2. Feingold KR, Wiley MH, Moser AH, Lear SR, Siperstein MD: *De novo* sterologenesis in intact primates. J Lab Clin Med 100:405–410, 1982
3. Feingold KR, Wiley MH, Wu J, MacRae G, Moser AH, Lear SR, Siperstein MD: Differences in *de novo* cholesterol synthesis between the intact male and female rat. Endocrinology 112:96–103, 1983
4. Pack GZ, Olsen TG, Yoder FW, Strauss JS, Downing DT, Pandya M, Buthus D, Battandier JA: Prolonged remission of cystic and coglombate acne with 13-*cis*-retinoic acid. N Engl J Med 300:329–333, 1979
5. Gomez EC, Moskowitz RJ: Effect of 13-*cis*-retinoic acid on the hamster flank organ. J Invest Dermatol 74:392–397, 1980
6. Jansen GR, Zanetti ME, Hutchison CF: Synthesis of cholesterol from glucose ^{14}C in the liver and extrahepatic tissues of the mouse. Arch Biochem Biophys 138:433–442, 1970
7. Turley SD, Andersen JM, Dietschy JM: Rates of sterol synthesis and uptake in the major organs of the rat *in vivo*. J Lipid Res 22:551–569, 1981
8. Van Scott EJ, Ekel TB, Averbach R: Determination of rate and kinetics of cell division in scalp hair. J Invest Dermatol 41:269–273, 1963
9. Brannan PG, Goldstein JL, Brown MS: 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase activity in human hair roots. J Lipid Res 16:7–11, 1975
10. Pochi PE, Strauss JS: Endocrinologic control of the development and activity of the human sebaceous gland. J Invest Dermatol 62:191–201, 1974
11. Pochi PE, Strauss JS: Sebaceous gland suppression with ethinyl estradiol and diethylstilbesterol. Arch Dermatol 108:210–214, 1973
12. Pochi PE, Strauss JS, Mescon H: Sebum secretion and urinary fractional 17-ketosteroids and total 17-hydroxycorticoid excretion in male castrates. J Invest Dermatol 39:475–483, 1962
13. Nicolaides N, Ansami MNA, Fu HC, Lindsay DG: Lipid composition of comedones compared with that of human skin surface lipids in acne patients. J Invest Dermatol 54:487–495, 1970
14. Downing DT, Pochi PE, Strauss JS: Variability in the chemical composition of human skin surface lipids. J Invest Dermatol 53:322–327, 1969
15. Elias PM: Epidermal lipids, barrier function, and desquamation. J Invest Dermatol 80(6)(suppl):44–49, 1983
16. Holick MF, Frommier JE, McNeill SC, Richland NM, Henley JW, Potts JT Jr: Photometabolism of 7-dehydrocholesterol to previtamin D_3 in skin. Biochem Biophys Res Commun 76:107–114, 1977

0022-202X/83/8104-0369$02.00/0
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Studies on the Role of Reactive Oxygen Species in Mediating Lipid Peroxide Formation in Epidermal Microsomes of Rat Skin

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The role of superoxide, hydrogen peroxide, and singlet oxygen in mediating nonenzymic and NADPH-supported enzymic lipid peroxidation in skin microsomes was investigated. Incubation of skin microsomes with NADPH and/or Fe^{+3}-ADP or ascorbate resulted in the formation of lipid peroxides. The epidermis was the major target site for microsomal lipid peroxide formation in skin. Enzymic peroxidation of epidermal microsomes required NADPH and was oxygen-dependent. Addition of the nonenzymic catalysts, Fe^{+3} and ADP, to the enzymic peroxidation system had an additive effect on the generation of lipid peroxide in epidermal microsomes. Epidermal microsomal lipid peroxidation was inhibited by singlet oxygen quenchers such as dimethylfuran, histidine, and β-carotene. Hydroxyl ion scavengers such as mannitol, benzoate, or ethyl alcohol and the enzymic scavenger of superoxide, superoxide dismutase, were all ineffective in this respect. Addition of EDTA, Mn^{+2}, cytochrome c^{+3}, and catalase to the NADPH-supported enzymic peroxidation system resulted in strong inhibition of lipid peroxide formation in skin. Glutathione or epidermal cytosol added alone or in combination to the NADPH-supported incubation system enhanced peroxidation of microsomal lipids. Vitamin E (α-tocopherol) inhibited lipid peroxidation. These results indicate that singlet oxygen may mediate lipid peroxide formation in epidermal microsomes.

The peroxidative destruction of lipid-rich biomembranes occurs ubiquitously among aerobic organisms and is regarded as one of the major causes associated with inflammation and tissue damage. Several lines of evidence indicate that peroxidation of unsaturated lipids not only causes deterioration of lipids but is highly catastrophic to the integrity of cellular membranes and membrane-bound enzymes [1–3]. The possibility that lipid peroxidation of biomembranes may be a mechanism whereby foreign chemicals exert their toxic effects, has also evoked considerable interest in understanding the mechanisms of this phenomenon in various biologic systems [2,3].

Several forms of reactive oxygen including superoxide (O_2^-), hydroxyl ion (OH^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) are generated in situ either as a result of various metabolic processes or following exposure to xenobiotics. The role of these reactive moieties in triggering lipid peroxidation of microsomal membranes of certain tissues including liver and lung has been well documented [1–4]. The association of these reactive moieties with peroxidative damage to membranes of the skin, a tissue that is quite vulnerable to the effects of active oxygen radicals, is poorly understood.

Skin is the largest body organ and provides the major interface between the body and its environment. The biologic role of skin in maintaining body homeostasis and protecting against the entry of noxious foreign chemicals, has generated a surge

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Abbreviations:
MDA: malondialdehyde
SOD: superoxide dismutase

of interest in understanding the mechanisms underlying various metabolic processes occurring in cutaneous tissue. In this regard, recent studies have clearly demonstrated that skin is an active site of the biotransformation of a variety of endogenous substances such as steroid hormones, cholesterol, and foreign xenobiotics including drugs and carcinogens (for review see [5]).

Recent observations suggest that lipid-rich membranes in skin can undergo active peroxidation by free radicals generated in vivo [6–12]. The aging of human skin is accompanied by morphologic changes that are identical to those found in various membranes damaged by lipid peroxidation [6]. The observation of premature dystrophic changes in epidermal cells repeatedly exposed to sunlight has led to the suggestion that this process is associated with lipid peroxidation [7]. Psoriatic epidermis has been shown to have increased xanthine oxidase activity, an enzyme capable of generating the superoxide anion radical [8]. Studies have also suggested that during keratinization the metabolism of prostaglandins in skin causes lipid peroxide formation [9,10]. In addition, cutaneous photosensitization resulting from exposure to certain chemicals and to solar radiation is thought to be partially mediated by peroxidative damage to lipid-rich membranes [11]. These observations strongly suggest that cellular membranes in skin could be a target for peroxidative damage and such damage could contribute to photosensitization, to cellular aging, and to UV damage in the skin. Yet, very few data are available relating to the mechanism underlying lipid peroxide formation in skin. This study was designed to characterize the role of reactive oxygen species in nonenzymic and NADPH-supported enzymic lipid peroxide formation in skin microsomal membranes. Our data indicate that such damage does occur and that this may be a model for in vitro assessment of oxidative damage to cutaneous tissue.

MATERIALS AND METHODS

Thiobarbituric acid, mannitol, β-carotene, sodium benzoate, xanthine, L-ascorbic acid, superoxide dismutase, xanthine oxidase, and catalase were purchased from Sigma Chemical Company, St. Louis, Missouri. All other chemicals were purchased at the highest available purity.

Animals

Sperm-positive Sprague-Dawley rats were obtained from Holtzman Rat Farm, Madison, Wisconsin, and shipped during the last trimester. Neonatal rats born in situ were allowed to suckle until the 4th day after birth. The advantages of using neonatal rats for studies of xenobiotic metabolism in skin have been discussed previously [13,14].

Preparation of Tissue

The neonatal rats were sacrificed by decapitation with surgical scissors. The head and extremities of the animal were removed and whole skin from the remaining body, which constitutes 80–90% of the total skin, was excised and immediately placed in ice-cold 0.15 M KCl. Each skin was placed epidermal side down on a covered glass Petri dish containing crushed ice. The skin was scraped with a sharp scalpel blade (Bard-Parker No. 20) to remove subcutaneous fat and muscle. For the separation of epidermis and dermis the method of Epstein et al [15] was adapted. The skins were floated in 0.1 M potassium phosphate buffer, pH 7.40, containing 10 mM dithiothreitol in a 100-ml glass beaker (4 skins in each glass beaker). The beaker was placed on a rotary shaker with gentle shaking in a cold room at 4°C. After 2 h of shaking the epidermis was peeled away from the dermis using forceps. The whole skin, epidermis, and dermis were washed using fresh 0.15 M KCl, blotted with paper towels, and carefully minced into small pieces (< 1 mm^3).

Homogenization and Subcellular Fractionation

All operations were done at 4°C. The minced tissues were added to 50-ml polyethylene beakers containing 4 volumes of 20 mM phosphate buffer, pH 7.4, containing 0.15 M KCl and subjected to 6 separate 10-s bursts of a Polytron Tissue Homogenizer (Brinkman Instruments) equipped with a ST-10 generator. There was a pause of 30 s between each burst to minimize heating of the tissue. This whole homogenate was then poured into the tube of a ground-glass conical tissue homogenizer fitted with a ground-glass pestle made to carefully defined tolerances with a clearance of 0.004–0.006 mm (Kontes Glass Co., Vineland, New Jersey) and was homogenized for 6 passes at 400 rpm using a rotary drill press. This whole homogenate was filtered through 2 layers of surgical gauze soaked in the homogenizing buffer using gentle vacuum with a Buchner funnel. The homogenate was centrifuged at 800 × g for 20 min in a Sorvall RC 2B refrigerated centrifuge using an SS-24 rotor. The pellet was washed gently and respun at 800 × g for 20 min. The pooled supernatants were then spun at 9000 × g for 20 min. The 9000 × g pellet was washed and respun for 20 min. The supernatant was centrifuged at 100,000 × g for 60 min in a Beckman LS-50 ultracentrifuge using a 50-Ti rotor. The washed microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 10^{-4} M $MgCl_2$.

Assay of Lipid Peroxidation

Lipid peroxidation in microsomal preparations from whole skin, dermis, or epidermis was assessed by measuring the formation of malondialdehyde (MDA) as described by Wright et al [16]. The following modifications were made. NADPH-mediated lipid peroxidation was measured by incubating 1.2–1.8 mg microsomal protein, 1 mM NADPH in 0.6 ml of Ca^{+2}-free 0.1 M phosphate buffer, pH 7.4, which contained 10^{-4} M $MgCl_2$, for 1 h at 37°C. Nonenzymic lipid peroxidation was studied by adding either Fe^{+3} ($FeCl_3$, 1 mM) and ADP (5 mM) or ascorbate (1 mM) to the above reaction mixture in place of NADPH. Blanks were prepared using microsomes only. The reaction was terminated by the addition of 0.6 ml of 10.0% (w/v) trichloroacetic acid. One milliliter of 0.5% (w/v) 2-thiobarbituric acid was added to the mixture which was then heated for 10 min in a boiling water bath. Samples were cooled under running tap water, centrifuged, and the levels of MDA were measured by reading the formation of color at 535 nm. The concentration of MDA was calculated by using a molar extinction coefficient of 1.5×10^5 M^{-1} [17].

RESULTS

Nonenzymic Lipid Peroxidation of Skin Microsomes

The effects of the addition of Fe^{+3} and ADP or ascorbate on epidermal microsomal lipid peroxide formation are presented in Table I. Addition of Fe^{+3} (1 mM) and ADP (5 mM) significantly enhanced the formation of MDA as compared to microsomes alone. Lipid peroxidation induced by Fe^{+3} and ADP was linear up to 75 min of incubation. Ascorbate was equally effective in stimulating lipid peroxidation in epidermal microsomes. As shown in Table I the magnitude of MDA formation was increased 2-fold when ascorbate was added along with Fe^{+3}–ADP.

Lipid Peroxidation in Microsomes Prepared From Epidermis, Dermis, and Whole Skin

To assess the comparative capacity of each structural component of the skin to undergo lipid peroxidation, we compared nonenzymic and NADPH-supported lipid peroxidation in epidermis and dermis as well as whole skin (Table II). NADPH significantly stimulated the formation of MDA in microsomes in each of the cutaneous structures analyzed. However, epidermis and whole skin were much more active in the production of MDA than was the dermis. Moreover, nonenzymic lipid peroxidation was also higher in epidermis and whole skin. With both NADPH and Fe^{+3} in the incubation system, MDA pro-

TABLE I. *Nonenzymatic lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Microsomes only	0.30 ± 0.02
+ Fe^{+3} (1 mM) + ADP (5 mM)	2.23 ± 0.13
+ ascorbate (1 mM)	2.50 ± 0.16
+ Fe^{+3} + ADP + ascorbate (above concentrations)	4.30 ± 0.28

Values for malondialdehyde (MDA) were obtained following incubation of epidermal microsomal suspension at 37°C for 60 min. The numbers are mean values for 4 experiments ± SE.

TABLE II. *Comparison of lipid peroxide formation in microsomes from whole skin, dermis, and epidermis*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)		
	Whole skin	Dermis	Epidermis
Microsomes	0.39 ± 0.02	0.33 ± 0.03	0.59 ± 0.04
+ NADPH (0.25 mM)	0.93 ± 0.07	0.46 ± 0.04	1.19 ± 0.18
+ NADPH (1 mM)	1.73 ± 0.13	0.79 ± 0.05	1.53 ± 0.11
+ ascorbate (1 mM)	2.06 ± 0.23	0.99 ± 0.10	1.79 ± 0.17
+ Fe^{+3} (0.5 mM) + ADP (5 mM)	2.19 ± 0.13	1.96 ± 0.18	1.79 ± 0.14
+ NADPH (1 mM) + Fe^{+3} (0.5 mM) + ADP (5 mM)	4.10 ± 0.32	2.65 ± 0.21	4.92 ± 0.33
Boiled microsomes	0.19 ± 0.02	0.13 ± 0.02	0.19 ± 0.03
Boiled microsomes + NADPH (1 mM)	0.19 ± 0.03	0.11 ± 0.02	0.17 ± 0.02

Values for malondialdehyde (MDA) were measured following incubation of the microsomal suspensions at 37°C for 60 min. The values are mean values for 4 experiments ± SE.

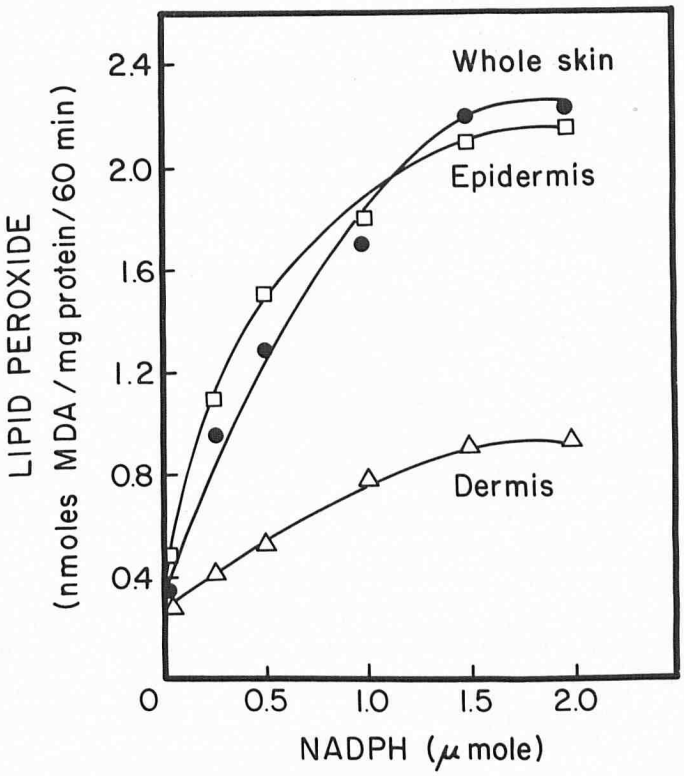

FIG 1. Dependence of microsomal lipid peroxidation on NADPH. The assay system consisted of 0.1 M phosphate buffer, pH 7.4, 1.2–1.6 mg microsomal protein, and indicated amounts of NADPH in a final volume of 0.6 ml. The reaction was carried out at 37°C for 60 min under air. MDA formation was estimated as described in the text.

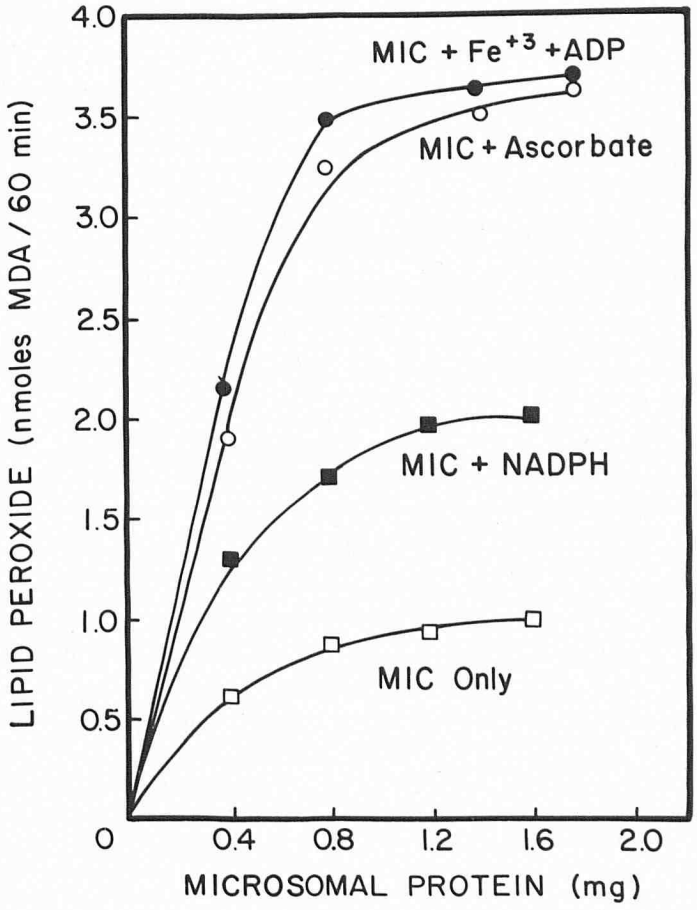

FIG 2. Dependence of enzymic and nonenzymic lipid peroxidation on epidermal microsomal protein. Assay system consisted of 0.1 M phosphate buffer, pH 7.4, 1 mM NADPH or 1 mM ascorbate or 0.5–5 mM Fe^{+3}–ADP, and indicated amounts of epidermal microsomal protein. The reaction was carried out at 37°C for 60 min under air. MDA formation was estimated as described in the text.

duction increased additively (Table II). The formation of MDA in microsomes from whole skin, dermis, and epidermis was dependent on the amount of NADPH. As shown in Fig 1, increasing the amount of NADPH beyond 1.5 μmol, had no further enhancing effect on MDA production from either microsomal preparation. Fig 2 shows that both the nonenzymic and the NADPH-catalyzed peroxidation of epidermal microsomes was linear over a range of 0.4–1.6 mg protein per ml. Nonenzymic and NADPH-supported lipid peroxidation in epidermal microsomes was linear up to an incubation time of 60 min (Fig 3).

Effect of Air, Oxygen, Carbon Monoxide, and Nitrogen on NADPH-Supported Lipid Peroxidation in Epidermal Microsomes

The relationship between oxygen consumption and lipid peroxidation has been established [1–3]. To evaluate the role of oxygen in augmentation of lipid peroxidation of epidermal microsomes, NADPH-supported lipid peroxidation was carried out under room air and under atmospheres of oxygen, carbon monoxide, or nitrogen. In the presence of oxygen, lipid peroxide formation increased substantially (70%) over that observed in the presence of room air. Nitrogen and carbon monoxide each markedly reduced lipid peroxidation (data not shown). This suggested a critical requirement for oxygen in lipid peroxide formation in epidermal microsomes.

Effect of Scavengers of Active Oxygen Species on Lipid Peroxide Formation by Epidermal Microsomes

Previous studies have demonstrated that O_2^-, OH , H_2O_2, and $^1O_2$, each can initiate lipid peroxide formation in microsomes [3,4,18]. We therefore investigated the effects of scavengers of

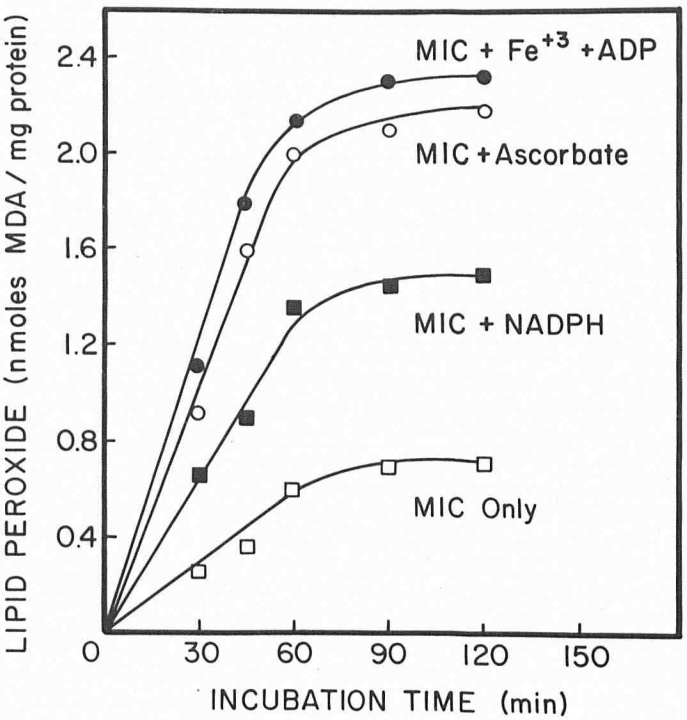

FIG 3. Enzymic and nonenzymic lipid peroxidation in epidermal microsomes as a function of time of incubation. Assay system consisted of 0.1 M phosphate buffer, pH 7.4, NADPH (1 mM) or ascorbate (1 mM) or Fe^{+3}–ADP (5 mM) and 1.6–2.0 mg epidermal microsomal protein. The reaction was carried out at 37°C for the times indicated in the figure. MDA formation was estimated as described in the text.

these reactive oxygen species on lipid peroxidation in skin microsomes. The data presented in Table III indicate that superoxide dismutase (SOD) which catalyzes the elimination of $O_2^-(O_2^- + O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$ had no effect on NADPH-supported enzymic lipid peroxide formation [19]. The enzyme also had no protective effect on the generation of lipid peroxides in either the Fe^{+3}–ADP or ascorbate-supported incubation system. Catalase which is an active scavenger of $H_2O_2(2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2$ provided significant protection against NADPH as well as Fe^+–ADP or ascorbate-supported lipid peroxide formation (Tables III, IV). MDA production in NADPH or Fe^+–ADP plus NADPH or the ascorbate-supported peroxidation systems was decreased as much as 50–60% at concentrations of 200 μg/ml and 500 μg/ml of catalase. Since xanthine oxidase produces both O_2^- and H_2O_2 during aerobic oxidation of xanthine, we investigated whether SOD or catalase had any effect on lipid peroxide formation in this system. As is evident from Table V, lipid peroxide formation in epidermal microsomes was substantially augmented by the addition of xanthine oxidase. SOD had no protective effect whereas addition of catalase significantly inhibited lipid peroxide formation (Table V).

Effect of Scavengers of Hydroxyl Radical and Singlet Oxygen on Lipid Peroxidation of Epidermal Microsomes

Since $OH^\cdot$ and $^1O_2$ are known to trigger lipid peroxide formation in microsomes, we next attempted to assess the effect of scavengers of these moieties on lipid peroxide formation. As evident from Table VI, none of the scavengers of $OH^\cdot$ including benzoate, mannitol, and ethyl alcohol afforded significant protection against lipid peroxide formation in epidermal microsomes [20,21]. Dimethylfuran and histidine, which are potent scavengers of $^1O_2$ [4,22,23], protected against the generation of lipid peroxides in the NADPH-supported system (Table VII) indicating that $^1O_2$ is also associated with the generation of lipid peroxides in epidermal microsomes.

TABLE III. *Effect of superoxide dismutase (SOD) on NADPH and ascorbate-induced lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.61 ± 0.12
+ SOD (25 μg/ml)	1.42 ± 0.13
+ SOD (50 μg/ml)	1.31 ± 0.11
+ catalase (200 μg/ml)	1.03 ± 0.03[a]
+ catalase (500 μg/ml)	0.60 ± 0.04[a]
Complete − NADPH + ascorbate (1 mM)	3.62 ± 0.20
+ SOD (25 μg/ml)	3.42 ± 0.30
+ SOD (50 μg/ml)	3.33 ± 0.16
+ catalase (200 μg/ml)	1.12 ± 0.09[a]
+ catalase (500 μg/ml)	0.81 ± 0.04[a]

Values for malondialdehyde (MDA) were obtained following incubation of microsomes in the following manner for 60 min at 37°C. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.3–1.8 mg microsomal protein, and 1 mM NADPH. Each observation is the mean ± SE of 3–4 experiments.

[a] $p < 0.01$.

TABLE IV. *Effect of superoxide dismutase (SOD) and catalase on lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.53 ± 0.13
Complete + Fe^{+3} (1 mM) + ADP (5 mM) − NADPH (1 mM)	2.60 ± 0.22
Complete + Fe^{+3} (1 mM) + ADP (5 mM)	4.90 ± 0.36
+ SOD (25 μg/ml)	4.72 ± 0.29
+ SOD (50 μg/ml)	4.71 ± 0.26
+ catalase (200 μg/ml)	3.10 ± 0.18[a]
+ catalase (500 μg/ml)	2.01 ± 0.17[a]
+ heated catalase	4.70 ± 0.30
+ heated SOD	4.65 ± 0.25
+ bovine serum albumin (500 μg/ml)	4.70 ± 0.26

Values for malondialdehyde (MDA) were obtained following incubation of the microsomal suspensions at 37°C for 60 min. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1 mM NADPH, and 1.2–1.9 mg epidermal microsomal protein. Data are collected for blanks which contained all the reactants excepting NADPH. Each observation is the mean ± SE of 4 experiments.

[a] $p < 0.01$.

Inhibition of NADPH-Supported Lipid Peroxidation in Epidermal Microsomes by Cytochrome c^{+3}, Mn^{+2}, and EDTA

The addition of cytochrome c^{+3} or EDTA to the complete incubation system inhibited MDA production by 50–60% (Table VII). These results are in agreement with those reported for liver and lung [23–26]. The inhibitory effect of cytochrome c^{+3} may be due to its ability to compete with cytochrome P-450 for reducing equivalents generated by NADPH-cytochrome c reductase. Inhibition of MDA production in skin microsomes by EDTA could be due to chelation of iron that is required for lipid peroxidation. The concentration-dependent inhibition of lipid peroxidation by Mn^{+2} could reflect formation of salt with the double bonds of lipids making it more resistant to peroxidative attack [23,27].

Effect of Epidermal Cytosol and Glutathione on Lipid Peroxidation of Epidermal Microsomes

Since the cytosolic fraction of cells contains a variety of substances that can influence lipid peroxidation in microsomes [28], we investigated the role of cytosolic proteins and glutathione on the production of epidermal microsomes. Addition of glutathione to the incubation system significantly stimulated

TABLE V. *Effect of superoxide dismutase (SOD) on xanthine oxidase-catalyzed lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.60 ± 0.21
+ Fe^{+3}-ADP	3.31 ± 0.41
+ Fe^{+3}-ADP + xanthine oxidase system[a]	6.49 ± 0.72
+ Fe^{+3}-ADP + xanthine oxidase system + SOD (40 μg/ml)	6.56 ± 0.51
+ Fe^{+3}-ADP + xanthine oxidase system + SOD (80 μg/ml)	6.50 ± 0.63
+ Fe^{+3}-ADP + xanthine oxidase system + catalase (200 μg/ml)	3.50 ± 0.32[b]
+ Fe^{+3}-ADP + xanthine oxidase system + catalase (400 μg/ml)	3.10 ± 0.15[b]
+ Fe^{+3}-ADP + catalase (400 μg/ml)	1.56 ± 0.11

Values for malondialdehyde (MDA) were obtained following incubation of epidermal microsomes at 37°C for 60 min. Complete system (1.0 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.6–2.2 mg microsomal protein, and 1 mM NADPH. Additions to the complete system were done as indicated. Each observation is the mean ± SE of 4 experiments.

[a] Xanthine (1.5 mM) + xanthine oxidase (200 μg/ml).

[b] $p < 0.01$.

TABLE VI. *Effect of scavengers of hydroxyl radicals on NADPH-supported lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	4.29 ± 0.33
+ benzoate (5 mM)	4.20 ± 0.26
+ benzoate (10 mM)	4.30 ± 0.27
+ mannitol (5 mM)	4.20 ± 0.25
+ mannitol (10 mM)	4.17 ± 0.22
+ EtOH (5 mM)	3.78 ± 0.31
+ EtOH (10 mM)	3.71 ± 0.28

Values for malondialdehyde (MDA) were obtained following incubation of epidermal microsomes at 37°C for 60 min. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.3–1.8 mg microsomal protein, 1 mM NADPH, Fe^{+3} (1 mM), and ADP (5 mM). The numbers are mean values of 4 experiments ± SE.

TABLE VII. *Effect of quenchers of singlet oxygen on lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.26 ± 0.10
+ histidine (5 mM)	0.69 ± 0.05[a]
+ histidine (10 mM)	0.50 ± 0.04[a]
+ dimethylfuran (5 mM)	0.79 ± 0.06[a]
+ dimethylfuran (10 mM)	0.60 ± 0.05[a]
+ cytochrome c^{+3} (0.1 mM)	0.75 ± 0.07
+ EDTA (1 mM)	0.90 ± 0.08
+ Mn^{+2} (1 mM)	0.85 ± 0.06
+ Mn^{+2} (5 mM)	0.55 ± 0.03

Values for malondialdehyde (MDA) were obtained by incubating microsomes at 37°C for 60 min. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.6–2.0 mg microsomal protein, and 1 mM NADPH. Data represent the mean ± SD of 4 experiments.

[a] $p < 0.05$.

the formation of lipid peroxides in microsomes (Table VIII). The addition of both the epidermal cytosol and glutathione had no further additive effect on MDA production in epidermal microsomes. Glutathione and cytosol had similar effects when either NADPH or Fe^{+3}–ADP was present in the incubation system.

Effect of Vitamin E and β-Carotene on Lipid Peroxidation

Antioxidants such as vitamin E and the carotenoids (β-carotene) are known to protect against lipid peroxidation of cellular membranes [1,29]. The results presented in Table IX

TABLE VIII. *Effect of glutathione and epidermal cytosol on NADPH- and Fe^{+}–ADP-mediated lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.62 ± 0.15
+ glutathione (1 mM)	2.50 ± 0.16
+ glutathione (5 mM)	3.08 ± 0.21
+ glutathione (5 mM) + cytosol (0.5 mg)	3.25 ± 0.18
Complete + Fe^{+3} (1 mM) + ADP (5 mM)	4.90 ± 0.36
Complete + Fe^{+3} + ADP + glutathione (5 mM)	6.70 ± 0.46
Complete + Fe^{+3} + ADP + glutathione (5 mM) + cytosol (0.5 mg)	6.85 ± 0.55

Values for malondialdehyde (MDA) were obtained by incubating microsomes at 37°C for 60 min. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.4–1.8 mg microsomal protein, and 1 mM NADPH. Each observation is the mean ± SE of 4 experiments.

TABLE IX. *Effect of β-carotene and α-tocopherol on NADPH-dependent lipid peroxidation in epidermal microsomes*

Incubations	Lipid peroxide formation (nmol MDA/mg protein/60 min)
Complete	1.26 ± 0.08
+ β-carotene (5 μM)	0.53 ± 0.03
+ β-carotene (10 μM)	0.39 ± 0.03
+ α-tocopherol (5 μM)	0.73 ± 0.05
+ α-tocopherol (10 μM)	0.59 ± 0.03
+ EtOH (20 μl)	1.21 ± 0.02

Values for malondialdehyde (MDA) were obtained by incubating microsomes at 37°C for 60 min. The complete system (0.6 ml) consisted of 0.1 M phosphate buffer, pH 7.4, 1.4–1.8 mg microsomal protein, and 1 mM NADPH. β-Carotene and α-tocopherol were suspended in 20 μl of 100% EtOH. Each observation is the mean ± SE of 3 experiments.

show that β-carotene, a potent quencher of singlet oxygen, afforded substantial protection against lipid peroxide formation in epidermal microsomes. Vitamin E (α-tocopherol), which interrupts free radical chain reactions, caused a 60% decrease in lipid peroxide formation when added at a concentration of 10 μM.

DISCUSSION

The data presented in this paper demonstrate that polyunsaturated lipids of skin microsomes undergo active peroxidation in the presence of oxygen and one or several catalysts. NADPH and certain nonenzymic catalysts such as Fe^{+3}–ADP and ascorbate can significantly enhance lipid peroxide formation in skin microsomes. Furthermore, our results indicate that the epidermis is a major target site in skin for the peroxidation of microsomal lipids. Enzyme-mediated peroxidation required NADPH and was critically dependent upon oxygen. Addition of the nonenzymic catalysts, particularly Fe^{+3}–ADP, to the NADPH-supported peroxidation system augmented the generation of lipid peroxides. Our results further suggest that lipid peroxidation of epidermal microsomes is strongly inhibited by quenchers of $^1O_2$ such as histidine, dimethylfuran, and β-carotene. Hydroxyl ion scavengers such as mannitol, benzoate, and ethanol had no significant effect on epidermal lipid peroxidation. SOD, a scavenger of the superoxide radical, had no effect on lipid peroxidation, whereas catalase that inactivates hydrogen peroxide significantly decreased lipid peroxide formation in epidermal microsomes.

The inhibition of lipid peroxidation by singlet oxygen quenchers such as dimethylfuran, histidine, and β-carotene strongly suggests that these agents protected against membrane peroxidation by scavenging $^1O_2$. Although the formation of $^1O_2$ in biologic systems is highly controversial (for review see

[1,30]), the inhibition of lipid peroxidation by quenchers of singlet oxygen is clearly suggestive of its participation in lipid peroxidation occurring in the skin.

EDTA, Mn^{+2}, and cytochrome c^{+3} each has been shown to inhibit lipid peroxidation in liver [26,31] and lung [23] microsomes. These agents also strongly inhibited enzyme and NADPH-supported lipid peroxidation in skin microsomes. It is possible that EDTA could inhibit lipid peroxidation by chelating Fe^{+3}. It is also possible that Mn^{+2} forms a salt complex with the unsaturated double bonds of lipids, making them less vulnerable to peroxidative attack. The inhibition of lipid peroxidation by cytochrome c^{+3} could relate to its competition with cytochrome P-450 for reducing equivalents from the reductase. Similar mechanisms for the inhibitory actions of these agents in liver and lung microsomes have also been suggested [23,26,31]. Our results indicate the likely involvement of cytochrome P-450 and NADPH cytochrome P-450 reductase in epidermal microsomal lipid peroxidation. In this regard previous studies from this laboratory have demonstrated the presence of NADPH-cytochrome P-450 reductase and cytochrome P-450 in epidermal microsomes [13,14].

It is known that the xanthine-xanthine oxidase system produces H_2O_2 and O_2^- by the Haber-Weiss reaction [32]. Since SOD was ineffective whereas catalase significantly inhibited the peroxidation reaction, it is possible that H_2O_2 served as a major agent which initiates or propagates peroxidation rather than O_2^-. This further strengthens our hypothesis that H_2O_2 and singlet oxygen can initiate lipid peroxidation in epidermal microsomes in the presence of either membrane-bound Fe^{+3} (cytochrome P-450) and/or externally added chelated Fe^{+3}.

Cytosolic factors are also known to alter the generation of peroxides in liver and lung microsomes. The enhancing effect of cytosol could be due, among other factors, to the presence of either glutathione or ascorbate or both. Addition of glutathione or ascorbate to the NADPH-supported microsomal peroxidation system accelerated the formation of lipid peroxides. Inhibition of microsomal lipid peroxidation in skin by vitamin E and by β-carotene suggest that these agents have substantial antioxidant effects in cutaneous tissue. This is of interest since β-carotene is known to have a photoprotective effect in the human disease erythropoietic protoporphyria. Other studies have shown that porphyrin photosensitivity in the skin is likely mediated by lipid peroxidation [11]. Our findings demonstrating the inhibitory effect of β-carotene on lipid peroxidation in epidermal microsomes support that hypothesis.

In conclusion, the data presented in this study suggest (i) lipid peroxidation occurs in skin and is an ongoing process that could participate in a variety of processes, (ii) singlet oxygen, H_2O_2, O_2^- and $OH^\cdot$ may be generated in skin microsomes either as a result of NADPH-cytochrome P-450 reductase activity or due to the presence of Fe^{+3} and ADP or xanthine oxidase, (iii) singlet oxygen and H_2O_2 are possibly the major mediators of the initiation or propagation of lipid peroxide formation in skin microsomes, (iv) catalase and singlet oxygen quenchers including histidine and β-carotene and α-tocopherol may provide important defenses against peroxidation of epidermal microsomes, and (v) epidermal cytosolic factors can augment lipid peroxide formation in epidermal microsomes. Since skin is directly exposed to UV radiation and to a wide variety of xenobiotics known to generate activated oxygen radicals, it is highly likely that these moieties are a major cause of certain forms of toxic damage in cutaneous tissue.

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REFERENCES

1. Bus JS, Gibson JR: Lipid peroxidation and its role in toxicology, Reviews in Biochemical Toxicology, vol 1. Edited by E Hodgson, JR Bend, RM Philpot. Amsterdam, Elsevier-North Holland, 1979, pp 125–149
2. Recknagel RO, Glende EA Jr: Lipid peroxidation: a specific form of cellular injury, Handbook of Physiology—Reactions to Environmental Agents. Edited by DHK Lee. Bethesda, Maryland, American Physiological Society, 1977, pp 591–601
3. Kellogg EWM III, Fridovich I: Superoxide, hydrogen peroxide and singlet oxygen in lipid peroxidation by xanthine oxidase system. J Biol Chem 250:8812–8817, 1975
4. King MM, Lai EK, McCay PB: Singlet oxygen production associated with enzyme catalyzed lipid peroxidation in liver microsomes. J Biol Chem 250:6496–6501, 1975
5. Bickers DR, Kappas A: The skin as a site of chemical metabolism, Extrahepatic Metabolism of Drugs and Foreign Compounds. Edited by TE Gram. Jamaica, New York, Spectrum Publications, 1980, pp 295–318
6. Serri F, Tosti A, Cerimele D, Fazzini ML, Villardita S, Compagano G: Studies on pathomechanism of chronic actinic dermatosis, Research in Pathobiology. Edited by A Castellani. New York/London, Plenum Press, 1977, pp 91–121
7. Serri F, Bartoli GM, Seccia A, Borrello S, Galeotti T: Age related mitochondrial lipoperoxidation in human skin. J Invest Dermatol 73:123–125, 1979
8. Kizaki H, Matsuo I, Sakurada T: Xanthine oxidase and guanase activities in normal and psoriatic epidermis. Clin Chim Acta 75:1–4, 1977
9. Aso K, Rabinowitz I, Farber EM: The role of prostaglandin E, cyclic AMP and cyclic GMP in proliferation of guinea pig ear skin stimulated by topical application of vitamin A acid. J Invest Dermatol 67:231–234, 1976
10. Lowe NJ, Stoughton RB: Effects of prostaglandin E_2 analogue on normal hairless mouse epidermal DNA synthesis. J Invest Dermatol 68:134–137, 1977
11. Harber LC, Bickers DR: Drug induced photosensitivity, Photosensitivity Diseases. Edited by LC Harber, DR Bickers. Philadelphia, Saunders, 1981, pp 189–223
12. Dixit R, Mukhtar H, Bickers DR: Evidence that lipid peroxidation in microsomal membranes of epidermis is associated with generation of hydrogen peroxide and singlet oxygen. Biochem Biophys Res Commun 105:546–552, 1982
13. Bickers DR, Dutta-Choudhury T, Mukhtar H: Epidermis: a site of drug metabolism in neonatal rat skin: studies on cytochrome P-450 content and mixed function oxidase and epoxide hydrolase activity. Mol Pharmacol 21:239–247, 1982
14. Mukhtar H, Bickers DR: Drug metabolism in skin: comparative activity of mixed function oxidases, epoxide hydrolase and glutathione-S-transferase in liver and skin of neonatal rat. Drug Metab Dispos 9:311–314, 1981
15. Epstein EH Jr, Munderloh NL, Fukuyama K: Dithiothreitol separation of newborn rodent dermis and epidermis. J Invest Dermatol 73:207–210, 1979
16. Wright JR, Colby HD, Miles PR: Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Arch Biochem Biophys 206:296–304, 1981
17. Utley HG, Bernheim F, Hochstein P: Effect of sulfhydryl reagents on peroxidation in microsomes. Arch Biochem Biophys 118:29–32, 1967
18. McCay PB, Poyer JL: Enzyme catalyzed free radicals as initiators of lipid peroxidation in biological membranes, The Enzymes of Biological Membranes. Edited by A Martonosi. New York, Plenum Press, 1976, vol 4, pp 239–246
19. Fridovich I: The biology of oxygen radicals. Science 201:875–880, 1978
20. Plaa GL, Witschi M: Chemicals, drugs and lipid peroxidation. Ann Rev Pharmacol Toxicol 16:125–141, 1976
21. Dorfman LM, Adams GE: Reactivity of the Hydroxyl Radical in Aqueous Solutions, National Bureau of Standards, Publication 46, Washington, DC, 1973
22. Fridovich I: Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J Biol Chem 245:4053–4057, 1970
23. Misra HP, Gorsky LD: Paraquat and NADPH dependent lipid peroxidation in lung microsomes. J Biol Chem 256:9994–9998, 1981
24. Matheson IBC, Etheridge DR, Kratowich NR, Lee J: The quenching of singlet oxygen by amino acids and proteins. Photochem Photobiol 21:165–171, 1975
25. Pryor WA: Free radical reactions and their importance in biochemical systems. Fed Proc 32:1862–1870, 1972
26. May HE, McCay PB: Reduced triphosphopyridine nucleotide oxidase catalyzed alteration of membrane phospholipids I. Nature of lipid alterations. J Biol Chem 243:2296–2305, 1968
27. Misra HP, Fridovich I: The univalent reduction of oxygen by reduced flavins and quinanes. J Biol Chem 247:188–192, 1972
28. Hochstein P, Ernster L: ADP-activated lipid peroxidation coupled to TPNH oxidase system of microsomes. Biochem Biophys Res

Commun 12:388–394, 1963
29. Demopoulos HB: The basis of free radical biology. Fed Proc 32:1859, 1973
30. Glende EA Jr, Recknagel RO: Biochemical basis for the in vitro prooxidant action of carbon tetrachloride. Exp Mol Pathol 11:172–185, 1969
31. Fong K-L, McCay PB, Poyer JL, Keele BB, Misra H: Evidence that peroxidation of lysosomal membranes is initiated by hydroxyl free radicals produced during flavin enzyme activity. J Biol Chem 248:7792–7797, 1973
32. Haber F, Weiss J: The catalytic decomposition of hydrogen peroxide by ion salts. Proc R S Edinburgh [A] 147:332–351, 1934

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A Role for Collagen Phagocytosis by Fibroblasts in Scar Remodeling: An Ultrastructural Stereologic Study

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A role for collagen phagocytosis and intracellular degradation by fibroblasts during remodeling activity has been suggested by studies on several connective tissues characterized by high rates of collagen turnover and remodeling. The possible importance of such activity in the normal remodeling of scar tissue has been studied by a quantitative ultrastructural stereologic measure of collagen phagocytosis by fibroblasts at various postwounding intervals in mouse skin scars. The results demonstrate a correlation between the peak periods of such phagocytic activity and the interval during which collagen fiber reorientation across the scar appears to take place.

Scar tissue is commonly conceived of as an inert mass of collagen representing the terminus of the healing process. However the scar is in fact a dynamic, metabolically active tissue that normally undergoes a series of maturational changes which are as yet incompletely understood. While the absolute amount of collagen reaches a maximum at 2–3 weeks [1,2], a progressive increase in tensile strength can be measured for 1 year or more postwounding. This increase has been attributed in part to an increased degree of covalent cross-linkages and transformation of these cross-linkages to more stable forms in the collagen molecule [3] and to a turnover and remodeling of collagen as demonstrated with biochemical [2] and scanning electron microscopic [4,5] techniques. This remodeling brings about a reorientation of fiber direction in scar tissue.

Remodeling of scar tissue demands degradation and synthesis of collagen. Over the past decade a number of studies have suggested that in connective tissues with high rates of collagen turnover, the fibroblast degrades this protein by a process of phagocytosis [6–15]. The purpose of this study was to quantitate the amount of fibroblast collagen phagocytosis occurring during scar tissue formation and maturation and to attempt to correlate this with the qualitative changes in the scar.

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MATERIALS AND METHODS

Ten young-adult male hairless mice (strain HRF-J) were used. Following ether anesthesia, longitudinal 2-cm incisions were made with fine-pointed scissors in 8 animals through dorsal skin and panniculus carnosis muscle to the right of midline extending caudally from the scapular region. The wounds were closed with surgical tape (3-M Co. Ltd.). Two animals served as controls.

The animals were sacrificed in pairs after 1 week, 3 weeks, 2 months, and 4 months postwounding by ether overdose. Wound tissue was easily distinguished at all time intervals and was excised with 2-mm margins of surrounding skin. Samples of unwounded skin were obtained from the pair of control animals. All specimens were bisected, with one half taken for light microscopic study and the other for study with the electron microscope.

For light microscopy each specimen was further bisected and processed using conventional technique. The blocks were sectioned parallel to the epidermis and perpendicular to the epidermis. Resulting sections were stained with hematoxylin and eosin and with Gomori's silver strain.

Specimens for electron microscopy were trimmed to produce 1-mm^3 blocks of tissue from the center of the scar or control tissue. The blocks were fixed by 5-h immersion in cold (4°C) 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. Following postfixation in 2% osmium tetroxide for 1 h, each block was dehydrated in graded ethanol solutions, embedded with a random orientation in a gelatin capsule containing Epon, and allowed to polymerize at 60°C for 3 days prior to sectioning.

One block was randomly selected and trimmed for fine sectioning from each animal and 70- to 90-nm sections were cut with a diamond knife mounted in an MT-2 Porter-Blum ultramicrotome. Each section was mounted on a 400-mesh copper grid, double-stained with uranyl acetate and lead citrate, and examined with a Philips EM-200 transmission electron microscope.

Systematic random sampling of each section was achieved by establishing the convention that the upper right-hand corner of each grid square was photographed [14]. Fields containing epithelial, vascular, nerve, or muscle tissue were excluded. Forty electron micrographs were obtained at random in this manner from one section from each animal and printed at a final magnification of 25,640.

The resulting prints were analyzed by a stereologic point counting method [14,16]. A coherent double lattice printed on a transparent acetate sheet was superimposed in register over each print. The coarse-grid pattern (13 × 13 mm) of the lattice was employed to determine the number of intersection points falling on either cell cytoplasm or extracellular collagen. Since the volume density [16] of phagocytosed collagen was expected to be of a smaller order of magnitude than that of cytoplasm or extracellular collagen, the measurement sensitivity was enhanced by using the fine-grid pattern (1.3 × 1.3 mm) of the lattice