T. (1976). Salicylates, copper complexes, free radicals and arthritis. J.Pharm. Pharmacol. 28, 666.

Rister, M., K. Bauermeister, U. Gravert & E. Gladtke. (1979). Superoxide dismutase and glutathione peroxidase in polymorphonuclear leukocytes. Eur. J. Pediat. 130, 127-136.

Roos, D. (1977). Oxidative killing of microorganisms by phagocytic cells. Trends Biochem. Sci. 2, 61-64.

Roycroft, J.H., W.B. Gunther & D.B. Menzel. (1977). Ozone toxicity: Hormone-like oxidation products from arachidonic acid by ozone-catalyzed autoxidation. Toxicol. Lett. 1, 75-82.

Saeed, S.A., W.J. McDonald-Gibson, J. Cuthbert, J.L. Copas, C. Schneider, P.J. Gardiner, N.M. Butt & H.O.J. Collier. (1977). Endogenous inhibitor of prostaglandin synthetase. Nature 270, 32-36.

Saeed, S.A. & B.T. Warren. (1965). On the mode of action and biochemical properties of anti-inflammatory drugs-I. Biochem. Pharmacol. 22, 1965-1969.

Salin. M.L. & J.M. McCord. (1974). Superoxide dismutases in polymorphonuclear leukocytes. J. Clin. Invest. 54, 1005-1009.

Salin, M.L. & J.M. McCord. (1975). Free radicals and inflammation. Protection of phagocytosing leukocytes by superoxide dismutase. J. Clin. Invest. 56, 1319-1323.

Sato, Y., N. Hotta, N. Sakamoto, S. Matsuoka, N. Ohishi & K. Yagi. (1979). Lipid peroxide level in plasma of diabetic patients. Biochem. Med. 21, 104-107.

Satoh, K. (1978). Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin. Chim. Acta 90, 37-43.

Sawicki, E., T.W. Stanley & H. Johnson. (1963). Comparison of spectrophotometric and spectrofluorometric methods for the determination of malonaldehyde. Anal. Chem. 35, 199-205.

Seligman, M., J. Mitamura, N. Shera & H.B. Demopoulos. (1979). Corticosteroid (methylprednisolone) modulation of photoperoxidation by ultraviolet light in liposomes. Photochem. Photobiol. 29, 549-558.

Sevanian, A., J.F. Mead & R.A. Stein. (1979). Epoxides as products of lipid autoxidation in rat lungs. Lipids 14, 634-643.

Sharma, S.C., H. Mukhtar, S.K. Sharma & C.R. Krishna Murt. (1972). Lipid peroxide formation in experimental inflammation. Biochem. Pharmacol. 21, 1210-1214.

Shen, T.Y. (1979). In: Anti-inflammatory Drugs. Handbook of Experimental Pharmacology. Vol. 50/II (JR Vane & SH Ferreira, eds.). Springer Verlag, Berlin, Heidelberg, New York. pp. 305-347.

Shim, B.S. (1976). Increase in serum haptoglobin stimulated by prostaglandins. Nature 259, 326-327.

Shutler, G.G., J.M. Langstaff, J.C. Jamieson & D.N. Burton. (1977). The effects of experimental inflammation on adaptive synthesis of rat liver fatty acid synthetase. Arch. Biochem. Biophys. 183, 710-717.

Slater, T.F. (1972). Free Radical Mechanisms in Tissue Injury. Pion Ltd., London. pp. 29-33.

Siegel, M.I., R.T. McConnell, S.L. Abrahams, N.A. Porter & P. Cuatrecasas. (1979). Regulation of arachidonate metabolism via lipoxygenase and cyclooxygenase by 12-HETE, the product of human platelet lipoxygenase. Biochem. Biophys. Res. Commun. 89, 1273-1280.

Siegel, M.I., R.T. McConnell & P. Cuatrecasas. (1979b). Aspirin-like drugs interfere with arachidonate metabolism by inhibition of the 12-hydroperoxy-5,8,11,14-eicosatetraenoic acid peroxidase activity of the lipoxygenase pathway. Proc. Nat. Acad. Sci. USA 76, 3774-3778.

Smith, S.J. (1977). Plasma proteins and colloid osmotic pressure following acute myocardial infarction (in Dutch). PhD. Thesis, Erasmus University Rotterdam, The Netherlands.

Sobański, H., J. Krupińska, B. Cebo, J. Mazur & A. Kieć-Dembińska. (1976). Antioxidants as agents potentiating the anti-inflammatory action of indomethacin. Acta Biol. Med. Germ. 35, 1547-1551.

Sorenson, J.R.J. (1976). Copper chelates as possible active forms of anti-arthritic agents. J. Med. Chem. 19, 135-148.

Spielberg, S.P., L.A. Boxer & J.M. Oliver. (1979). Oxidative damage to the neutrophils in glutathione synthetase deficiency. Br. J. Haematol. 42, 215-223.

Stuyvesant, V.W. & W.B. Jolley. (1967). Anti-inflammatory activity of d-α-tocopherol (vitamin E) and linoleic acid. Nature 216, 585-586.

Suematsu, T., T. Kamada, H. Abe S. Kikuchi & K. Yagi. (1977). Serum lipoperoxide level in patients suffering from liver diseases. Clin. Chim. Acta 79, 267-270.

Summerfield, F.W. & A.L. Tappel. (1978). Enzymatic synthesis of malonaldehyde. Biochem. Biophys. Res. Commun. 547-552.

Svingen, B.A., J.A. Buege, F.O. O'Neal & S.D. Aust. (1979). The mechanism of NADPH-dependent lipid peroxidation. The propagation of lipid peroxidation. J. Biol. Chem. 254, 5892-5899.

Swern, D. (1961). In: Autoxidation and Antioxidants. Vol. 1 (WO Lundberg, ed.). J. Wiley and Sons, New York. pp. 2-49.

Tauber, A.I. & B.M. Babior. (1977). Evidence for hydroxyl radical production by human neutrophils. J. Clin. Invest. 60, 374-379.

Terashita, Z.I., K. Nishikawa, S Terao, M. Nakagawa & T. Hino. (1979). A specific prostaglandin I_2 synthetase inhibitor, 3-hydroxy-3-methyl-2-phenyl-3H-indole. Biochem. Biophys. Res. Commun. 91, 72-78.

Thomás, G. & G.B. West. (1973). Amino acids and inflammation. Br. J. Pharmacol. 47, 662P.

Thomson, J.F. (1962). Radiation Protection in Mammals. Reinhold Publ. Co., New York. p. 168.

Tobias, L.D. & J.G. Hamilton. (1979). The effect of 5,8,11,14-eicosatetraynoic acid on lipid metabolism. Lipids 14, 181-193.

Trachte, G.J., A.M. Lefer, D. Aharony & J.B. Smith. (1979). Potent constriction of cat coronary arteries by hydroperoxides of arachidonic acid and its blockade by anti-inflammatory drugs. Prostaglandins 18, 909-914.

Trang, L.E., E. Granström & O. Lövgren. (1977). Levels of prostaglandin $F_{2\alpha}$ and E_2 and thromboxane B_2 in joint fluid in rheumatoid arthritis. Scand. J. Rheumatol. 6, 151-154.

Van Gool, J., J. Schreuder & N.C.J.J. Ladiges. (1974). Inhibitory effect of foetal α2-globulin, an acute phase protein, on carrageenin oedema in the rat. J. Pathol. 112, 245-262.

Vapaatalo, H. & J. Parantainen. (1978). Prostaglandins: Their biological and pharmacological role. Med. Biol. 56, 163-183.

Voelkel, E.F., L. Levine, C.A. Alper & A.H. Tashjian Jr. (1978). Acute phase reactants ceruloplasmin and haptoglobin and their relationship to plasma prostaglandins in rabbits bearing the VX_2 carcinoma. J. Exp. Med. 147, 1078-1088.

Von Euler, U.S. (1935). A depressor substance in the vesicular gland. J. Physiol. 84, 21P.

Weinstein, J. & B.H.J. Bielski. (1979). Kinetics of the interaction of HO_2 and O_2^- radicals with hydrogen peroxide. The Haber-Weiss reaction. J. Am. Chem. Soc. 101, 58-62.

Weiss, S.J., G.W. King & A.F. Lobuglio. (1977). Evidence for hydroxyl radical generation by human monocytes. J. Clin. Invest. 60, 370-373.

Whitehouse, M.W. (1977). Some biochemical complexities of inflammatory disease affecting drug action. Agents Actions Suppl. 2, 135-147.

Willoughby, D.A. (1978). Inflammation. Endeavour 2, 57-65.

Wills, E.D. (1966). Mechanism of lipid peroxide formation in animal tissues. Biochem. J. 99, 667-676.

Wills, E.D. (1969a). Lipid peroxide formation in microsomes. The role of non-haem iron. Biochem. J. 113, 325-332.

Wills, E.D. (1969b). Lipid peroxide formation in microsomes. Relationship of hydroxylation to lipid peroxide formation. Biochem. J. 113, 333-341.

Wills, E.D. (1971). Effects of lipid peroxidation on membrane-bound enzymes of endoplasmatic reticulum. Biochem. J. 123, 983-991.

Willson, R. (1977). Zinc: A radical approach to disease. New Scient. 76, 558-560.

Wolff, D.G. & W.R. Bidlack. (1976). The formation of carbon monoxide during peroxidation of microsomal lipids.

Younes, M., E. Lengfelder, S. Zienau & U. Weser. (1978). Pulse radiolytically generated superoxide and copper(II)-salicylates. Biochem. Biophys. Res. Commun. 81, 576-580.

Younes, M. & C.P. Siegers. (1980). Lipid peroxidation as a consequence of glutathione depletion in rat and mouse liver. Res. Commun. Chem. Pathol. Pharmacol. 27, 119-128.

SUMMARY

The investigations which have led to this thesis, are concerned with the role of free radicals and lipid peroxidation in inflammation. Free radicals are relatively reactive toward other molecules because they have an unpaired electron in the outer orbital. This reactivity is advantageous in the chemical processes involved in polymer production. Free radical reactions also occur within the body, for instance, during enzymatic processes. Generally, the free radicals generated by enzymes remain closely associated with the protein and are not often released, but there are exceptions. Xanthine oxidase, an enzyme converting xanthine to uric acid, liberates superoxide radicals during its enzymatic actions. These radicals are very reactive and they are not very specific in choosing molecules to react with. This implies that the body should be equipped with lines of defence against free radicals and harmful products generated during free radical reactions. These lines of defence exist of enzymes and compounds which react with preformed radicals to render them less harmful to biologically important molecules.

Free radical formation may lead to undesired effects, when too may radicals are formed within a relative short period and in states of inefficiency of the lines of defence. An example of undesired reactions is the reaction of free radicals with lipids, leading to a 'chain' of reactions which may be self-supporting for several minutes and this sequence of reactions is called 'lipid peroxidation'. As a consequence, membranes may loose their structure and fluidity and harmful products may also be formed. Antioxidants, for instance vitamin E, inhibit lipid peroxidation and protect the cell against damage due to this process.

Virtually all cells of the body have lipoxygenases and cyclo-oxygenase, enzymes catalyzing the formation of peroxides from specific polyunsaturated fatty acids. The endoperoxides formed as a result of cyclo-oxygenase action, are precursors of prostaglandins which are local hormones involved in a number of physiological processes (e.g. platelet aggregation, protection of vascular endothelium). During inflammation, prostaglandins promote the acute phase of this process due to the stimulatory effects on oedema formation. Aspirin-like drugs inhibit the biosynthesis of prostaglandins and, as a consequence, the acute phase of inflammation. This is

one reason for the therapeutical applications of these drugs as anti-inflammatory agents. However, aspirin-like drugs are markedly ineffective against rheumatoid arthritis, characterized by chronic inflammation of the synovia which leads to the formation of enormous amounts of collagen. In view of this lack of effects, many investigators made efforts to develop alternative drugs. One of the results has been the successful therapeutical application of immune modulators against rheumatoid arthritis.

Another approach in the development of new drugs is the study of free radical formation during phagocytosis and its implication for the inflamed area. Free radical generation by phagocytosing granulocytes and macrophages may lead to tissue damage and depolymerization of synovial fluid. Furthermore, free radicals may induce lipid peroxidation by affecting polyunsaturated fatty acids embedded in cell membranes or in the free form. The products formed have a certain degree of toxicity for living cells and tissues. The investigations which have been described in this thesis have been performed from the point of view that lipid peroxidation is involved in inflammation.

The introduction of this thesis is concerned with the mechanisms of induction of lipid peroxidation by free radicals, the products formed, the measurement of lipid peroxidation and the ways via which the process may be prevented or inhibited. Furthermore, some interactions have been described between free radical-induced lipid peroxidation and the formation of prostaglandins. The second part of the introduction describes the interactions of free radicals and lipid peroxidation with inflammation, while much attention has been paid to the role of the acute phase proteins. Ceruloplasmin is especially important in this context as it is a scavenger of superoxide radicals and as it is the major serum antioxidant. Furthermore, ceruloplasmin has been shown to be effective as an anti-inflammatory agent in animal models of inflammation. In this respect it is obvious that the liver is involved in the systemic response on a remote localized injury, because it synthesizes the acute phase reactants and undergoes lipid peroxidation in an enhanced rate. Malonaldehyde is a stable end product of lipid peroxidation and it has been used for many decades as a monitor of lipid peroxidation in food technology as well as in the study of biological systems. Malonaldehyde has also been used as a monitor of lipid peroxidation in the experiments included in this thesis.

As malonaldehyde is also formed during the biosynthesis of prosta-

glandins which play an important role in inflammation, attention has been initially focussed on the conversion of radioactively labelled arachidonate to prostaglandins and related substances.

Appendix 1 is concerned with the development of an inflammation model in the rat, which enables accurate injections of arachidonate into the inflamed area (granuloma) and the perfusion of metabolites, without the necessity of surgically traumatizing the animal or sacrificing it. The newly developed model, a teflon chamber provided with two cannulae, has many appaerances in common with the model of cannulated sponge implants with respect to inflammatory parameters.

The metabolism of arachidonate in vivo by granulomatous tissue has been described in Appendix 2. The metabolism studies with the newly developed chamber model reveal that prostaglandin (PG) E_2 and hydroxy-fatty acids are main metabolites at any stage of granuloma formation. These results are in marked contrast with the findings of other authors working with in vitro systems. The metabolism of arachidonate in the chamber model is very similar to arachidonate metabolism in the affected joints of patients with rheumatoid arthritis, for which the model is thought to be relevant.

Appendix 3 is concerned with the pharmacological aspects of inflammation because indomethacin was the drug of choice to investigate the effects of nonsteroidal anti-inflammatory drugs on arachidonate metabolism and granuloma formation. PGE_2 biosynthesis and granuloma formation may be independently influenced by indomethacin in view of the inhibition of PGE_2 formation in combination with unaffected granuloma growth. Furthermore, in the acute phase of inflammation indomethacin inhibited the formation of the lipoxygenase product, HETE, which had not been previously described at that moment. Indomethacin could never fully suppress metabolite formation from arachidonic acid, suggesting nonenzymatic formation of products.

The formation of malonaldehyde at the site of inflammation, independently of the biosynthesis of prostaglandins is given in Appendix 4. Thus, it is very likely that nonenzymatic lipoperoxidation occurs during inflammation. Enhanced levels of malonaldehyde were observed in the blood of rats during inflammation and this was probably due to enhanced hepatic formation in view of elevated levels of malonaldehyde in livers from these rats.

As lipid peroxidation in vitro in liver homogenates has been described to interfere with oxidative drug metabolism by the cytochrome P-450 system, the in vivo metabolism of the model drug, aminopyrine, has been

studied and the results have been presented in Appendix 5. During inflammation, aminopyrine metabolism is more inhibited in rats with a stronger inflammatory response.

Appendix 6 is concerned with decresed levels of antioxidants in the liver. Thus, catalase activity and the levels of reduced glutathione and ascorbic acid were decreased, but superoxide dismutase activity was not significantly affected. Although the mechanisms behind these observations have not yet been elucidated, it is obvious that there is some kind of oxidant stress in the liver during remote localized inflammation.

Finally, Appendix 7 describes the possibility of pharmacologically influencing granulomatous inflammation by free radical scavengers and antioxidants. Catalase was a potent anti-inflammatory drug as it was effective against exudation and granuloma formation. Superoxide dismutase, however, was inactive, which might have been anticipated in view of the low chemical reactivity of the superoxide radical. Combinations of catalase and superoxide dismutase were more potent than catalase alone as inhibitors of granuloma formation. As a consequence, scavengers of hydroxyl radicals were investigated, but without effect. That this does not simply justify the conclusion that hydroxyl radicals are not important in inflammation is emphasized by our observation that an iron chelator was an effective inhibitor of granuloma formation. In in vitro experiments, iron chelators inhibit hydroxyl radical formation and subsequent depolymerization of synovial fluid.

The results have been evaluated in the discussion, whereas pharmacological consequences of the use of antioxidants as anti-inflammatory agents have been given. The possible advantages of these compounds, either alone or in combination with frequently used nonsteroidal anti-inflammatory drugs, have been considered and suggestions for future research have not been omitted.

SAMENVATTING

Het onderzoek dat in dit proefschrift staat beschreven betreft de rol van vrije radicalen en lipoperoxidatie bij ontsteking. Vrije radicalen zijn atomen of moleculen met een ongepaard elektron in de buitenste schil, waardoor deze een grote reaktiviteit bezitten. Deze reaktiviteit kan nuttig zijn wanneer via vrije-radicaal mechanismen wordt getracht een kunststof te synthetiseren, of welke chemische stof dan ook. Ook in het lichaam komen vrije-radicaal reakties voor, bijvoorbeeld tijdens enzymatische processen. In het algemeen laat het enzym de ontstane radicalen niet los, maar er zijn enzymen bekend die dit echter wèl doen. Een voorbeeld is xanthine oxidase, een enzym dat xanthine omzet in urinezuur. Tijdens deze omzetting ontstaan superoxide radicalen. Omdat deze radicalen zo reaktief zijn en bovendien niet erg specifiek in het kiezen van stoffen waar ze mee reageren, heeft het lichaam een aantal 'verdedigingsmechanismen' tegen vrije radicalen en schadelijke produkten die ontstaan tijdens vrije-radicaal reakties. Deze mechanismen bestaan uit enzymen en stoffen, die met grote snelheid met gevormde radicalen reageren, nog vóór deze met biologisch belangrijke moleculen kunnen reageren en de radicalen worden hierbij omgezet in minder schadelijke produkten.

Worden er op een bepaald moment teveel radicalen gevormd of schieten de beschermende mechanismen in hun doel tekort, dan kunnen de gevormde radicalen aanleiding geven tot ongewenste reakties. Een voorbeeld hiervan is de reaktie van radicalen met lipiden, waarbij een soort kettingreaktie ontstaat die zichzelf enige tijd in stand kan houden en deze serie reakties wordt aangeduidt als 'lipoperoxidatie'. Als gevolg van lipoperoxidatie verliezen membranen hun struktuur en fluïditeit en worden voor de cel schadelijke stoffen gevormd. Antioxidantia, zoals bijvoorbeeld vitamine E, remmen lipoperoxidatie en beschermen dus de cel tegen de schadelijke gevolgen ervan.

Het lichaam is in staat om in vrijwel alle cellen specifieke peroxiden te maken, via lipoxygenases en cyclo-oxygenase. Dit laatste enzym is erg belangrijk, omdat de endoperoxiden die het maakt uit speciale meervoudig onverzadigde vetzuren worden omgezet in prostaglandines. Deze prostaglandines zijn betrokken bij tal van fysiologische processen (o.a. aggregatie van bloedplaatjes, bescherming van de vaatwand). Ook bij ontstekingen spelen ze een rol, zij het dat ze het ontstekingsproces verergeren, doordat ze oedeem-

vorming in de beginfase van een ontsteking stimuleren. Aspirine-achtige farmaca remmen de vorming van prostaglandines en voorkomen daardoor een verergering van de ontsteking. Dit is een van de redenen dat deze kategorie van verbindingen toepassing vindt als ontstekingremmers. Tegen rheumatoide arthritis, gekenmerkt door een chronische ontsteking van de synovia die gepaard gaat met de vorming van grote hoeveelheden bindweefsel, zijn deze farmaca echter weinig aktief. Dit heeft ertoe geleid dat vele onderzoekers hun aandacht zijn gaan richten op het ontwikkelen van farmaca met andere werkingsmechanismen. Dit heeft onder andere tot gevolg gehad dat tegenwoordig modulatoren van het immuunsysteem worden gebruikt in de therapie van rheumatoide arthritis.

Een andere benadering in de ontwikkeling van nieuwe farmaca is de bestudering van de vorming van vrije radicalen tijdens de fagocytose door granulocyten en macrofagen en de gevolgen hiervan voor ontstoken weefsels. Vrije radicalen kunnen weefselbeschadiging veroorzaken en leiden tot depolymerisatie van synoviaalvloeistof. Ook kunnen ze aanleiding geven tot peroxidatie van meervoudig onverzadigde vetzuren in celmembranen of in vrije vorm aanwezig, waarbij talloze produkten ontstaan die een zekere mate van toxiciteit bezitten. De onderzoekingen die in dit proefschrift zijn beschreven gaan uit van de veronderstelling dat lipoperoxidatie een rol speelt bij het ontstekingsproces.

In de inleiding is beschreven hoe vrije radicalen lipoperoxidatie kunnen induceren, welke produkten er worden gevormd, hoe lipoperoxidatie kan worden gemeten en hoe dit proces kan worden voorkomen of geremd. Bovendien is ingegaan op de interakties tussen lipoperoxidatie en de biosynthese van prostaglandines. In het tweede deel van de inleiding is omschreven op welke wijze lipoperoxidatie en vrije radicalen invloed kunnen hebben op het ontstekingsproces en is ingegaan op de rol van de akute fase eiwitten, waarvan ceruloplasmine belangrijk is met het oog op het wegvangen van vrije radicalen. In ontstekingsmodellen heeft ceruloplasmine een ontstekingremmende werking. Het is duidelijk gebleken dat de lever een rol speelt bij een ontsteking die zich ver buiten de lever afspeelt, immers de lever maakt de akute fase eiwitten en het is beschreven dat de lever in verhoogde mate malonaldehyde vormt tijdens zo'n ontsteking. Malonaldehyde is een stof die een stabiel eindprodukt is van lipoperoxidatie en de vorming ervan wordt al tientallen jaren gebruikt als maat voor de peroxidatieve omzetting van lipi-

den, zowel in de voedingsmiddelen technologie als in het onderzoek van biologische systemen. Zo ook in de in dit proefschrift beschreven experimenten.

Omdat malonaldehyde óók gevormd wordt tijdens de biosynthese van prostaglandines, die zoals juist gezegd, belangrijk zijn bij ontsteking, is in eerste instantie gekeken naar de omzetting van radioaktief gemerkt arachidonzuur naar prostaglandines en analoga.

Appendix 1 beschrijft de ontwikkeling van een ontstekingsmodel bij de rat dat het mogelijk maakt om op eenvoudige wijze arachidonzuur nauwkeurig in het ontstoken gebied (granuloma weefsel) te injecteren en de metabolieten te verkrijgen, zonder dat daarbij het dier opgeofferd hoeft te worden. Het ontwikkelde model, een geïmplanteerde teflon kamer voorzien van canules, is vergeleken met een reeds bestaand ontstekingsmodel en het blijkt hiermee in grote lijnen overeen te komen.

In Appendix 2 wordt beschreven dat het metabolisme van arachidonzuur door granulomaweefsel in vivo, met behulp van het nieuw ontwikkelde ontstekingsmodel, voornamelijk leidt tot de vorming van hydroxy-vetzuren en prostaglandine (PG) E_2, in elk stadium van granulomavorming. De beschreven resultaten zijn volkomen verschillend van de resultaten die andere onderzoekers verkregen uit in vitro experimenten. Bovendien blijkt het metabolisme van arachidonzuur door het experimentele granuloma sterk overeen te komen met dat in de gewrichten van patienten met rheumatoide arthritis, waarvoor het model overigens wordt geacht representatief te zijn.

De farmacologische beinvloeding van de granulomateuze ontsteking en het metabolisme van arachidonzuur wordt beschreven in Appendix 3. De vorming van prostaglandine E_2 blijkt te kunnen worden geremd zonder dat daardoor het ontstekingsproces wordt geremd. Bovendien bleek dat indomethacine de vorming van lipoxygenase-afhankelijke produkten te kunnen remmen, wat tot dat moment nog niet eerder beschreven was. Indomethacine was niet in staat om de vorming van metabolieten voor honderd procent te remmen, zodat een niet-enzymatische omzetting waarschijnlijk was.

Appendix 4 beschrijft de vorming van malonaldehyde op de plaats van ontsteking en deze blijkt onafhankelijk te zijn van de vorming van prostaglandines. Dit maakt het zeer aannemelijk dat nonenzymatische lipoperoxidatie optreedt. Verhoogde spiegels van malonaldehyde werden gemeten in het bloed van ratten met een ontsteking. Dit is mogelijk afkomstig van de lever, omdat ook hierin verhoogde lipoperoxidatie optreedt tijdens een ontsteking ver buiten de lever.

Omdat lipoperoxidatie in in vitro experimenten vaak gepaard gaat met een remming van het farmaconmetabolisme in de lever, werd het metabolisme van het modelfarmacon aminopyrine bestudeerd. Dit staat beschreven in Appendix 5. Het metabolisme van aminopyrine in vivo blijkt sterker geremd naarmate de ontsteking heviger is.

Appendix 6 beschrijft de veranderingen in de antioxidant status van de lever tijdens een buiten de lever gelokaliseerde ontsteking. Het blijkt dat de hoeveelheid gereduceerd glutathion zeer sterk afneemt, terwijl ook de hoeveelheid ascorbinezuur en de katalase aktiviteit zijn afgenomen. Dit benadrukt de veranderingen die zich in de lever voltrekken, hoewel het mechanisme ervan niet duidelijk is.

Appendix 7, tenslotte, beschrijft de farmacologische beinvloeding van een granulomateuze ontsteking met behulp van vrije-radicaal scavengers en antioxidantia. Katalase had in het gebruikte ontstekingsmodel sterk anti-inflammatoire eigenschappen, terwijl superoxide dismutase niet effektief bleek te zijn. Op grond van de geringe reaktiviteit van het superoxide anion kan dit overigens wel worden verwacht. Combinaties van katalase en superoxide dismutase waren sterker anti-inflammatoir dan katalase alleen en dit gaf aanleiding tot het testen van scavengers van hydroxyl radicalen. Daar deze geen effekt hadden op het ontstekingsproces, mag niet zonder meer worden aangenomen dat hydroxyl radicalen geen rol spelen bij een ontsteking. Dit wordt nog eens benadrukt door het feit dat een ijzerchelator, een stof die in vitro de vorming van hydroxyl radicalen voorkomt, het ontstekingsproces kon remmen.

In de discussie worden de resultaten gewogen en wordt ingegaan op de farmacologische betekenis van het gebruik van antioxidantia als ontstekingremmers, hetzij alleen, hetzij in combinatie met reeds veelvuldig toegepaste aspirine-achtige farmaca en worden suggesties gegeven voor nader onderzoek.

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Peter Cull, in:
The Pharmacology of Inflammation
W.G. Spector & D.A. Willoughby
England Univ. Press Ltd
Frome and London, 1968.

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