Symposium

Papers from the symposium on
Cancer — A molecular event

The papers appearing here as a unit publication were presented at a special symposium held late in 1983 at Lake Geneva, Wisconsin. Its purpose was to review and discuss lipid oxidation, antioxidants and selenium as factors modifying the process of carcinogenesis. National and international experts presented data dealing with these issues during the 2½ day meeting. The data presented indicated that intakes of lipid, antioxidants and selenium all are factors that modify the incidence of cancer in human beings and in experimental models. Possible mechanisms by which these factors modify susceptibility to cancer also were addressed. About 150 persons representing the fields of nutrition, chemistry, biochemistry, medicine, food technology and other, more specialized disciplines, attended the conference. We also would like to thank the American Oil Chemists' Society for its generous support. Furthermore the generous contributions from Hoffman LaRoche, Campbell Soup Co., Best Foods-CPC, the Eastman Chemical Products Health and Nutrition Division, and the Archer-Daniels Midland Company were of great assistance to the success of the conference.

John A. Milner
Edward G. Perkins,
Chairmen

URL 05432

Anticarcinogenic Effect of Selenium in the Dimethylbenz(a)anthracene-Induced Mammary Tumor Model in Rats

CLEMENT IP, Department of Breast Surgery and Breast Cancer Research Unit, Roswell Park Memorial Institute, Buffalo, NY 14263

ABSTRACT

Using the dimethylbenz(a)anthracene-induced mammary tumor model in rats, our studies indicated that there was a dose-response relationship between dietary selenium supplementation and the inhibition of mammary carcinogenesis. The degree of inhibition was proportional to the level of dietary selenium up to 5 ppm, at which point toxicity in the form of a reduction in weight gain was evident. Moreover, it was observed that the chemopreventive efficacy of selenium was influenced by the dose of carcinogen as well as the fat intake of the animals. By supplementing selenium for defined periods of time, we concluded that selenium inhibited both the initiation and the promotion phases of chemical carcinogenesis, and that a continuous intake of selenium was necessary to achieve maximal suppression of tumor growth. In an attempt to improve the efficacy of lower levels of selenium, we conducted another series of experiments in which selenium and vitamin E were tested in combination. Results showed that although vitamin E alone had no prophylactic effect against tumorigenesis, it potentiated the ability of selenium to inhibit the development of mammary tumors. Further investigation suggested that the anticarcinogenic action of selenium could not be explained by its antioxidant function in lipid peroxidation. On the other hand, vitamin E might be able to provide a more favorable environment against oxidant stress to assist selenium in exerting its inhibitory effect through some other mechanisms.

INTRODUCTION

There is increasing evidence that selenium has a protective effect against tumorigenesis in laboratory animals. References to current experimental reports in the literature concerning selenium and cancer have been summarized by Dr. Shamberger in this conference. Most of these studies involve the use of inorganic selenium supplements either in the drinking water or in the diet at a concentration ranging from 0.5 to 6 ppm (mg/kg). These levels are considerably higher than the nutritional requirement of about 0.1 ppm established by the NRC for animals. A comparison of the results from several laboratories indicates that mice may be more sensitive to selenium inhibition of tumorigenesis than rats.

The breast cancer models that have been shown to be

responsive to selenium chemoprevention include both virus- and chemical carcinogen-induced mammary tumors (8,9,12, 18,21,22,26,27,32,33,35,36). Since selenium is effective in suppressing mammary neoplastic development induced by both methylnitrosourea and dimethylbenz(a)anthracene, it is unlikely that the primary action of selenium is exerted via changes in carcinogen metabolism. A report from Medina's laboratory (21) indicates that selenium markedly inhibits mammary tumorigenesis in BALB/cfC3H mice (MuMTVS positive), but has little effect on the incidence of neoplastic transformation in preneoplastic outgrowth lines or the growth rate of primary mammary tumors transplanted subcutaneously in BALB/c mice (MuMTVS negative). Thus there seems to be a decreasing sensitivity to selenium mediated inhibition as cells progress from normal to preneoplastic to neoplastic. This is in contrast to 2 other reports in which selenium was found to be effective in retarding the growth of a canine mammary tumor line in athymic nude mice (34) and the MT-W9B transplantable mammary tumor in W/F rats (10).

Our work has involved primarily the mammary tumor model induced by 7,12-dimethylbenz(a)anthracene (DMBA) in female Sprague-Dawley rats. The studies described in the present paper were designed to address the following questions. (a) What are the factors that influence the anticarcinogenic efficacy of selenium? (b) Are the different types of lesions found in the mammary gland subsequent to carcinogen administration equally sensitive to selenium inhibition? (c) How does the time and duration of selenium supplementation affect tumorigenesis? (d) Is selenium cytotoxic when present at high levels? (e) Can the chemopreventive effectiveness of selenium be improved by combining it with another agent? (f) Is the anticarcinogenic action of selenium related to its function in regulating the activity of selenium-dependent glutathione peroxidase? Details of the experimental protocol have been published previously (6,8,9,12). In all our studies, selenium in the form of sodium selenite was added to semi-purified synthetic diets.

Anticarcinogenic Efficacy of Selenium: Influence of Fat Intake and Carcinogen Dosage

The objective of the first experiment is to test the effect of graded levels of dietary selenium on mammary carcinogenesis, and to determine if the optimal level of supplementation depends on the dose of the carcinogen and the fat intake of the animals. Table I shows the effect of various levels of selenium supplementation on tumorigenesis in rats that were fed either a 5% or a 25% corn oil diet and given either 5 or 10 mg of DMBA at 50 days of age (7). Selenium supplementation of both diets was started from weaning and continued until the end of the experiment 22 weeks after DMBA administration. Mammary tumor pathology is defined according to the criteria of Young and Hallowes (38). In rats treated with DMBA at 50 days of age, over 90% of the tumors obtained are adenocarcinomas. Only adenocarcinomas are reported unless otherwise stated.

At 0.1 ppm of selenium, which is considered to meet the nutritional requirement of rats (control level), tumor incidence was higher in the 25% fat group than in the 5% fat group. We found that selenium had to be raised to 1.5 ppm before its chemopreventive effect became noticeable. The degree of inhibition was proportional to the level of dietary selenium up to 5 ppm, at which point a slight reduction in weight gain (about 10%) was evident. This decrease in growth was due to a lower food intake. Pair-feeding experiments, however, indicated that reduced food consumption alone was not sufficient to account for the striking suppression of tumorigenesis in those rats treated with 5 ppm of selenium (results not shown). In general, the selenium-mediated inhibitory responses included a lower tumor incidence, a reduction in tumor yield and a longer latency period. It should be noted that the anticarcinogenic efficacy of selenium was diminished by a larger dose of carcinogen. Moreover, selenium was unable to counteract completely the enhancing effect of fat in mammary carcinogenesis, since rats on a high-fat diet still developed more tumors than those on a low-fat diet at comparable levels of selenium supplementation.

TABLE I

Effect of Selenium Supplementation on DMBA-Induced Mammary Tumorigenesis in Rats Fed Either a 5% or a 25% Corn Oil Diet

Experiment	Dietary group	Selenium in diet (ppm)	Initial body wt.[a] (g)	Final body wt. (g)	Tumor incidence	Total no. of tumors	Average latency period[b] (days)
A 5 mg DMBA	5% fat	0.1	152 ± 2[c]	290 ± 6[c]	12/30 (40.0%)	26	92 ± 7[c]
		0.5	150 ± 2	292 ± 6	11/30 (36.7%)	23	89 ± 6
		1.5	151 ± 3	289 ± 6	9/31 (29.0%)	19	97 ± 7
		2.5	150 ± 3	288 ± 7	7/29 (24.1%)	10	109 ± 8
	25% fat	0.1	153 ± 2	294 ± 6	21/30 (70.0%)	65	85 ± 6
		0.5	155 ± 3	290 ± 6	20/29 (68.9%)	66	86 ± 6
		1.5	151 ± 2	290 ± 7	16/29 (55.2%)	41	92 ± 7
		2.5	151 ± 3	288 ± 7	10/30 (33.3%)	21	106 ± 7
B 10 mg DMBA	5% fat	0.1	154 ± 3	294 ± 7	21/30 (70.0%)	71	71 ± 6
		2.5	155 ± 3	290 ± 7	13/29 (44.8%)	32	81 ± 6
		5.0	139 ± 3	259 ± 6	7/30 (23.3%)	15	95 ± 7
	25% fat	0.1	156 ± 2	289 ± 6	30/30 (100%)	135	65 ± 5
		2.5	155 ± 3	290 ± 7	23/30 (76.7%)	85	73 ± 6
		5.0	142 ± 3	255 ± 6	16/29 (55.2%)	46	88 ± 7

[a]At the time of DMBA administration.
[b]Time between DMBA administration and the appearance of the first palpable tumor.
[c]Mean ± S.E.

URL 05433

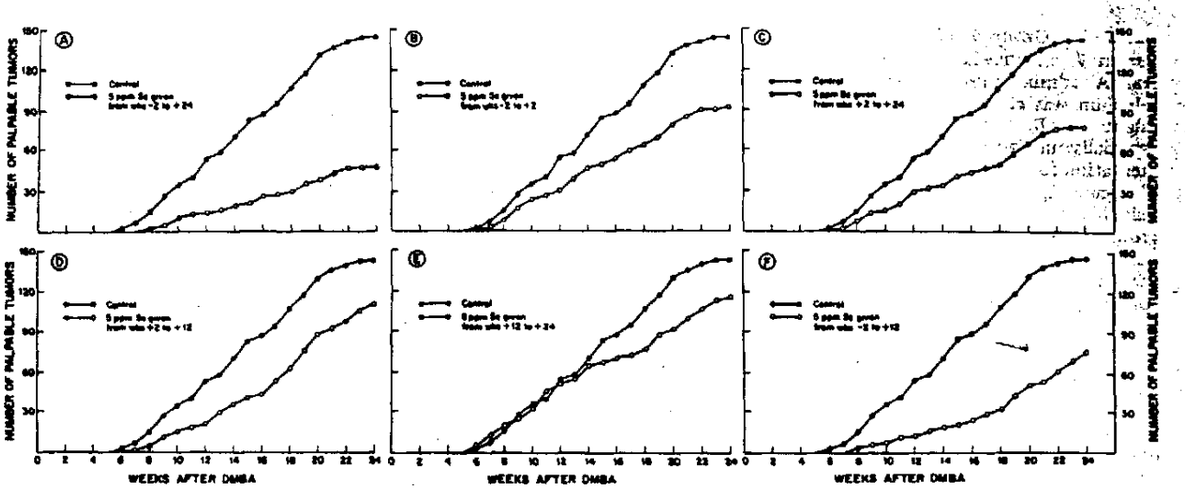


FIG. 1. Effect of selenium supplementation (5 ppm) for various periods of time on the development of palpable mammary tumors. The time of DMBA administration (50 days of age) was taken as 0; minus and plus signs represent the time in weeks before and after DMBA administration. The schedule of selenium supplementation is indicated in panels A to F. The control group (0.1 ppm selenium) is reproduced in each panel for comparison. There were 35 rats/group.

Effect of Selenium Supplementation on the Development of Hyperplastic Alveolar Nodules (HANs) and the Formation of Adenocarcinomas and Fibroadenomas in Older Rats

HANs are a form of dysplasia in the mammary gland produced subsequent to carcinogen treatment. Normally they can be detected before the appearance of palpable tumors. There is, however, some controversy as to the precancerous nature of these lesions in the rat model. In the present experiment, these nodules were identified and counted in stained whole mount preparation of the mammary gland (2) obtained from rats that were killed 8 weeks after DMBA (5 mg dose given at 50 days of age). The average number of HANs found per rat was 18.0 ± 3.6 in the 0.1 ppm selenium control group and 7.1 ± 1.6 in the 2.5 ppm selenium supplemented group ($P < 0.05$). Further investigation is necessary to evaluate the usefulness of this system in assessing the inhibitory effect of selenium in the early stages of neoplastic transformation.

Age is an important factor in the induction of mammary cancer in rats (1,4,23,28). The animals are most susceptible to carcinogenesis between 50-60 days of age and become more and more resistant as they get older. Consequently, the incidence is very low in rats treated with DMBA when they are over 100 days old. Moreover, there is a proportionate increase in fibroadenoma formation in this experimental model. By feeding animals a high fat diet and using a pulse-dose protocol, we were able partially to overcome the resistance of these older rats to mammary tumorigenesis induced by DMBA (7).

We were interested to find out whether selenium was equally effective in inhibiting the development of adenocarcinomas and fibroadenomas in rats that were maintained on a 20% corn oil diet and were given DMBA when they were 120 days of age. A multiple dose schedule was adopted with the administration of 5 mg of DMBA per week for 4 consecutive weeks. Selenium supplementation (2.5 ppm in the diet) was initiated immediately after the first dose of DMBA. Animals were killed 24 weeks after the last dose. Results in Table II show that the number of adenocarcinomas was reduced by 50% in the selenium-treated group. Interestingly, this was not accompanied by a comparable

TABLE II

Effect of Selenium Supplementation on Induction of Mammary Adenocarcinomas and Fibroadenoma in Adult Female Rats

Dietary selenium (ppm)	No. of rats	No. of adenocarcinomas	No. of fibroadenomas
0.1	30	29	18
2.5	30	14	16

Rats were given 5 mg of DMBA per week for 4 consecutive weeks; the first dose was given when the rats were 120 days of age.

suppression of fibroadenoma formation. These lesions generally appeared later in the course of the experiment. It is unclear at this time whether the immunogenicity or pathogenesis of the different tumor types have an effect on their responsiveness to selenium inhibition.

Prophylaxis of Mammary Neoplasia by Selenium Supplementation

In the first experiment described above, selenium was given for the entire duration of the study, and it was not possible to ascertain at which time point selenium was most effective in cancer chemoprevention. In order to answer this question, we conducted a new series of experiments in which the effect of selenium supplementation during the initiation and promotion (or proliferation) phases of DMBA-induced mammary carcinogenesis was examined.

In this experiment, 245 rats were divided randomly into 7 groups of 35 each. All were fed a high fat ration (25% corn oil) since diets rich in fat are known to promote the development of mammary neoplasia. Control rats in Group 1 received 0.1 ppm of selenium, while Groups 2 to 7 were supplemented with 5 ppm of selenium in the diet for various periods of time as indicated below. The time of DMBA administration (50 days of age) was taken as 0; minus and plus signs represent the time in weeks before and after DMBA administration (10 mg), respectively. The schedule of selenium treatment in Groups 2 to 7 was as follows: Group 2, −2 to +24; Group 3, −2 to +2; Group 4,

URL 05434

+2 to +24; Group 5, +2 to +12; Group 6, +12 to +24; and Group 7, −2 to +12. All rats were killed 24 weeks after DMBA administration. The reason for using 5 ppm of selenium was that we were afraid the inhibitory response might not be detected with a lower level of selenium, especially in those groups that received selenium supplementation for only a short period of time.

Figure 1, panels A to F, shows the time course of palpable mammary tumor development in the different groups. The control group (Group 1) is reproduced in each panel for comparison. Table III summarizes the final tumor incidence and the total tumor yield in Groups 1 to 7. The following conclusions can be drawn after careful analysis of the data. (a) A continuous intake of selenium is necessary to achieve maximal inhibition of tumorigenesis, such as in rats that were supplemented with selenium for the longest period of time (−2 to +24 weeks, Fig. 1A; Group 2 in Table III). (b) Selenium can inhibit both the initiation and promotion phases of carcinogenesis. This is suggested by the observation that a decrease in tumorigenesis was evident when selenium was supplemented either around the time of DMBA administration (−2 to +2 weeks, Fig. 1B; Group 3 in Table III) or during the proliferation phase of tumor development (+2 to +24 weeks, Fig. 1C; Group 4 in Table III). (c) The inhibitory effect of selenium in the early promotion phase probably is reversible, since we found that the chemopreventive response was severely diminished when selenium supplementation was limited from +2 to +12 weeks (Fig. 1D; Group 5 in Table III). (d) In rats that were supplemented with selenium from +12 to +24 weeks, there was only an insignificant reduction in the number of tumors found (Fig. 1E; Group 6 in Table III), suggesting that the efficacy of selenium is much attenuated when it is given long after carcinogenic injury. It should be pointed out that the schedule of dividing the promotion phase into 2 parts was an arbitrary one and should not be construed as the distinction of 2 separate events with identifiable phenotypic manifestation, but rather as a temporal relationship in terms of tumor development (early versus late).

TABLE III

Effect of Selenium Supplementation (5 ppm in the Diet) for Various Periods of Time on DMBA-Induced Mammary Tumorigenesis

Group[a]	Period of selenium supplement[b] (week)	Tumor incidence	Total no. of tumors	% of inhibition
1	none	97.1%	152	–
2	−2 to +24	45.7%	52	65.8%
3	−2 to +2	71.6%	101	33.5%
4	+2 to +24	68.5%	89	41.4%
5	+2 to +12	85.7%	126	17.1%
6	+12 to +24	91.4%	124	18.4%
7	−2 to +12	57.1%	80	47.4%

[a]Rats were given 10 mg of DMBA intragastrically. There were 35 rats per group.

[b]The time of DMBA administration was taken as 0; minus and plus signs represent the time in weeks before and after DMBA administration, respectively.

TABLE IV

Effect of Selenium Treatment In Vitro on Labeling Index of Mammary Explants Cultured with DMBA and on Subsequent Tumorigenesis in W/F Rats Following Transplantation

Selenium treatment[a]	Labeling index in explants[b]	Rats with tumors[c]
None	15.3 ± 3.1%	12/25
10^{-6} M	13.5 ± 2.4%	10/25
5×10^{-6} M	12.8 ± 2.0%	8/25
10^{-5} M	8.7 ± 1.4%	4/25
5×10^{-5} M	3.2 ± 0.5%	2/25

[a]Selenium in the form of sodium selenite was used.

[b]Mammary explants were incubated with DMBA (1 μg/ml) in the presence of insulin, estradiol, progesterone and prolactin for the first 3 days. On the fourth day, the culture was replenished with fresh hormone-supplemented medium but without DMBA. ^{3}H-Thymidine was added to the culture on day 6. Labeling was allowed to continue for 24 hr before the explants were fixed for autoradiography.

[c]Mammary explants were transplanted in the subscapular fat pad of isologous hosts using day 7 culture.

Cytotoxic Effect of High Levels of Selenium

In order to better evaluate if high levels of selenium have any cytotoxic effect, we proceeded to use the DMBA-treated mammary transplant technique in which organ cultures were incubated with different concentrations of selenium (as sodium selenite) before grafting to hosts for observation of tumorigenesis. Details of this procedure have been described previously (11). Mammary explants from female W/F rats were exposed to DMBA (1μg/ml) in the presence of insulin, estradiol, progesterone and prolactin for the first 3 days. On the fourth day, fresh hormone-supplemented medium without DMBA was replenished and the culture was continued for 3 more days. Selenium was present during the entire period of the culture. Explants were then transplanted in the subscapular fat pad of isologous hosts. Results are shown in Table IV.

When selenium in the culture was increased from 10^{-6} to 5×10^{-5} M there was a gradual inhibition of tumorigenesis following transplantation of the DMBA-treated mammary explants. Only 2 out of 25 rats developed tumors upon receiving the transplants that had been exposed to 0.05 mM of selenium in the medium. In contrast, 12 out of 25 rats in the control group developed tumors (no added selenium in the medium). The proliferative activity of the culture also was determined immediately before transplantation. Tritiated thymidine was added to the medium on day 6. Labeling was allowed to continue for 24 hr before the explants were fixed for autoradiography (13). It can be seen from Table IV that high levels of selenium markedly suppressed DNA synthesis in the culture. Those explants that had a low proliferative rate also had a low potential to develop into tumors when grafted to the recipients. The present finding thus provides a model to study the cytotoxic effect of selenium and its chemopreventive action in the initiation phase of neoplastic transformation.

Improvement of Selenium Chemoprevention by Combination with Vitamin E

Since high levels of selenium (e.g. 5 ppm) lead to a slight depression in growth of the animals, we have been trying to improve the anticarcinogenic efficacy of lower levels of selenium by combining it with other agents. Our experience with vitamin E proved to be most promising. The rationale for selecting vitamin E is two-fold. First, selenium and vitamin E share in common the role of endogenous antioxidants. Second, there is ample evidence in the literature which shows that they have a sparing effect on each other in the prevention of several nutritional deficiency diseases.

In this experiment, rats were fed a 20% corn oil diet containing 0.1 ppm selenium and 50 mg vitamin E per kg of diet (NRC recommended requirement). Additional selenium (2.5 ppm) and vitamin E (DL-α-tocopheryl

URL 05435

acetate, 1,000 mg/kg of diet) were tested singly and in combination. Rats were maintained on a high polyunsaturated fat diet, thus enabling us to evaluate the efficacy of the vitamin E and selenium combination treatment under a more vigorous condition of oxidant stress. Selenium was supplemented in the diet for the entire duration of the experiment, while additional vitamin E was present for various lengths of time, depending on the experimental design. The reason for adopting this protocol is that we have found previously that a continuous intake of selenium is necessary to achieve a maximal inhibitory response. By supplementing vitamin E for a defined period either around the time of or after DMBA administration, we can examine the effect of vitamin E during the initiation and promotion phases of mammary carcinogenesis.

In the first animal carcinogenicity study, both selenium and vitamin E were added to the diet starting 2 weeks before DMBA administration (10 mg at 50 days of age) and continued until the animals were sacrificed 25 weeks later. Figure 2 illustrates the per cent incidence of rats with palpable tumors as a function of time in the 4 experimental groups. Selenium supplementation (Group 2) led to a modest reduction compared to the controls (Group 1); the difference, however, was not statistically significant. Vitamin E by itself had no effect (Group 3), but a combination of selenium and vitamin E resulted in the only significant inhibitory response (Group 1 *vs* Group 4, $P < 0.05$).

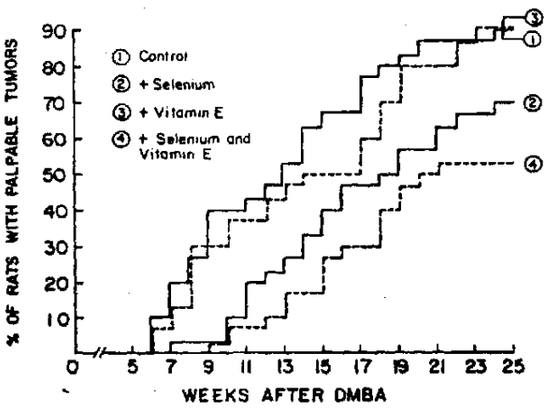


FIG. 2. Effect of selenium and/or vitamin E supplementation on the cumulative palpable mammary tumor incidence in rats fed a 20% corn oil diet. The control diet contained 0.1 ppm of selenium and 50 mg of vitamin E per kg of diet. Additional selenium and vitamin E were present at 2.5 ppm and 1,000 mg/kg of diet, respectively.

Table V summarizes the total tumor yield and the data on the number of tumors per tumor-bearing rat and the time of first tumor appearance. Rats supplemented with selenium produced fewer tumors (90 in Group 2 *vs* 132 in Group 1, $P < 0.01$), whereas those given vitamin E did not manifest any meaningful reduction (Group 3). In contrast, rats supplemented with both selenium and vitamin E developed the least number of tumors (Group 4), with a tally even lower than that of the selenium-supplemented group (difference between Group 2 and Group 4 was statistically significant, $P < 0.05$). These observations suggested that vitamin E, although ineffective by itself, was able to potentiate the anticarcinogenic action of selenium.

We decided to ascertain if vitamin E exerted its effect on the initiation or promotion phase of DMBA-induced mammary carcinogenesis. In the second experiment, vitamin E was tested only in combination with selenium. Selenium was supplemented in the diet from −2 to +24 weeks, while vitamin E was supplemented for different periods of time: −2 to +24 weeks, −2 to +2 weeks, and +2 to +24 weeks. Results in Table VI show that vitamin E enhanced the prophylactic effect of selenium only when it was present in the post-initiation or promotion phase (Groups 3 and 5). Supplementation with vitamin E around the time of DMBA administration (−2 to +2 weeks) produced no beneficial effect (Group 4).

Effect of Selenium and/or Vitamin E on Lipid Peroxidation and Glutathione Peroxidase Activity

In view of the well known antioxidant property of both selenium and vitamin E, we proceeded to investigate their effects on the peroxidative potential of the mammary tissue. Lipid peroxidation was measured by the thiobarbituric acid method (24). The principal reactant is considered to be malondialdehyde (MDA), which is produced by lipid

TABLE V

Effect of Selenium and/or Vitamin E Supplementation on DMBA-Induced Mammary Carcinogenesis

Group[a]	Dietary supplement[b]	Rats with tumors[c]	Tumor incidence[d] (%)	Total no. of tumors[e]	Tumors per tumor-bearing rat[f]	Latency period[g] (wk)
1	None	28	93	132	4.7 ± 0.4	12.2 ± 1.0
2	Selenium (Se)	23	77	90	3.9 ± 0.4	15.0 ± 1.1
3	Vitamin E	27	90	122	4.5 ± 0.3	13.7 ± 1.0
4	Se + Vit E	18	60	60	3.3 ± 0.3	15.7 ± 0.9

In this experiment, both selenium and vitamin E were supplemented starting 2 weeks before DMBA administration and continued until the animals were sacrificed.

[a]There were 30 rats per group. All rats received 10 mg DMBA i.g. at 50 days of age and were killed 25 weeks later.

[b]Selenium (Se) and/or vitamin E (Vit E) were supplemented in the diet at a concentration of 2.5 mg/kg and 1,000 mg/kg, respectively.

[c]Include rats with nonpalpable tumors discovered at autopsy.

[d]Only Group 4 is statistically different from Group 1 ($P < 0.05$).

[e]Groups 2 and 4 are different from Group 1 ($P < 0.01$). Group 4 is different from Group 2 ($P < 0.05$).

[f]Values are expressed as mean ±S.E. Only Group 4 is different from Group 1 ($P < 0.05$).

[g]Latency period is denoted as the time between DMBA administration and the appearance of the first palpable tumor. Values are expressed as mean ±S.E. Groups 2 and 4 are different from Group 1 ($P < 0.05$).

URL 05436

C. IP

TABLE VI

Effect of Selenium and/or Vitamin E Supplementation on DMBA-Induced Mammary Carcinogenesis

Group[a]	Dietary supplement[b]	Duration of vitamin E supplementation[c] (wk)	Rats with tumors[d]	Tumor incidence[e] (%)	Total no. of tumors[f]	Tumors per tumor-bearing rat[g]	Latency period[h] (wk)
1	None	–	23	92	113	4.9 ± 0.4	10.1 ± 1.0
2	Selenium (Se)	–	18	72	73	4.1 ± 0.3	12.3 ± 1.1
3	Se + Vit E	−2 to +24	12	48	36	3.0 ± 0.3	13.7 ± 1.1
4	Se + Vit E	−2 to +2	19	76	66	3.5 ± 0.4	12.8 ± 1.0
5	Se + Vit E	+2 to +24	14	56	40	2.9 ± 0.3	12.5 ± 1.1

In this experiment, additional selenium was present in the diet for the entire duration of the study in Groups 2 to 5, while vitamin E was present for different periods of time.

[a]There were 25 rats per group. All rats received 10 mg DMBA i.g. at 50 days of age and were killed 24 weeks later.

[b]Selenium (Se) and/or vitamin E (Vit E) were supplemented in the diet at a concentration of 2.5 mg/kg and 1,000 mg/kg, respectively. Additional selenium was supplemented starting 2 weeks before DMBA administration and continued until the end of the experiment. Vitamin E was present for different periods of time as indicated in column 3.

[c]The time of DMBA administration was taken as time 0; minus and plus signs represent the time in weeks before and after DMBA administration, respectively.

[d]Includes rats with nonpalpable tumors discovered at autopsy.

[e]Only Groups 3 and 5 are statistically different from Group 1 ($P < 0.01$).

[f]Groups 2, 3, 4 and 5 are all different from Group 1 ($P < 0.05$). Groups 3 and 5 are different from Group 2 ($P < 0.02$).

[g]Values are expressed as mean ±S.E. Groups 3, 4 and 5 are different from Group 1 ($P < 0.02$). Groups 3 and 5 are different from Group 2 ($P < 0.05$).

[h]Latency period is denoted as the time between DMBA administration and the appearance of the first palpable tumor. Values are expressed as mean ±S.E. Only Group 3 is different from Group 1 ($P < 0.05$).

TABLE VII

Effect of Selenium and/or Vitamin E Supplementation on Lipid Peroxidation and Selenium-Dependent Glutathione Peroxidase (GSH-Px) Activity in the Mammary Fat Pad

Group[a]	Dietary supplement[b]	Lipid peroxidation[c]	Se-dependent GSH-Px[d]
1	None	355 ± 30	44 ± 3
2	Selenium (Se)	320 ± 28	52 ± 4
3	Vitamin E	142 ± 12[e]	41 ± 3
4	Se + Vit E	121 ± 10[e]	54 ± 4

[a]There were 8 rats per group. All rats received 10 mg DMBA i.g. at 50 days of age and were killed 2 mo later.

[b]Selenium and/or vitamin E (Vit E) were supplemented in the diet at a concentration of 2.5 mg/kg and 1,000 mg/kg, respectively. Both selenium and vitamin E supplementations were started 2 weeks before DMBA administration and continued until the animals were sacrificed.

[c]Values are expressed as nmol MDA formed/g tissue, mean ±S.E.

[d]Hydrogen peroxide was used as the substrate to assay for the Se-dependent GSH-Px activity. Values are expressed as nmol NADPH oxidized/min/mg protein, mean ±S.E.

[e]Statistically different from Groups 1 and 2 ($P < 0.001$).

peroxidation and released upon heating the sample in an acid medium. Results in Table VII show that vitamin E significantly suppressed peroxidation, whereas selenium had no effect. A combination of selenium and vitamin E did not result in further inhibition compared to vitamin E alone. With respect to the selenium-dependent glutathione peroxidase activity as measured by the coupled assay of Paglia and Valentine (25), selenium supplementation produced only a slight but insignificant increase. This suggests that in control rats receiving 0.1 ppm of selenium, the enzyme already is operating at near maximal capacity. Additional selenium will not further increase its activity, since the enzyme protein becomes the limiting factor.

DISCUSSION

The present study confirms previous findings by other investigators that selenium supplementation above dietary requirement inhibits tumorigenesis. In addition, our observations lead to the conclusion that the optimal level of selenium for manifestation of this protective effect depends on the dose of the carcinogen and the nutritional status of the animal, specifically in relation to fat intake. Higher selenium supplementation is necessary to neutralize the insult produced by a larger dose of DMBA. Moreover, we found that selenium was unable to counteract completely the enhancing effect of dietary fat in mammary carcinogenesis, since rats fed a high fat diet still developed more tumors than those fed a low fat diet at comparable selenium intake level. By supplementing selenium for defined lengths of time, we showed that selenium can inhibit both the initiation and promotion phases of carcinogenesis and that a continuous intake of selenium is necessary to achieve maximal inhibition of tumorigenesis.

In the present study, we found that additional selenium supplement failed to increase glutathione peroxidase activity in the mammary tissue. Selenium is an integral component of this enzyme which is involved in the destruction of hydroperoxides (5). Our observation is in agreement with the report by Lane and Medina (14). These results suggest that the anticarcinogenic action of selenium may not be mediated by its antioxidant function via glutathione peroxidase. Vitamin E is a much more potent antioxidant than selenium. Our experiment using a combination of vitamin E and selenium indicates that although the suppression of lipid peroxidation by vitamin E alone is not sufficient to inhibit tumor formation, vitamin E may provide a favorable environment against oxidant stress to facilitate selenium in exerting its anticarcinogenic action through some other mechanisms.

Little information is available on the mode of action of selenium. Reports from Griffin's laboratory showed that selenium impedes activation and accelerates detoxification of 2-acetylaminofluorene (3,16). Other evidence that supports a role for selenium in the initiation phase include protection of liver DNA against single-strand breaks induced by 2-acetylaminofluorene (37) and facilitation of the repair process (15). Pharmacological levels of seleni

URL 05437

have been reported to potentiate the immune response of the host (29-31). Exposure to high concentrations of selenium is known to inhibit DNA synthesis (20) such that cells are blocked in the S-G2 phase of the cell cycle (17). A modulation of mitochondrial function by selenium also has been suggested as one of the early effects of growth inhibition (19). It is likely that selenium may be acting through several mechanisms. Any working hypothesis concerning the mode of action of selenium should accommodate the observation that selenium inhibits both virus- and chemical carcinogen-induced tumors and that it is effective during the proliferative or promotion phase of tumorigenesis.

ACKNOWLEDGMENT

This work was supported by grant CA 27706 from the National Cancer Institute, NIH.

REFERENCES

1. Dao, T.L., Science 165:810 (1969).
2. Dao, T.L.; D. Sinha, S. Christakos and R. Varela, Cancer Res. 35:1128 (1975).
3. Daoud, A.H., and A.C. Griffin, Cancer Lett. 5:231 (1978).
4. Haslam, S.Z., Int. J. Cancer 23:374 (1979).
5. Hoekstra, W.G., Fed. Proc. 34:2083 (1975).
6. Horvath, P.M., and C. Ip, Cancer Res. 43, In press.
7. Ip, C., Ibid. 40:2785 (1980).
8. Ip, C., Ibid. 41:2683 (1981).
9. Ip, C., Ibid. 41:4386 (1981).
10. Ip, C.; M.M. Ip and U. Kim, Cancer Lett. 14:101 (1981).
11. Ip, C., and D. Sinha, Ibid. 11:277 (1981).
12. Ip, C., and D. Sinha, Carcinogenesis 2:435 (1981).
13. Koyama, H.; D. Sinha and T.L. Dao, J. Natl. Cancer Inst. 48:1671 (1972).
14. Lane, H.W., and D. Medina, Cancer Res. 43:1558 (1983).
15. Lawson, T., and D. Birt, Proc. Am. Assoc. Cancer Res. 22:93 (1981).
16. Marshall, M.V.; M.S. Arnott, M.M. Jacobs and A.C. Griffin, Cancer Lett. 7:331 (1979).
17. Medina, D.; H.W. Lane and C.M. Tracey, Cancer Res. 43:2460 (1983).
18. Medina, D.; H. Lane and F. Shepherd, Anticancer Res. 1:377 (1981).
19. Medina, D.; F. Miller, C.J. Oborn and B.B. Asch, Cancer Res. 43:2100 (1983).
20. Medina, D., and C.J. Oborn, Cancer Lett. 13:333 (1981).
21. Medina, D., and F. Shepherd, Ibid. 8:241 (1980).
22. Medina, D., and F. Shepherd, Carcinogenesis 2:451 (1981).
23. Meranze, D.R.; M. Gruenstein and M.B. Shimkin, Int. J. Cancer 4:480 (1969).
24. Ohkawa, H.; N. Ohishi and K. Yagi, Anal. Biochem. 95:351 (1979).
25. Paglia, D.E., and W.N. Valentine, J. Lab. Clin. Med. 70:158 (1967).
26. Schrauzer, G.N.; J.E. McGinness and K. Kuehn, Carcinogenesis 1:199 (1980).
27. Schrauzer, G.N.; D.A. White and C.J. Schneider, Bioinorg. Chem. 8:387 (1978).
28. Sinha, D., and T.L. Dao, J. Natl. Cancer Inst. 64:519 (1980).
29. Spallholz, J.E.; J.L. Martin, M.L. Gerlach and R.H. Heinzerling, Proc. Soc. Exp. Biol. Med. 143:685 (1973).
30. Spallholz, J.E.; J.L. Martin, M.L. Gerlach and R.H. Heinzerling, Infect. Immun. 8:841 (1973).
31. Spallholz, J.E.; J.L. Martin, M.L. Gerlach and R.H. Heinzerling, Proc. Soc. Exp. Biol. Med. 148:37 (1975).
32. Thompson, H.J., and P.J. Becci, J. Natl. Cancer Inst. 65:1299 (1980).
33. Thompson, H.J.; L.D. Meeker, P.J. Becci and S. Kokoska, Cancer Res. 42:4954 (1982).
34. Watrach, A.M.; J.A. Milner and M.A. Watrach, Cancer Lett. 15:137 (1982).
35. Welsch, C.W.; M. Goodrich-Smith, C.K. Brown, H.D. Greene and E.J. Hamel, Carcinogenesis 2:519 (1981).
36. Whanger, P.D.; J.A. Schmitz and J.H. Exon, Nutrition & Cancer 3:240 (1982).
37. Wortzman, M.S.; H.J. Besbris and A.M. Cohen, Cancer Res. 40:2670 (1980).
38. Young, S., and R.C. Hallowes, in Pathology of Tumors in Laboratory Animals, ed., V.S. Turusov, Vol. 1, Tumors of the Rat, Part 1. Lyon:IARC (1973), p. 31.

[Received June 20, 1984]

URL 05438

Role of Lipids in Tumorigenesis

K.K. CARROLL[1], Department of Biochemistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1

ABSTRACT

High fat diets are associated with increased mortality from cancer at various sites, including breast, colon, rectum, prostate, ovary and pancreas. Additional evidence for this association has been obtained for some sites (e.g. mammary gland and colon) by studies on experimental animals. Dietary polyunsaturated fats increase the yield of mammary tumors in rats more effectively than saturated fats, apparently because of their higher content of essential fatty acids. Saturated and polyunsaturated fats are about equally effective in enhancing the yields of colon tumors in rats. Dietary fat appears to act as a promoter rather than an initiator of tumorigenesis, although the exact mechanisms of action are not known. Mammary tumorigenesis in rats can be inhibited by reducing the level of dietary fat after a period of promoting with a high fat diet. Decreasing the present high levels of fat in American diets might help to reduce cancer incidence and mortality.

INTRODUCTION

Evidence has been accumulating in recent years that diet, particularly dietary lipids, plays an important role in tumorigenesis. This evidence has been reviewed in a number of publications, most of which consist of proceedings of symposia or workshops (1-6). A comprehensive review of diet, nutrition and cancer also has been prepared recently by a Committee of the Assembly of Life Sciences, National Research Council (7). This report has stimulated considerable controversy, some of which has been published by the Council for Agricultural Science and Technology (8).

On the basis of their review of the literature, the Committee on Diet, Nutrition, and Cancer concluded "that of all the dietary components it studied, the combined epidemiological and experimental evidence is most suggestive for a causal relationship between fat intake and the occurrence of cancer." The purpose of the present article will be to review briefly some of the evidence on which this conclusion was based. Further details may be found in the original report (7) and in other publications referred to above (1-6).

EPIDEMIOLOGICAL DATA

Intercountry comparisons have shown positive correlations between the amount of fat available for consumption and mortality from cancer at various sites in the body, including breast, colon, rectum, prostate, ovary and pancreas (9). Data for breast and colon are illustrated in Figure 1 (10). Cancer at other sites, such as liver and stomach, is not positively correlated with dietary fat, but cancers that do show this positive correlation are responsible for a large proportion of cancer deaths in the United States and Canada (11).

The data on which such correlations are based are subject to many inaccuracies, but it seems clear that mortality from cancers of the breast and colon is much lower in a country like Japan than in the United States, since good records are maintained in both countries. Furthermore, differences such as these appear to be related to environment rather than heredity, since mortality from breast cancer and colon cancer in Japanese immigrants in the United States increases with time until it approaches that of the American population as a whole. Similar shifts in mortality patterns have been observed in other immigrant populations (12).

[1]Career Investigator of the Medical Research Council of Canada.

STUDIES ON EXPERIMENTAL ANIMALS

The existence of a positive correlation between cancer mortality and dietary fat does not necessarily mean that they are causally related. However, supporting evidence has come from studies on experimental animals. Mammary cancer develops more readily in animals fed high fat diets compared to those fed low fat diets (9,10), and similar observations have been made for intestinal tumors (13). Recent studies also have indicated that dietary fat can influence the development of pancreatic tumors in rats (14).

In our studies on mammary tumorigenesis, diets high in polyunsaturated fats produced a marked increase in tumor yield, compared to low fat diets or diets high in saturated fats (9). It appears that there is a requirement for essential fatty acids as well as for a high fat diet, and that the more saturated fats are ineffective because they contain insufficient amounts of essential fatty acids (15). The two requirements may be related, since at lower levels of dietary fat a higher degree of polyunsaturation was required to produce an increase in tumor yield (16). Dietary polyunsaturated fat also was observed to enhance the yield of pancreatic tumors more effectively than saturated fat (14), but the type of fat appears to be less imporant in the case of intestinal tumors (13).

In human populations, breast cancer mortality correlates best with total dietary fat intake (10,17), and it seems probable that the dietary fat in most countries would be sufficiently unsaturated to provide the necessary amounts of essential fatty acids. Thus, one might expect that the amount of fat in the diet would have a greater influence on breast cancer than the type of fat, as observed in the epidemiological data.

MECHANISM OF ACTION

Cancer is a multi-step process, and the evidence indicates that dietary fat affects the promotional stage rather than initiation of tumorigenesis (9,16). High fat diets increase the yield of spontaneous mammary tumors as well as those induced by various carcinogens, and there is no indication that the fat itself is acting as a carcinogen. The enhancement in tumor yield can still be observed when animals are fed a high fat diet only after treatment with a carcinogen capable of inducing mammary tumors (9).

The exact mechanism by which dietary fat affects tumorigenesis is not known, but a number of possibilities have been suggested (13,15,16,18). In the case of mammary tumors, this might involve an alteration in hormonal balance, whereas dietary fat may affect intestinal tumors by stimulating production of bile acids, some of which have been shown to act as tumor promoters (13). Other possibilities include effects on the composition and properties of cellular membranes, effects on the immune system, and increased production of peroxidized products or biologically active compounds, such as prostaglandins, derived from polyunsaturated fatty acids (15).

Whatever the mechanism, evidence that dietary fat acts as a promoter of carcinogenesis suggests that the process can be influenced at later stages by the fat content of the diet. Experiments with rats have, in fact, shown that mammary tumorigenesis can be inhibited by reducing the level of dietary fat after a period of promoting with a high fat

URL 05439

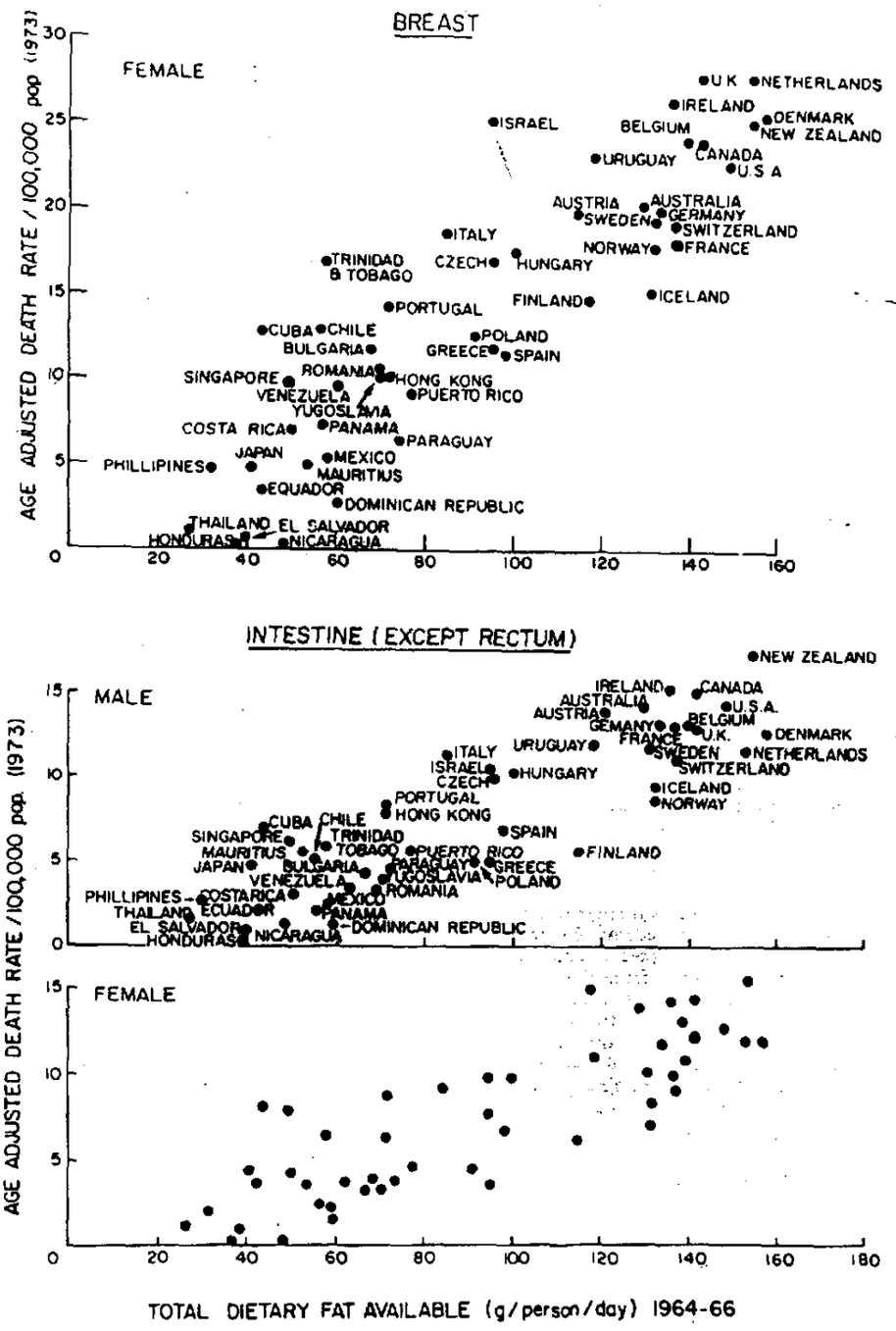


FIG. 1. Correlations between total dietary fat available and age-adjusted death rates from breast cancer and intestinal cancer. Reproduced from Carroll (10).

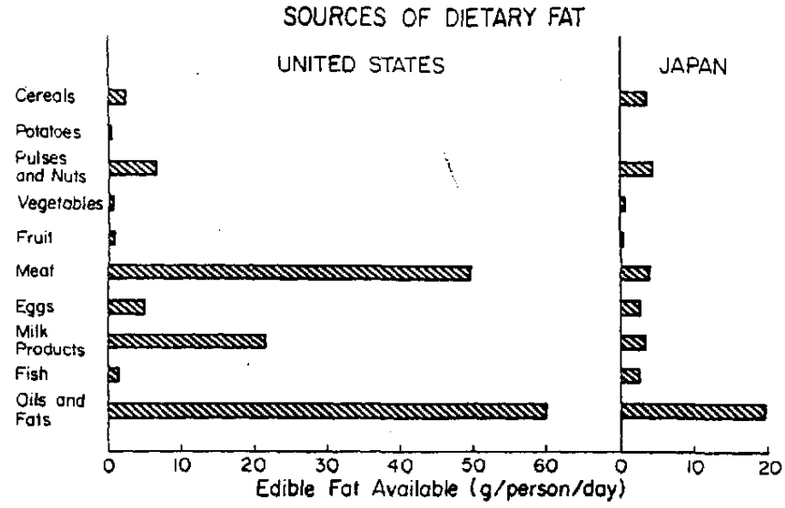


FIG. 2. Sources of available dietary fat in the United States and Japan. Reproduced from Carroll and Hopkins (21).

diet (16). A more recent study in our laboratory showed that decreasing the dietary fat from 20% to 10% by weight (i.e. from about 40% to 20% of calories) reduced the yield of tumors to about the same extent as complete removal of fat from the diet (19). If breast cancer in humans is similarly influenced by dietary fat, it might be possible to improve the prognosis of breast cancer patients by reducing their fat intake, with the aim of inhibiting development and proliferation of metasteses which constitute one of the major problems in breast cancer. In this connection, it is of interest that Japanese women not only have a lower incidence of breast cancer than American women, but also experience a lower recurrence rate when cancer develops (20).

SOURCES OF DIETARY FAT

In order to devise diets of lower fat content, information on the sources of dietary fat is required. As illustrated in Figure 2, visible fats and oils used as spreads, cooking fats and salad oils, are the largest source of fat in the American diet. The next major source is meat, but the amount indicated in the diagram may overestimate its contribution, since fat often is trimmed from meat and also can be lost during cooking. Dairy products are the only other major source of dietary fat in the United States diet. Foods such as eggs and nuts are relatively high in fat, but are not consumed in sufficient quantities to make a large contribution to the total intake (Fig. 2).

PROS AND CONS OF DECREASING FAT INTAKE

Aside from its possible effects on carcinogenesis, a reduction in dietary fat intake has been advocated for some time as a means of reducing the incidence of cardiovascular disese (22). There also have been suggestions that other chronic diseases of Western civilization may be largely due to consumption of a diet that is high in fat and relatively low in complex carbohydrate and fiber (23).

It is important to consider, however, whether there may be disadvantages in reducing dietary faty intake. The main function of dietary fat is as a source of calories, but it also provides essential fatty acids and fat-soluble vitamins, and facilitates absorption of fat-soluble vitamins from the gut.

There are some segments of the population, such as infants under one year of age, for whom a high fat diet is desirable. The rapid growth occurring at this stage of life requires a high caloric intake relative to body weight, and milk is relatively high in fat. It therefore would appear unwise to reduce the fat intake of infants.

Fat enhances the flavor and texture of food and by stimulating the appetite may help to maintain weight in elderly people or people with debilitating diseases, where weight loss is a problem. High fat diets also may be appropriate for people engaged in heavy manual labor or working in a cold environment, who thus require a relatively high caloric intake. For sedentary, middle-aged individuals, however, some reduction in the level of dietary faty probably is desirable and may decrease the likelihood of developing some of the chronic diseases, including cancer, that are common in our society.

Attempts to reduce dietary fat intake inevitably will involve eating less of some of the high fat foods referred to above. This could be achieved by using less fat as spreads and salad oils, reducing the amount of fat used in cooking, and eating less high fat meat and dairy products. It is important, however, to maintain a balanced diet in order to avoid deficiencies of required nutrients.

Normal humans eating a varied diet are unlikely to become deficient in essential fatty acids, but this possibilty still must be considered (24). Deficiencies of fat-soluble vitamins also are possible, but could be avoided by use of dietary supplements. Foods such as meat and dairy products contain many essential non-fatty nutrients and thus contribute to a well-balanced diet. As pointed out earlier, some high fat foods such as eggs, which also contain valuable essential nutrients, do not contribute unduly to the total fatty intake because they are consumed in relatively small amounts.

In closing, it should be emphasized that there are as yet insufficeient data to conclude that a reduction in dietary fat will lead to a decrease in cancer incidence and mortality, although the evidence points in that direction. More work is needed to assess the relative advantages and disadvantages of reducing the level of fat in the American diet. At pre-

URL 05441

sent, it appears that many individuals would benefit from a lower dietary fat intake, but care should be taken to achieve this by adjusting the relative proportions of low and high fat foods so as to maintain a well-balanced diet.

ACKNOWLEDGMENT

The National Cancer Institute of Canada supported this work.

REFERENCES

1. Workshop on Fat and Cancer, edited by D.J. Fink and D. Kritchevsky, Cancer Res. 41:3677 (1981).
2. IUNS Workshop on Nutrition and Cancer, Nutr. Cancer 2:197 (1981).
3. Nutrition and Cancer: Etiology and Treatment, edited by G.R. Newell and N.M. Ellison, Raven Press, New York, 1981.
4. Molecular Interrelations of Nutrition and Cancer, edited by M.S. Arnott, J. van Eys and Y.-M. Wang, Raven Press, New York, 1982.
5. Dietary Fats and Health, AOCS Monograph 10, edited by E.G. Perkins and W.J. Visek, American Oil Chemists' Socity, Champaign, IL, 1983.
6. Workshop Conference on Nutrition in Cancer Causation and Prevention, Cancer Res. Suppl. 43:2385s (1983).
7. Diet, Nutrition, and Cancer, Report of a Committee on Diet, Nutrition and Cancer, Assembly of Life Sciences, National Research Council, National Academy Press, Washington, DC, (1982).
8. Diet, Nutrition, and Cancer: A Critique, Special Publication No. 13, Council for Agricultural Science and Technology, Ames, IA, 1982.
9. Carroll, K.K., and H.T. Khor, Progr. Biochem. Pharmacol. 10:308 (1975).
10. Carroll, K.K., J. Environ. Pathol. Toxicol. 3(4):253 (1980).
11. World Health Statistics Annual, Vital Statistics and Causes of Death, World Health Organization, Geneva, 1980.
12. Gori, G.B. Diet and Nutrition in Cancer Causation. Nutr. Cancer 1:5 (1978).
13. Reddy, B.S., L.A. Cohen, G.D. McCoy, P. Hill, J.H. Weisburger and E.L. Wynder, Adv. Cancer Res. 32:237 (1980).
14. Roebuck, B.D., J.D. Yager Jr. and D.S. Longnecker, Cancer Res. 41:888 (1981).
15. Carroll, K.K. G.J. Hopkins, T.G. Kennedy and M.B. Davidson, Progr. Lipid Res. 20:685 (1981).
16. Carroll, K.K., The role of dietary fat in carcinogenesis, in Dietary Fats and Health, AOCS Monograph 10, edited by E.G. Perkins and W.J. Visek, American Oil Chemists' Society, Champaign, IL, 1983, p. 710.
17. Carroll, K.K., Cancer Res. 35:3374 (1975).
18. Hopkins, G.J., and C.E. West, Life Sci. 19:1103 (1976).
19. Carroll, K.K., and R. Kalamegham, Lipid components and cancer, in Environmental Aspects of Cancer: The Role of Macro and Micro Components of Foods, edited by E.L. Wynder, G.A. Leveille, J.H. Weisburger, and G.E. Livingston. Food Nutrition Press, Inc., Westport, CT, 1983, p. 101.
20. Wynder, E.L., and L.A. Cohen, Nutr. Cancer 3:195 (1982).
21. Carroll, K.K., and G.J. Hopkins, Lipids 14:155 (1979).
22. Rationale of the Diet-Heart Statement of the American Heart Association. Report of the AHA Nutrition Committee, Arteriosclerosis 2:177 (1982).
23. Burkitt, D.P., S. Afr. Med. J. 61:1013 (1982).
24. Holman, R.T., JAOCS 55:774A (1978).

[Received June 20, 1984]

URL 05442

The Biochemistry of Selenoproteins

ROGER A. SUNDE, Department of Nutrition and Food Science, College of Agriculture, University of Arizona, Tucson, AZ 85721

ABSTRACT

There currently are 7 known bacterial selenoenzymes. All but thiolase contain selenocysteine (Se-Cys), presumably at the active site, and all but thiolase catalyze oxidation-reduction reactions. Selenide appears to be a central intermediate in selenium (Se) metabolism in animals, and it may be the precursor used for formation of the Se-Cys moiety in glutathione peroxidase (GSH-Px). The incorporation of Se into GSH-Px appears to occur via a post-translational mechanism, but the nature and extent of Se-Cys formation in higher animals has not been established. GSH-Px deficiency remains a logical explanation for a number of Se-deficiency signs, but other known selenoproteins and other functions may match up with defects apparently not prevented by GSH-Px.

INTRODUCTION

Many of the topics and the compounds discussed in this symposium in association with carcinogenesis reappear when considering the biochemistry of selenium. The discovery in the early 1970's that Se was an integral part of the enzyme GSH-Px (1) provided new support for the antioxidant theory of vitamin E function and, along with the discovery of superoxide dismutase (2), suggested that oxidative and/or free radical attack on cellular components may be responsible for the toxicity of a number of drugs. Some of these prooxidant or procancerous species are even more toxic when administered to Se-deficient animals, indicating that the biochemical functions of Se may be related to the processes involved in carcinogenesis.

Some of the anti-cancer activity of Se undoubtedly is related to the cytotoxicity of inorganic Se. The pharmacological effects of Se excess will not be the subject of this article. Instead, this article will review the biochemistry of Se and the effects of Se deficiency in animals as it relates to the biochemical functions of Se. The main sections of this review will discuss (1) bacterial selenoenzymes, (2) Se-Cys and GSH-Px, (3) Se metabolism, (4) GSH-Px function, (5) some of the selenoproteins and (6) functions of Se in animals apparently not related to GSH-Px. Recent reviews in related areas of Se biochemistry are available (3-6).

The story concerning Se essentiality in the US began in the late 1940's, when Klaus Schwarz came to the US from Germany. In Germany he had been studying dietary liver necrosis, a disease that could be prevented by either dietary vitamin E or the sulfur amino acids (7). After arriving in the US, he found that rats fed diets based on American brewer's yeast did not develop this disease. Rats fed torula yeast-based diets, however, developed liver necrosis in 3 to 4 weeks. Schwarz then isolated the organic factor present in American brewer's yeast that would protect against dietary liver necrosis, calling it factor-3 (8). In 1957 he identified Se as the crucial component in factor-3 (9). Factor-3 never has been fully characterized, but Schwarz and coworkers (10) have reported that several dialkyl diselenides have activities equivalent to factor-3 and were more active than

inorganic selenite.

Mills (11) discovered an enzyme in red blood cells (RBC) that would protect the cell against peroxidative damage. GSH-Px destroys hydrogen peroxidase and in the process oxidizes 2 molecules of glutathione (GSH) to GSSG. A protective sequence in the RBC and other cells uses glucose, glucose-6-phosphate dehydrogenase and glutathione reductase to maintain intracellular GSH concentrations (12). Adequate intracellular GSH is necessary, because the V_{max} of GSH-Px is first-order with respect to GSH (13). Little and O'Brien (14) showed that GSH-Px, unlike catalase, also would destroy hydroperoxides; this ability makes GSH-Px a unique peroxidase within animal cells. Scott et al. (15) showed that Se was essential for the growth of the chick but, as with the rat, only when the diet was deficient in vitamin E. The demonstration that Se was essential in the face of adequate dietary vitamin E came in 1969 when Thompson and Scott (16) reported that chicks, fed a diet deficient in Se but supplemented with vitamin E, developed a degeneration of the pancreas. Se alone was shown to be essential for that rat in 1969 (17).

Bacterial Selenoproteins

Pinsent (18) reported that selenite along with molybdate was necessary for the optimum development of formate dehydrogenase activity when *E. coli* was grown aerobically in nitrate-free media even though Se and molybdenum (Mo) had no effect on growth. In hindsight, this was the first suggestion of a Se-dependent enzyme. At latest count, 7 bacterial proteins have been identified as selenoenzymes.

Formate Dehydrogenase. Formate dehydrogenase of *E. coli* was shown in 1972 (19) to be a Se-containing enzyme. This enzyme catalyzes the oxidation of formate to CO_2 with the concomitant reduction of nitrate to nitrite. With a molecular weight of 600,000 daltons, formate dehydrogenase has four 110,000 dalton subunits each containing one atom of Se. The Se has been shown to be present as Se-Cys (20). In addition to 4 g-atom of Se, each mole of the enzyme also contains 4 g-atom of Mo, 56 g-atom of nonheme iron (Fe), 53 g-atom of acid-labile sulfur (S), and 4 moles of cytochrome b (21). A number of slightly different formate dehydrogenases have been identified from different bacteria, including some that do not require Se (5).

Glycine Reductase. The second bacterial enzyme identified as a selenoenzyme was glycine reductase from Clostridia (22). Glycine reductase has 3 types of subunits including a 12,000 dalton Se-containing subunit called protein A. The other 2 subunits are membrane-bound; Fe co-purifies with one of these subunits (23). Glycine reductase, as well as formate dehydrogenase, is inhibited by the thiol specific reagents iodoacetate and iodoacetamide (22,21). Cone et al. (24) used these reagents to alkylate Se, and demonstrated that the Se in protein A was present as Se-Cys. The enzyme catalyzes the reductive deamination of glycine to acetate and ammonia, and also involves a substrate-level phosphorylation of ADP (25). A variety of donor substrates can provide reducing equivalents for the reaction. The enzyme presumably goes through a selenophosphate intermediate (5), and thus the nuclear spin of ^{77}Se-labeled glycine reductase might help researchers to identify the intermediates in phosphorylation reactions.

After a gap of several years, a number of other bacterial enzymes were identified as Se-containing enzymes. In none of these cases, however, did bacteria grown under usual conditions produce sizable quantities of the selenoenzymes, nor did Se deficiency impair growth of these organisms in typical media. It is only when one critically selects the media for the bacteria that there is a strict requirement for Se. Most of the bacteria are grown anerobically so an electron sink, such as nitrate in the case of formate dehydrogenase, is necessary for growth.

Nicotinic Acid Hydroxylase. The third bacterial enzyme shown to be a selenoenzyme was nicotinic acid hydroxylase from *Clostridium barkeri* (26). The enzyme adds water across a double bond of nicotinic acid and then the ring is dehydrated to form the 6-oxo derivative. This reaction is the first step in the metabolism of nicotinic acid to ammonia, pyruvate, acetate and CO_2. The enzyme contains nonheme Fe, S and a flavin moiety in addition to Se.

Xanthine Dehydrogenase. Xanthine dehydrogenase, an enzyme with a molecular weight of 300,000 daltons, was the fourth bacterial selenoenzyme to be discovered (27). This Clostridial enzyme oxidizes xanthine, purines or aldehydes, with concomitant reduction of dyes, ferricyanide or oxygen. The enzyme also contains Mo, Fe, S and flavin.

Thiolase. The fifth bacterial enzyme shown to be a selenoenzyme was thiolase (28). The enzyme is 160,000 daltons in size with a 40,000 dalton Se-containing subunit. This is the enzyme involved in the cleavage of acetoacetyl CoA to 2 acetyl CoA molecules during β-oxidation of fatty acids. In *Clostridium kluyveri* (a fatty acid-producing bacteria), the enzyme apparently acts in a synthetic role to catalyze the condensation of acetyl CoA to form acetoacetyl CoA. The typical thiolase isolated from bacteria or from pigs contains a cysteine at the active site, and the amino acid sequence at the active site of these enzymes was shown to be Lys-Val-Cys-Ala-Ser (29). In the selenothiolase, however, the Se is present as selenomethionine (Se-Met) (30). Depending on the S/Se ratio in the media they found either that only a little Se-dependent thiolase was present or that 50-60% of the thiolase activity was due to the Se-form. In no case did Hartmanis and Stadtman find preparations with only Se-dependent thiolase activity.

The presence of Se-Met rather than Se-Cys at the active site of the Se-dependent thiolase is difficult to rationalize because of the difference in mRNA codons for methionine (Met) and cysteine (Cys), and thus presumably Se-Met and Se-Cys. A point mutation could not change a Cys to a Met but a frame shift toward the 5'-end of the mRNA would shift Val-Cys codons (GUA-UGU) to a Met (AUG) codon. It is interesting that this is the only selenoenzyme discovered to date not involved in an oxidation-reduction reaction, and the only selenoenzyme shown to contain Se-Met rather than Se-Cys; this exception strongly reinforces the idea that a Se-Cys moiety in an enzyme serves as an electron-carrying intermediate during redox reactions.

Se-Dependent Hydrogenase. In 1982 Yamazaki (31) discovered a Se-dependent hydrogenase from *Methanococcus vannielli.* This enzyme has a molecular weight of 340,000 daltons, and the Se is present in a 42,000 dalton subunit. There are a reported 3.8 g-atom Se per mole of enzyme, which apparently is one per subunit. Most interestingly, Yamazaki (32) recently has reported that 2 g-atom nickel (Ni) per mole of enzyme co-purify with the hydrogenase activity. The enzyme catalyzes the hydrogenation using H_2 gas of a variety of substrates such as methyl viologen. 8-hydroxy-5-deazaflavin is its natural acceptor substrate. The Se has been reported to be present as Se-Cys (31).

W-dependent Formate Dehydrogenase. The seventh enzyme on the list is a tungsten-dependent formate dehydrogenase from *Clostridium thermoaceticum* (33). The molecular weight of this formate dehydrogenase appears to be 340,000 daltons and thus about half of the usual 600,000 daltons, but it still is unclear whether tungsten (W) is simply substituting for the 2g-atom Mo per mole that would be present in a formate dehydrogenase of this size. Two Se-Cys are present per 340,000 dalton enzyme. Yamamoto et al. (34) recently have reported that this protein has a strict re-

URL 05443

quirement for W rather than Mo for full activity, and that it contains (on a g-atom basis) 2 W, 2 Se, 36 Fe and about 50 S per mole.

SELENOCYSTEINE/GLUTATHIONE PEROXIDE

Both Se-Cys and selenomethionine (Se-Met) are found in the tissues of plants grown on Se-containing soils. Those plants that tolerate high Se levels in the soil and that accumulate Se, such as several species of Astragalus (the milk vetch genus), sequester the Se in rare amino acids such as methyl selenocysteine and selenocystathionine (35). Monogastric animals, however, do not accumulate Se-Met in their tissues when fed diets supplemented with inorganic Se (36,37), and Olson and Palmer (38) found only small but nonetheless detectable quantities of Se-Cys (as 2,7 diamino-4-thio-5-selenaoctanedioic acid) in rats fed selenite. These trace amounts of Se-Cys were thought at first to be unimportant, but with the discovery that the bacterial selenoenzymes contained Se-Cys, the presence of Se-Cys in animal tissues has assumed new importance.

Se-Cys in GSH-Px

The form of Se in reduced GSH-Px was shown to be Se-Cys in 1978 by Forstrom et al. (39) and Wendel et al. (40). ^{75}Se-labeled GSH-Px was purified, reduced with GSH, alkylated with iodoacetate, hydrolyzed in 6 N HCl, and then subjected to automated amino acid analysis. The ^{75}Se was found to co-elute with authentic carboxymethylselenocysteine (CM-Se-Cys) when chromatographed on the long column of a Beckman 121 amino acid analyzer. CM-Se-Cys eluted approximately 7 min after CM-Cys and between aspartate and threonine. Edman degradation of peptides obtained by tryptic digestion of GSH-Px indicated that the Se-Cys was incorporated into the peptide backbone of the enzyme (41). Ladenstein et al. (42) crystallized the selenoenzyme and used X-ray crystallography to establish the structure of GSH-Px at 2.8 A resolution. The Se is present 35 residues from the N-terminal end of the bovine erythrocyte enzyme and at residue 41 of the rat liver enzyme (43). A refined structure at 2.0 A resolution has indicated that the dimer is the functional unit and that there are 2 active (GSH-binding) sites per tetramer (44). The mechanism used to form and incorporate Se into GSH-Px thus has become one of the important areas of Se research.

Se Incorporation

There are 2 postulated mechanisms—translational and post-translational—for inserting Se into GSH-Px. If Se-Cys is incorporated during translation by direct insertion into the peptide backbone of the enzyme facilitated by a Se-Cys-specific tRNA, then there should be no interference with this process by the other Se metabolites. If the insertion of Se is post-translational—after the peptide backbone has been synthesized beyond the 35th/41st residue—then other metabolites of Se would interfere with the incorporation of ^{75}Se from ^{75}Se-Cys into GSH-Px.

We set out to directly test these 2 hypotheses using the isolated, erythrocyte-free perfused liver (45). An erythrocyte-free perfusate was used because red blood cells rapidly take up selenite, metabolize it to selenide, and release Se$^{=}$ back into the plasma (46). Se-adequate livers were perfused for 4 hr with a perfusate that consisted of glucose, individual amino acids mirroring the composition of rat fibrinogen, insulin, cortisol, antibiotics and heparin in Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin. Liver viability was assessed by bile duct cannulation and measuring the rate of bile production.

Each liver was perfused with 6.8 ng Se/g liver. Under these conditions, approximately 30% of the cytosolic ^{75}Se was incorporated into GSH-Px within 4 hr. Sephadex G-150 chromatography of the liver supernatant yielded ^{75}Se and GSH-Px activity peaks that co-eluted at a molecular weight corresponding to 90,000 daltons. The other ^{75}Se peaks (void volume, 40,000-dalton and low molecular-weight peak) apparently were due to proteins with affinity for ^{75}Se, as these peaks were reduced substantially when the supernatant was dialized prior to chromatography. When the pooled GSH-Px-containing fractions were purified further with carboxymethy cellulose chromatography, the GSH-Px activity and ^{75}Se co-eluted, well ahead of the major protein peak, and with a specific activity (^{75}Se/EU) across the peak the same as across the Sephadex G-150 peak. This indicated that Sephadex G-150 chromatography alone was sufficient to separate ^{75}Se-labeled GSH-Px from other ^{75}Se-containing or ^{75}Se-binding proteins (45).

If an individual lobe of the perfused liver is ligated and removed at various times during the perfusion, the time-course of ^{75}Se incorporation into GSH-Px can be obtained. A linear rate of ^{75}Se incorporation was observed in liver from both Se-adequate and Se-deficient rats. The rate in the Se-deficient liver was 1/3 that observed in the Se-adequate liver, even though there was no detectable GSH-Px activity in the chromatograms of Se-deficient liver supernatant. In intact rats, ^{75}Se incorporation was delayed 2 to 3 hr in Se-deficient rats, whereas detectable ^{75}Se incorporation was observed within 30 min of ^{75}Se administration in Se-adequate rats (47). This delay is consistent with the time required for mRNA induction. Cycloheximide pretreatment of both Se-adequate and Se-deficient rats completely blocked ^{75}Se incorporation into GSH-Px, indicating that detectable quantities of a pre-GSH-Px protein, into which Se can be inserted, do not accumulate even in Se-deficient rat liver (47).

An isotope dilution technique was used next to differentiate between the ability of various Se compounds to provide Se for GSH-Px synthesis. A 100-fold excess of unlabeled selenite or selenide very effectively eliminated ^{75}Se incorporation from [^{75}Se] selenite, whereas unlabeled selenocystine ($Se\text{-}Cys_2$) was relatively ineffective (45). This experiment demonstrated that selenite and selenide were more readily metabolized than was $Se\text{-}Cys_2$ to the form used for incorporation into GSH-Px. When conditions were reversed and [^{75}Se] $Se\text{-}Cys_2$ was used, a 9-fold excess of unlabeled selenite or selenide was far more effective than unlabeled $Se\text{-}Cys_2$ in decreasing the incorporation of ^{75}Se into GSH-Px. These experiments thus demonstrate that inorganic forms of Se, such as selenite or selenide, are more readily metabolized than is $Se\text{-}Cys_2$ to the form of Se used for insertion into GSH-Px. The results further suggest that a post-translational modification of another amino acid residue is the mechanism for formation of the Se-Cys residue present in GSH-Px. For instance, nucleophilic attack of selenide on the β-carbon of serine, cysteine or dehydroalanine in the peptide backbone of the enzyme would result in formation of a Se-Cys residue in GSH-Px (46). The insertion of Se could be facilitated by a specific GSH-Px Se-Cys synthetase, or at the substrate level by a moiety such as a selenopersulfide of GSH (GS-SeH).

Evidence Supporting Translational Se Incorporation

In 1979 Hawkes et al. (48) reported preliminary results suggesting that Se-Cys was incorporated directly into GSH-Px using a Se-Cys-specific tRNA. Hawkes et al. (49) described the isolation of ^{75}Se-Cys-acylated tRNA that was prepared from ^{75}Se-Cys in a cell-free system or from [^{75}Se] selenite in a rat liver slice system. Only 15% of the

URL 05444

^{75}Se-Cys from the acylated tRNA prepared from selenite was shown to be authentic Se-Cys, and the authors stated that as much as 61% of the ^{75}Se in acylated tRNA prepared from liver slices incubated with ^{75}Se-Cys was in a form other than Se-Cys. ^{75}Se incorporation into GSH-Px from selenite was blocked by puromycin or cycloheximide in the liver slice system, and dilution of [^{75}Se] selenite by a 6-fold excess of Se as $Se\text{-}Cys_2$ reduced ^{75}Se incorporation by only 50%. These results obtained with the liver slice system are thus in agreement with those of Sunde and Hoekstra (45, 47). Using the cell-free system, however, ^{75}Se from Se-Cys-tRNA was more efficient (on a percentage basis) than were Se-Cys or selenite in providing Se for GSH-Px synthesis. This result suggests that Se-Cys is inserted into GSH-Px in a tRNA-mediated process. Recent reports from the same laboratory, however, have indicated that 82% of the ^{75}Se incorporation from ^{75}Se-Cys was not inhibited by cycloheximide in this rabbit reticulocyte system (50), and that Se-Cys and Cys in this system were in direct competition for incorporation into TCA-insoluble material (51).

Se-Cys apparently is a rare amino acid in both bacteria and in higher animals. Most rare amino acids are formed post-translationally (52), and all 64 codons have been unambiguously assigned to the common amino acids or to initiation/termination codons. The recent report of Wilhelmsen et al. (51) further shows that Se-Cys can be incorporated into general proteins (which normally do not contain Se-Cys) by the same mechanism that inserts Cys. Thus the post-translational hypothesis remains the more logical of the 2 postulated mechanisms explaining Se insertion into GSH-Px.

Post-translational Processing of GSH-Px

The distribution of Se and GSH-Px activity in rat liver indicated that a majority (60%) of liver GSH-Px was found in the cytosol and that 28% was localized in the mitochondria (53,54). Using antibodies prepared against rat liver GSH-Px, Yoshimura et al. (55) showed that GSH-Px protein was localized predominantly in the cytoplasm of the parenchymal cells—the endothelial cells and the Kuppfer cells were devoid of immunoreactive protein. Yoshida et al. (56) used immunochemical analysis to demonstrate the virtual absence of immunochemically recognizable GSH-Px in liver from Se-deficient rats. When Se-deficient rats were administered 50 μg Se IV, 5 hr were required before GSH-Px was detected immunochemically. These experiments, confirming the results of Sunde and Hoekstra (47), demonstrated that a pre-GSH-Px protein does not accumulate in the liver of Se-deficient rats. Recently Vojgt and Autor (57) reported preliminary results indicating that the initial gene product from total rat liver RNA, translated in a cell-free rabbit reticulocyte system, was an immunoreactive protein of 28,000 daltons, as compared to 23,000 daltons for mature GSH-Px. These results clearly suggest that more than just Se-insertion occurs during maturation of GSH-Px.

SELENIUM METABOLISM

Se metabolism in higher animals parallels sulfur (S) metabolism as long as the Se is bound to carbon in an animo acid form (Fig. 1). Thus Se-Met is converted to Se-Cys which, in turn, is then degraded to release inorganic Se. As in S metabolism, the conversion of inorganic Se into an amino acid form does not occur readily in monogastric animals (38). In this diagram of Se metabolism, we have proposed that inorganic Se in the -2 valence state, as HSe^- or a similar compound, is the form inserted into a pre-GSH-Px protein to form the active enzyme (58).

Se Chemistry

Se compounds have higher redox potentials than the corresponding S compounds. Thus Se metabolism in animals

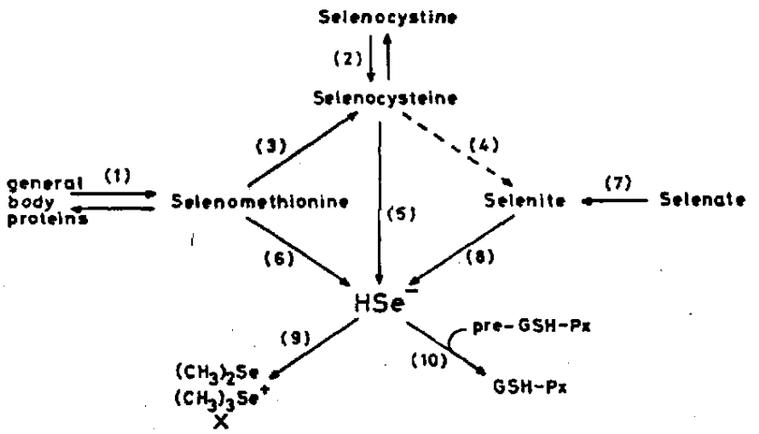


FIG. 1. Proposed Metabolism of Selenium. Reaction 1, incorporation of selenomethionine into general body proteins in place of methionine; Reaction 2, reduction of selenocystine to selenocysteine by GSH or by thioltransferase; Reaction 3, transsulfuration pathway analogous to sulfur metabolism; Reaction 4, selenite release from selenocysteine, analogous to sulfur metabolism, probably does not occur because of the high reduction potentials of selenium compounds; Reaction 5, selenocysteine lyase; Reaction 6, transamination pathway analogous to bacterial methionine-γ-lyase and to the methionine transamination pathway; Reaction 7, selenate reduction to selenite in animals may occur via APSe or PAPSe intermediates analogous to sulfur metabolism; Reaction 8, GSH/glutathione reductase catalyzed reduction of selenite to selenide; Reaction 9, methylation of selenide to produce the excretory compounds dimethyl selenide, trimethyl selenonium ion or unknown form(s) (X) which correspond to toxic, adequate or suboptimal levels of selenium intake; Reaction 10, hypothetical GSH-Px selenocysteine synthetase.

URL 05445

tends toward reduction, whereas S metabolism generally is oxidative (3). Selenate thus is reduced to selenite and then selenite is reduced to selenide in a series of GSH-dependent steps (59). The redox potentials further suggest that selenide rather than selenite would be the expected inorganic form released during Se-Cys degradation (45). Se is much more acidic than S; the pK of Se-Cys is 5.3, whereas Cys has a pK of 8.3 (60). This means that at physiological pH Se-Cys is mostly deprotonated whereas Cys is protonated. Enzymes that act on these substrates would be expected to act differentially on Se-Cys and Cys because of the charge on the side chain of Se-Cys. Se in Se-Met, in contrast, is covalently bound between 2 carbon atoms. The covalent radii of Se and S are approximately the same (1.17 versus 1.03 A). Thus, Se-Met would be expected to substitute for Met as a substrate for those enzymes that metabolize Met. Met and Se-Met are absorbed from the intestine by the same system (61,62), Se-Met readily acylates Met-tRNA with a K_m much the same as for Met (63), and is incorporated into proteins in place of Met in both *E. coli* and rat liver (64,65). Huber and Criddle (66) grew bacteria on selenate supplemented media, and replaced 70-75% of the Met residues in β-galactosidase with Se-Met without affecting the V_{max}. The only difference was that the Se-substituted enzyme was slightly more susceptible to heat denaturation.

Se-Met Biopotency

Met, unlike the other essential amino acids, still is catabolized to a relatively large extent even when it is the limiting amino acid in the diet (67), and as the level of Met in the diet increases, the percentage as well as total amount of Met catabolism increases. Because Se-Met apparently is an excellent analog for Met, Se-Met catabolism might be expected to be affected similarly.

To test whether the level of dietary Met would affect the proportion of Se from Se-Met that was available for GSH-Px synthesis, Se-deficient rats were repleted with various levels of dietary Se for 7 days while being fed different levels of dietary Met. Biopotency was quantitated by measuring the increase in tissue GSH-Px activity elicited by the 7 days of Se-Met or selenite repletion (68). We found that selenite biopotency was unaffected by the level of dietary Met, but that Se-Met biopotency for GSH-Px synthesis was reduced dramatically when the diet was suboptimal in Met. These results, indicating Se-Met incorporation into protein when the diet was suboptimal in Met, could explain the observations of Cary et al. (69), that muscle Se was raised in rats fed Se-Met as compared to rats fed selenite. These experiments show that Se-Met has 2 fates in the animal; it can be incorporated into general body proteins in place of Met or it can be catabolized to release Se which will then be available for GSH-Px synthesis.

It is important to note that what we are looking at is the biopotency of these Se compounds—that is, the ability of these forms of Se to raise tissue GSH-Px activity in a deficient rat. The level that gives maximal response in repleting a deficient rat (0.5 ppm Se as selenite) (68) is different from the dietary level necessary to maintain tissue GSH-Px levels (0.1 ppm Se as selenite) (70). Secondly, the plateau in liver GSH-Px activity above 0.5 ppm Se means that Se is no longer the rate-limiting factor for the synthesis of GSH-Px.

Nature of Tissue Se

During the conference there was some discussion about the proportion of Se that could be accounted for by GSH-Px. We conducted some experiments with radioactively labeled sheep that examined the nature of the Se in sheep liver and blood (71). Liver and erythrocyte GSH-Px was purified, and the specific radioactivity (dpm/EU) or the total Se specific activity (μg Se/Eu) of the purified GSH-Px was used to estimate the percentage of the liver or erythrocyte Se that could be accounted for by GSH-Px. For ovine liver only 10 to 15% of the total liver Se could be accounted for by GSH-Px, and 25% of the liver supernatant Se was present as GSH-Px. In contrast, 75% of the whole blood Se and 100% of the erythrocyte Se were accounted for by GSH-Px. These sheep were injected and dietarily supplemented with Se as selenite to induce maximal levels of GSH-Px. Animals with a lower Se status might be expected to have a greater proportion of the Se in liver present as GSH-Px, although the absolute amount of Se might be less. The form of the non-GSH-Px Se may have been Se-Met (formed by rumen bacteria from endogenous Se released into the rumen) or the non-GSH-Px Se may have been present in some of the other selenoproteins (discussed below). In a separate experiment, when rats were labeled with ^{75}Se, and the liver GSH-Px purified through several ion exchange steps, the constant ratio of ^{75}Se/EU across the GSH-Px peak was used to estimate Se distribution in rat liver. We found that 60% of rat liver Se was accounted for by GSH-Px and 70% of the liver supernatant Se was accounted for by GSH-Px. Levander et al. (54) earlier reported similar results for rat liver mitochondrial GSH-Px. It seems logical to assume that those tissues with high levels of GSH-Px, such as rat liver or sheep erythrocytes, might have a greater proportion of the Se accounted for by GSH-Px than tissues with lower GSH-Px activities, such as sheep liver.

Se-Cys Biopotency

The same biopotency technique was used to compare the metabolism of Se-Met, $Se\text{-}Cys_2$ and selenite (58). Yasumoto et al. (72) reported that vitamin B_6 deficiency would decrease the biopotency of Se-Met, but that selenite biopotency was unaffected. At first glance this result would make sense if the activities of the B_6-dependent enzymes involved in Met/Se-Met catabolism were so depressed that Se-Met catabolism was restricted. The transaminases and cystathionase involved in S/Se metabolism would be such B_6-dependent enzymes. In our experiments, however, we found that there was no difference in the biopotency of selenite, $Se\text{-}Cys_2$ or Se-Met at either 0.2 or 1.0 ppm Se, when control rats were restricted to the food intake of B_6-deficient rats and the diet was supplemented with Met. In our experiments, the B_6 deficiency was sufficient to impair growth by 37% and to depress cystathionase activity by more than 50%, whereas in the experiment of Yasumoto et al. (72) no growth depression was reported. In addition, Se was supplemented in those experiments at a level (2 ppm) where Se clearly was no longer rate limiting for GSH-Px synthesis in our previous experiments (68). The reason for the impaired Se-Met biopotency in the experiments of Yasumoto et al. (72) remains unclear.

Se-Met and Se-Cys Metabolism

In Figure 1 Se-Met has 2 fates, either incorporation into general body proteins in place of Met (reaction 1), or degradation. Se-Met degradation can follow the transsulfuration pathway (reaction 3) leading to Se-Cys (73). Alternatively, Se-Met may be transaminated and decarboxylated, with final release of methane selenol (reaction 6) in a manner similar to the Met transamination pathway described by Steele and Benevenga (74). In bacteria a methionine-γ-lyase activity has been shown to use Se-Met as a substrate and to release methane selenol (75). Methane selenol catabolism would yield methane and selenide. Se-Cys might be degraded with release of selenite (reaction 4) in a manner similar to the degradation of Cys, but the high reduction potential of Se compounds suggests that direct release of

URL 05446

selenide would occur instead. Soda and coworkers (76) found such an enzymatic activity in rat liver, Se-Cys lyase, that specifically catabolizes Se-Cys to alanine and selenide (reaction 5). As suggested by the lower pK's for selenols as compared to thiols, Cys was found to be a poor substrate for this enzyme. The normal cellular concentrations of free Se-Cys are most likely well below the K_m of Se-Cys lyase, so low concentrations of Se-Cys can be present inside cells (76). By analogy with Cys the adequate intracellular levels of GSH should readily reduce Se-Cys_2 to Se-Cys (reaction 2) either nonenzymatically (77) or catalyzed by the low molecular weight thioltransferases (78).

Inorganic Se Metabolism

Selenate is reduced to selenite (reaction 7) presumably via APSe or PAPSe intermediates similar to the APS and PAPS intermediate involved in sulfate reduction (79). The GSH/glutathione reductase/NADPH pathway involved in the reduction of selenite to selenide (reaction 8) has been well characterized by Hsieh and Ganther (59), as has the excretory methylation of selenide (reaction 9). When moderate levels of Se are ingested the selenide is methylated to form $(CH_3)_3Se^+$ (trimethyl selenonium ion) which is excreted in the urine (80). With acute Se toxicity, $(CH_3)_2Se$ (dimethyl selenide) is formed; this volatile compound is expired via the lung (81). Trimethyl selenonium ion may not be the major urinary form when the diet is deficient or marginally deficient in Se (82). The reductive pathway of Hsieh and Ganther (59) is important not only in terms of Se excretion, but also because it is the pathway used to metabolize selenite rapidly to selenide in erythrocytes and other tissues (46). This pathway also may be critically important in depleting lens GSH and thus causing selenite-induced cataract (83).

Selenide

Hydrogen selenide thus appears to be a central compound in Se metabolism (58). Selenide may be the chemical form that binds to a number of proteins, perhaps proteins with exposed thiols, to yield the various non-GSH-Px ^{75}Se peaks that are present in gel filtration profiles of liver supernatant. As postulated by Diplock (84), selenide also could be the chemical form that binds to microsomes. While selenide is highly toxic (85), the intracellular toxicity of this species may be lessened by its non-specific binding to proteins. Finally, HSe^- or a close metabolite may be the form inserted post-translationally into a pre-GSH-Px polypeptide to form the active GSH-Px subunits (reaction 10) (45). The small amount of Se-Cys usually detected in animal tissues (38) may be only from selenoenzyme synthesis.

GSH-Px FUNCTION

The discovery that GSH-Px was a Se-containing enzyme (1) and that GSH-Px destroyed hydroperoxides (14) as well as H_2O_2 provided strong evidence for the antioxidant theory of vitamin E function. The discovery of superoxide dismutase (2) also helped to demonstrate that superoxide and other activated oxygen species exist in vivo and that peroxidant damage could affect cells. It is unfortunate, perhaps, that in almost every case of Se deficiency, one can logically explain the signs of selenium deficiency in terms of an absence of GSH-Px which leads to peroxidative damage (86). The protective mechanisms of cells are compartmentalized within various subcellular components (6). A deficiency disease that is prevented by either vitamin E or Se, such as dietary liver necrosis in the rat, suggests that the origin of the toxic molecular species is in the cytosol where GSH-Px is localized, but that the target may be in the lipid (and vitamin E) soluble membrane. The metabolism of various drugs, for instance, leads to the production of superoxide either in the membranous or the aqueous compartments of the cell (87). Superoxide in turn could lead to the formation of lipid hydroperoxides, singlet oxygen or hydroxy radicals, all species capable of causing cell damage. In the membrane, vitamin E could quench lipid hydroperoxides or singlet oxygen. Superoxide and H_2O_2, in a modified Haber-Weiss-type reaction catalyzed by iron, can react to form hydroxy radical (88). Superoxide dismutase and GSH-Px thus can act in concert to minimize the formation of these aqueous activated oxygen species.

Compartmentalization

The chick provides an excellent illustration of the compartmentalization of protection in animal cells. A chick fed a diet deficient in the sulfur amino acids, very low in vitamin E and perhaps with an excess of unsaturated fatty acids, will develop muscular dystrophy (89). Met supplementation together with a small level of dietary ethoxyquin will prevent the development of this nutritional disease. Se supplementation will sometimes delay but will never prevent muscular dystrophy (90). With ethoxyquin and Met administration, chicks still develop another disease—exudative diathesis. Exudative diathesis is an increased permeability of the capillaries that results in the accumulation of a bluish-green fluid under the ventral skin of the chick. Either vitamin E or Se can prevent the development of this disease (91,15). If deficient chicks are supplemented with Se, they develop encephalomalacia, a degenerative brain condition (92). The brain is a tissue with a high lipid content, and it seems that the water soluble GSH-Px is unable to protect the lipids from peroxidation. If the chicks are supplemented with vitamin E but not Se, they will develop pancreatic atrophy (previously called fibrosis or degeneration) (93). This condition is not prevented by normal levels of vitamin E, but recent reports (94) indicate that pharmacological levels (550 ppm) of Vitamin E (as well as BHT, DPPD or ascorbate) will prevent the disease. Interestingly, Se-Met is reported to be 4 times as effective as selenite for the prevention of pancreatic atrophy (95). This may relate to the high anabolic rate of the pancreas, or to a non-GSH-Px role for Se. With each of the diseases prevented by Se, the signs of the disease can be explained logically by an absence of GSH-Px. That does not rule out the possibility of other important biological functions for Se.

GSH-S-Transferase

GSH-S-transferase may also have a protective role in the tissues of some species because of its hydroperoxidase activity (137,96,97). Burk et al. (98) perfused livers from Se-deficient rats with organic peroxides and demonstrated that GSH-S-transferase would destroy the peroxides and release GSSG into the perfusate. This work strongly suggests that GSH-S-transferase may have a biological role in tissues with low GSH-Px activity. These 2 enzymes, however, are not equivalent because GSH-Px also destroys H_2O_2 and GSH-S-transferase also conjugates compounds with GSH.

SELENOPROTEINS LOOKING FOR A FUNCTION

10K Muscle Selenoprotein

It would seem unusual if there existed only one function for Se in higher animals and yet a handful of selenoenzymes in bacteria. Researchers have been looking without success for alternative selenoproteins and for other functions for Se. In 1972 Pedersen et al. (99) reported a 10,000 dalton selenoprotein which they found to be lacking in lambs suf-

URL 0544?

fering from muscular dystrophy. Subsequent purification and identification of this protein has not been successful (100,101), although it recently was reported that the Se is present as Se-Cys (102). The classification of this protein as a selenoprotein must therefore remain tentative until more results are obtained.

80K Selenoprotein

A second selenoprotein is the ^{75}Se binding protein first identified by Herrman (103) in the plasma of rats. Burk and Gregory (104) used DEAE-Sephadex chromatography to further purify GSH-Px-containing fractions from gel filtration chromatography and found a non-GSH-Px ^{75}Se binding protein that was retained by DEAE-Sephadex, whereas GSH-PX passed straight through the column. This protein, called ^{75}Se-P, had an apparent molecular weight of 79,000 and 83,000 daltons when isolated from rat plasma and rat liver, respectively. Elution with NaCl released the ^{75}Se in several peaks. Motsenbocker and Tappel (105-107) have published a series of papers in which they examined the appearance and fate of this protein. Initially, they reported a 45,000 dalton Se-containing subunit of an 80,000 dalton plasma protein, but they recently have reestimated these molecular weights as 53,000 and 85,000 (108). The Se is reported to be present as Se-Cys, and Motzenbocker and Tappel have suggested that it serves as a transport protein that carries Se from the liver to the kidney and other tissues. They also have reported a 75,000 dalton ^{75}Se-binding protein in kidney that may be the same protein as the plasma/liver protein (106). A two-subunit transport protein, carrying only one atom of Se per 80,000 dalton protein and with the Se attached covalently to a residue in the peptide backbone, would be thermodynamically very expensive, so the nature and role of this ^{75}Se-binding protein await further characterization.

Burk and Gregory (104) have found a greater percentage of liver ^{75}Se binds to ^{75}Se-P in liver from Se-deficient rats as compared to Se-adequate rats. Three hours after injection of a low (0.76 μg) dose of ^{75}Se, they found 8% of the liver ^{75}Se in GSH-Px in Se-adequate rats as compared to 1.5% in Se-deficient rats; 1.6% of the liver ^{75}Se was present in ^{75}Se-P in Se-adequate rats as compared to 3.5% in Se-deficient rats. At 72 hr, the labeling pattern of neither GSH-Px nor ^{75}Se-P had changed significantly in the Se-deficient rats whereas the percentage of the ^{75}Se in GSH-Px in the Se-adequate rats had increased 3-fold. The exact nature of this Se-binding peak remains unclear, but this approach seemingly will provide a better picture of Se metabolism in animals.

Sperm Selenoprotein

Se was reported in 1973 to be required for the production of normal sperm (109,110), and these defects now can be associated with a 17,000 dalton Se-protein in rat sperm discovered by Calvin (111) and with a 20,000 dalton Se-protein in bovine sperm discovered by Pallini and Bacci (112). The selenoprotein is localized in the midpiece region of the sperm in association with the mitochondrial helix. In Se-adequate mice the mitochondria exist as regular, rectangular organelles located next to a central lumen. Using electron microscopy, a few aberrations are observed in the regular mitochondrial structure in sperm midpiece from first-generation Se-deficient mice, and sperm from second generation Se-deficient mice have severely disrupted mitochondrial organization (113). The SDS-insoluble sheath surrounding the mitochondria contains a protein with a high Cys content, and the Se is present in this Cys-rich structural protein (114). The form of the Se in this protein has not been characterized, but a selenoamino acid moiety would be a likely possibility.

130K Plasma Protein

One of the more recent selenoproteins to be identified was a 130,000 dalton protein from rat plasma. The protein was identified by Gasiewicz and Smith (46), and it appears that both Cd and Se are necessary for the labeling of this protein. Importantly, this protein has served to identify the form of Se released from erythrocytes. When rat plasma is incubated with cadmium and selenite and without erythrocytes, this protein is not observed. If erythrocytes are placed in the media, however, the protein becomes labeled with cadmium and Se; if hydrogen selenite is bubbled through the media during the incubation, the protein also becomes labeled. This work thus finally has identified the product released from erythrocytes as selenide.

Se-Cys Proteins

Hawkes et al. (115) have separated a number of ^{75}Se-labeled selenoproteins by chromatography from blood, liver, kidney, testes, skeletal muscle, lung, heart and epididymus of rats. The rats were isotopically equilibrated with ^{75}Se by providing $^{75}SeO_3^=$ in the drinking water for 5 mo. They reported that over 80% of the Se was present as Se-Cys, and roughly 50% of the total body Se was present as GSH-Px Se. Using DEAE sephacel chromatography with a buffer containing 0.1% triton-X-100 and 7M urea, they have identified 8 different sizes of proteins that bind ^{75}Se—>89,000, 46,000, 36,000, 26,000 (=GSH-Px), 20,100, 15,000, 9800, and 6800 dalton proteins. Ion exchange chromatography has further identified 9 different charge forms. Because protein(s) of one molecular weight can have several charge forms, they have estimated that there may be as many as 19 to 23 different selenoproteins. Hawkes, Wilhelmsen and Tappel (116) find only a 36,000 dalton protein in the plasma, whereas Motsenbocker and Tappel (108) have reported that the major plasma selenoprotein is 85,000 daltons with a 53,000 dalton subunit. Because these results are not in agreement, the conclusions of these experiments must remain tentative until confirmed by more rigorous methods.

FUNCTIONS LOOKING FOR A SELENOPROTEIN

Heme Metabolism

The role of Se in heme metabolism was one of the first areas to be studied in an attempt to find a non-GSH-Px biological function for Se. Burk and Masters (117) reported that phenobarbitol injection did not increase the concentration of cytochrome P450 in Se-deficient rats. When they examined the various enzymes involved in heme metabolism they found that heme synthesis was relatively unaffected by Se deficiency but that heme oxygenase activity was increased 8-fold by Se deficiency in the rat (118). A number of other mineral imbalances also alter heme metabolism (119), so it still is not clear whether this is a specific effect of Se. The stimulation in heme catabolism apparently is not due to a direct effect of Se deficiency in heme catabolism but because Se-deficient liver has a defect in its ability to use heme for the assembly of heme proteins (118).

Drug Metabolism

The toxicity of paraquat and diquat are elevated with Se-deficiency. Burk et al. (120) found that diquat was more toxic than paraquat, and that both were far more toxic to Se-deficient rats than to Se-adequate rats, as assessed by survival time or ethane evolution. An acute lethal dose of

URL 05448

diquat or paraquat, however, did not cause elevated ethane evolution in Se-adequate rats, suggesting that peroxidation is an important aspect of the toxicity in Se-deficient rats. Both vitamin E and Se deficiency increased the toxicity of paraquat (87), but vitamin E deficiency in the rat did not increase the toxicity of diquat or paraquat as dramatically as did Se deficiency (120). A single 50 μg Se injection of selenite 10 hr prior to sacrifice did not substantially raise measured lung or liver homogenate GSH-Px activity, but 50 μg Se administration 6 or 10 hr prior to diquat administration substantially increased survival time and decreased ethane evolution. These authors have interpreted these results to indicate that GSH-Px is not responsible for the protective effect of Se.

In contrast to rats, dietary vitamin E (100 ppm) supplementation of E- and Se-deficient chicks did not decrease the toxicity of paraquat or nitrofurantoin (121,122). Dietary Se supplementation, however, at 0.04 ppm Se allowed 90% survival in 8-day old chicks administered 175 mg paraquat/kg without significantly elevating plasma GSH-Px. GSH-Px activity in other tissues was not measured. Thus, these drug toxicity studies in both the chick and the rat suggest that GSH-Px may not be the important protective factor, but these experiments have not conclusively eliminated the possibility that crucial increases in GSH-Px at the specific site of attack by the toxic species are the protecting biochemical mechanism.

Transsulfuration

Another metabolic role for Se may be in the conversion of Met to Cys. Bunk and Combs (123) reported that Se-deficient chicks fed a crystalline amino acid diet for 28 days had negligible weight gains and high mortality even though they were supplemented with 0.8 or 1.2% D,L-Met. Replacement of part of the dietary Met with Cys increased weight gain 4-fold and reduced mortality from 65 to 40% without altering the incidence of pancreatic atrophy. Se supplementation (0.05 ppm as selenite) increased weight gain 8-fold and completely eliminated the mortality and pancreatic atrophy, thus suggesting that there was an inability to convert Met to Cys in Se-deficient chicks. These experiments were conducted with a fast-growing strain of broilers; the apparent inability to convert Met to Cys with Se deficiency was not observed in a slower growing strain of leghorn chicks (124). The lack of Se response in a slower-growing strain of broilers suggests that this defect could be related to the protein (Met) requirement rather than directly to Se deficiency.

Immune Response

As with a number of other essential elements, animals with Se-deficiency have been reported to have an impaired immune response. Ducks deficient in vitamin E and Se were less able to resist malarial infection than were vitamin E and Se supplemented ducks (125). The neutrophils from Se-deficient rats and cattle were shown to phagocytize bacteria readily but were not able to kill the bacteria as readily as Se-adequate controls (126,127,128). Arthur et al. (129) have shown that toxic oxygen radical production was reduced in Se-deficient neutrophils. A lack of GSH-Px—either normally acting as a protective enzyme or as an integral part of the bacteriocidal mechanism—may be responsible for this Se effect.

Arachidonate Metabolism

Hydroperoxides are intermediates in the metabolism of arachidonic acid to prostaglandins, thromboxanes and leucotrienes. Because of the lack of specificity for the peroxide substrate, GSH-Px was suggested as an important enzyme in prostaglandin metabolism (130). Prostaglandin endoperoxide synthetases also have peroxidase activity, so GSH-Px would not be essential for prostaglandin metabolism (131). Bryant and Bailey (132) reported that the relative products of arachidonate metabolism were altered by Se deficiency, and they suggested that GSH-Px (and thus Se) may have a specific role in platelet metabolism of essential fatty acids.

White Nail Beds

A recent report (133) documenting Se-deficiency in a child receiving total parenteral nutrition adds white fingernail beds to the list of symptoms of Se deficiency in humans—the others are chronic muscle pain, elevated plasma enzymes indicative of tissue damage, and Keshan disease in China (134). Some 25 months after the initiation of parenteral nutrition, the entire fingernail bed of all fingers from both hands was observed to be white even though the nails were fully developed and normal. Addition of Se to the IV fluid (97 μg Se as selenite per day) restored the beds to their normal pink state. White nail beds have been reported in patients with cirrhosis (135), but not in all patients with Se deficiency concurrent with total parenteral nutrition (136), suggesting that this condition may be due to liver necrosis caused by an absence of GSH-Px, or it may be due to lack of another Se function.

REFERENCES

1. Rotruck, J.T., A.L. Pope, H.E. Ganther, A.B. Swanson, D.G. Hafeman and W.G. Hoekstra, Science 179:588 (1973).
2. McCord, J.M., and I. Fridovich, J. Biol. Chem. 244:6049 (1969).
3. Levander, O.A., in Trace Elements in Human Health and Disease, Vol. II, (Prasad, A.S., ed.) Academic Press, N.Y., 1976, pp. 135.
4. Robinson, M.F., and C.D. Thomson, Nutr. Abst. Rev. 53:3 (1983).
5. Stadtman, T.C., Ann. Rev. Biochem. 49:93. Ann. Rev. Inc. N.Y., 1980.
6. Sunde, R.A., and W.G. Hoekstra, Nutr. Rev. 38:265 (1980).
7. Weichselbaum, T.E., Quart. J. Exptl. Physiol. 25:363 (1935).
8. Schwarz, K. Proc. Soc. Exp. Biol. Med 78:852 (1951).
9. Schwarz, K., and C.M. Foltz, J. Amer. Chem. Soc. 79:3292 (1957).
10. Schwarz, K., L.A. Porter and A. Fredga, Bioinorg. Chem. 3:145 (1974).
11. Mills, G.C., J. Biol. Chem. 229:189 (1957).
12. Cohen, G., and P. Hochstein, Biochemistry 2:1420 (1963).
13. Flohe, L., G. Loschen, W.A. Gunzler and E. Eichele, Physiol. Chem. 353:987 (1972).
14. Little, C., and P.J. O'Brien, Biochem. Biophys. Res. Commun. 31:145 (1968).
15. Scott, M.L., J.G. Bieri, G.M. Briggs and K. Schwarz, Poul. Sci. 36:1155 (1957).
16. Thompson, J.N., and M.L. Scott, J. Nutr. 97:335 (1969).
17. McCoy, K.E.M., and P.H. Weswig, Ibid. 98:383 (1969).
18. Pinsent, J., Biochem. J. 57:10 (1954).
19. Shum, A.C., and J.C. Murphy, J. Bacteriol. 110:447 (1972).
20. Jones, J.B., G.L. Dilworth and T.C. Stadtman, Arch. Biochem. Biophys. 195:255 (1979).
21. Enoch, H.G., and R.L. Lester, J. Biol. Chem. 250:6693 (1975).
22. Turner, D.C., and T.C. Stadtman, Arch. Biochem. Biophys. 154:366 (1973).
23. Tanaka, H., and T.C. Stadtman, J. Biol. Chem. 254:447 (1979).
24. Cone, J.E., R.M. del Rio, J.N. Davis and T.C. Stadtman, Proc. Natl. Acad. Sci. (USA) 73:2659 (1976).
25. Stadtman, T.C., P. Elliott and L. Tiemann, J. Biol. Chem. 231:961 (1958).
26. Imhoff, D., and J.R. Andreesen, FEMS Microbiol. Lett. 5:155 (1979).
27. Wagner, R., and J.R. Andreesen, Arch. Microbiol. 121:255 (1979).
28. Hartmanis, M., Fed. Proc. 39:1772 (1980).
29. Gehring, U., and F. Lynen, The Enzymes 7:391 (1972).
30. Hartmanis, M.G.N., and T.C. Stadtman, Proc. Natl. Acad. Sci. (USA) 79:4912 (1982).

URL 05449

31. Yamazaki, S., J. Biol. Chem. 257:7926 (1982).
32. Yamazaki, S., Fed. Proc. 42:2267 (1983).
33. Yamamoto, I., S-M Liu, T. Saiki and L.G. Ljungdahl, Fed. Proc. 41:888 (1982).
34. Yamamoto, I., T. Saiki, S-M. Liu and L.G. Ljundahl, J. Biol. Chem. 258:1826 (1983).
35. Shrift, A., Fed. Proc. 20:693 (1961).
36. Cummins, L.M., and J.L. Martin, Biochemistry 6:3162 (1967).
37. Jenkins, K.J., Can. J. Biochem. 46:1417 (1968).
38. Olson, O.E., and I.S. Palmer, Metabolism 25:299 (1976).
39. Forstrom, J.W., J.J. Zakowski and A.L. Tappel, Biochemistry 17:2639 (1978).
40. Wendel, A., B. Kerner and K. Graupe, in Functions of Glutathione in Liver and Kidney (Sies, H., and A. Wendel, eds.), Springer-Verlag, Berlin, 1978, pp. 107-113.
41. Zakowski, J.J., J.W. Forstrom, R.A. Condell and A.L. Tappel, Biochem. Biophys. Res. Commun. 84:248 (1978).
42. Ladenstein, R., O. Epp, K. Bartels, A. Jones, R. Huber and A. Wendel, J. Mol. Biol. 134:199 (1979).
43. Condell, R.A., and A.L. Tappel, Biochim. Biophys. Acta 709:304 (1982).
44. Epp, O., R. Ladenstein and A. Wendel, Eur. J. Biochem. 133:51 (1983).
45. Sunde, R.A., and W.G. Hoekstra, Biochem. Biophys. Res. Commun. 93:1181 (1980).
46. Gasiewicz, T.A., and J.C. Smith, Chem. -Biol. Interact. 23:171 (1978).
47. Sunde, R.A., and W.G. Hoekstra, Proc. Soc. Exp. Biol. Med. 165:291 (1980).
48. Hawkes, W.C., D.E. Lyons and A.L. Tappel, Fed. Proc. 38:820 (1979).
49. Hawkes, W.C., D.E. Lyons and A.L. Tappel, Biochim. Biophys. Acta 699:183 (1982).
50. Wilhelmsen, E.C., W.C. Hawkes and A.L. Tappel, Fed. Proc. 42:534 (1983).
51. Wilhelmsen, E.C., W.C. Hawkes and A.L. Tappel, Fed. Proc. 42:2152 (1983).
52. Uy, R., and F. Wold, Science 198:890 (1977).
53. Green, R.C., and P.J. O'Brien, Biochim. Biophys. Acta 197:31 (1970).
54. Levander, O.A., V.C. Morris and D.J. Higgs, Biochem. Biophys. Res. Commun. 58:1047 (1974).
55. Yoshimura, S., N. Komatsu and K. Watanabe, Biochim. Biophys. Acta 621:130 (1980).
56. Yoshida, M., K. Iwami and K. Yasumoto, Agric. Biol. Chem. 46:41 (1982).
57. Voigt, J.M., and A.P. Autor, Fed. Proc. 42:806 (Abstract) (1983).
58. Sunde, R.A., W.K. Sonnenburg, G.E. Gutzke and W.G. Hoekstra, in Trace Element Metabolism in Man and Animals-4 (Howell, J.M., J.M. Gawthorne and C.L. White, eds.), Australian Academy of Science, Canberra, Aust., 1981, pp. 165.
59. Hsieh, H.S., and H.E. Ganther, Biochim. Biophys. Acta 497:205 (1977).
60. Huber, R.E., and R.S. Criddle, Arch. Biochem. Biophys. 122:164 (1967).
61. McConnell, K.P., and G.J. Cho, Am. J. Physiol. 208:1191 (1965).
62. McConnell, K.P., and G.J. Cho, Am. J. Physiol. 213:150 (1967).
63. Hoffman, J.L., K.P. McConnell, and D.R. Carpenter, Biochim. Biophys. Acta 199:531 (1970).
64. McConnell, K.P., and J.L. Hoffman, Proc. Soc. Exp. Biol. Med. 140:638 (1972).
65. McConnell, K.P., and J.L. Hoffman, FEBS Lett. 24:60 (1972).
66. Huber, R.E., and R.S. Criddle, Biochim. Biophys. Acta 141:587 (1967).
67. Aguilar, T.S., A.E. Harper and N.J. Benevenga, J. Nutr. 102: 1199 (1972).
68. Sunde, R.A., G.E. Gutzke and W.G. Hoekstra, J. Nutr. 111:76 (1981).
69. Cary, E.E., W.H. Allaway and M. Miller, J. An. Sci. 36:285 (1973).
70. Hafeman, D.G., R.A. Sunde and W.G. Hoekstra, J. Nutr. 104:580 (1974).
71. Sunde, R.A., H.E. Ganther and W.G. Hoekstra, Fed. Proc. 37:757 (1978).
72. Yasumoto, K., K. Iwami and M. Yoshida, J. Nutr. 109:760 (1979).
73. Esaki, N., T. Nakamura, H. Tanaka, T. Suzuki, Y. Morino and K. Soda, Biochemistry 20:4492 (1981).
74. Steele, R.D. and N.J. Benevenga, J. Biol. Chem. 254:8885 (1979).
75. Esaki, N., H. Tanaka, S. Uemura, T. Suzuki and K. Soda, Biochemistry 18:407 (1979).
76. Esaki, N., T. Nakamura, H. Tanaka and K. Soda, J. Biol. Chem. 257:4386 (1982).
77. Flohe, L., and W.A. Gunzler, in Glutathione: Metabolism and Function (Arias, I.M., and W.B. Jakoby, eds.), Raven Press, N.Y., 1976, pp. 17.
78. Axelsson, K., S. Eriksson and B. Mannervik, Biochemistry 17:2978 (1978).
79. Shrift, A., in Organic Selenium Compounds: Their Chemistry and Biology (Klayman, D.L., and W.H.H. Gunther, eds.) Wiley and Sons, Inc. New York, 1973, pp. 763.
80. Palmer, I.S., D.D. Fischer, A.W. Halverson and O.E. Olson, Biochim. Biophys. Acta 177:336 (1969).
81. Ganther, H.E., Biochemistry 5:1089 (1966).
82. Nahapetian, A.T., M. Janghorbani and V.R. Young, J. Nutr. 113:401 (1983).
83. Bunce, G.E., and J.L. Hess, Exp. Eye Res. 33:505 (1981).
84. Diplock, A.T., Critical Reviews in Toxicology 4:271 (1976).
85. Dudley, H.C., and J.W. Miller, J. Ind. Hyg. Toxicol. 23:470 (1941).
86. Hoekstra, W.G., Fed. Proc. 34:2083 (1975).
87. Bus, J.S., S.D. Aust and J.E. Gibson, Res. Commun. Chem. Pathol. Pharmacol. 11:31 (1975).
88. Fridovich, I., Ann. Rev. Biochem. 44:147 (1975).
89. Dam, H., I. Prange and E. Sondergaard, Acta Pathol. Microbiol. Scand. 31:172 (1952).
90. Scott, M.L., Nutr. Abst. Rev. 32:1 (1962).
91. Dam, H., and J. Glavind, Nature 143:810 (1939).
92. Dam, H., G.K. Nielsen, I. Prange and E. Sondergaard, Nature 182:802 (1958).
93. Thompson, J.N., and M.L. Scott, J. Nutr. 100:797 (1970).
94. Whitacre, M.E., G.F. Combs Jr. and R.S. Parker, Fed. Proc. 42:928 (Abstract) (1983).
95. Cantor, A.H., M.L. Langevin, T. Noguchi and M.L. Scott, J. Nutr. 105:106 (1975).
96. Lawrence, R.A., and R.F. Burk, J. Nutr. 108:211 (1978).
97. Prohaska, J.R., and H.E. Ganther, Biochem. Biophys. Res. Commun. 76:437 (1977).
98. Burk, R.F., K. Nishiki, R.A. Lawrence and B. Chance, J. Biol. Chem. 253:43 (1978).
99. Pedersen, N.D., P.D. Whanger, P.H. Weswig and O.H. Muth, Bioinorg. Chem. 2:33 (1972).
100. Whanger, P.D., N.D. Pedersen and P.H. Weswig, Biochem. Biophys. Res. Commun. 53:1031 (1973).
101. Black, R.S., M.J. Tripp, P.D. Whanger and P.H. Weswig, Bioinorg. Chem. 8:161 (1978).
102. Beilstein, M.A., M.J. Tripp and P.D. Whanger, J. Inorg. Biochem. 15:339 (1981).
103. Herrman, J.L., Biochim. Biophys. Acta 500:61 (1977).
104. Burk, R.F., and P.E. Gregory, Arch. Biochem. Biophys. 213:73 (1982).
105. Motsenbocker, M.A., and A.L. Tappel, Biochim. Biophys. Acta 704:253 (1982).
106. Motsenbocker, M.A., and A.L. Tappel, Biochim. Biophys. Acta 709:160 (1982).
107. Motsenbocker, M.A., and A.L. Tappel, Biochim. Biophys. Acta 719:147 (1982).
108. Motsenbocker, M.A., and A.L. Tappel, Fed. Proc. 42:533 (1983).
109. Wu, S.H., J.E. Oldfield, P.D. Whanger and P.H. Weswig, Biol. Reprod. 8:625 (1973).
110. Brown, D.G., and R.F. Burk, J. Nutr. 103:102 (1973).
111. Calvin, H.I., J. Exp. Zool. 204:445 (1978).
112. Pallini, V., and E. Bacci, J. Submicrosc. Cytol. 11:165 (1979).
113. Wallace, E., G.W. Cooper and H.I. Calvin, Gamete Res. 7:389 (1983).
114. Calvin, H.I., G.W. Cooper and E. Wallace, Gamete Res. 4:139 (1981).
115. Hawkes, W.C., and A.L. Tappel, Biochim. Biophys. Acta 739:225 (1983).
116. Hawkes, W.C., E.C. Wilhelmsen and A.L. Tappel, Fed. Proc. 42:928 (1983).
117. Burk, R.F., and B.S.S. Masters, Arch. Biochem. Biophys. 170:124 (1975).
118. Correia, M.A., and R.F. Burk, Biochem. J. 214:53 (1983).
119. Maines, M.D., and A. Kappas, Science 198:1215 (1977).
120. Burk, R.F., R.A. Lawrence and J.M. Lane, J. Clin. Invest. 65:1024 (1980).
121. Peterson, F.J., G.F. Combs Jr., J.L. Holtsman and R.P. Mason, J. Nutr. 112:1741 (1982).
122. Combs, G.F. Jr., and F.J. Peterson, J. Nutr. 113:538 (1983).
123. Bunk, M.J., and G.P. Combs Jr., Proc. Soc. Exp. Biol. Med. 167:87 (1981).
124. Halpin, K.M., and D.H. Baker, Fed. Proc. 42:668 (1983).

125. Yarrington, J.T., C.K. Whitehair and R.M. Corwin, J. Nutr. 103:231 (1973).
126. Serfass, R.E., and H.E. Ganther, Nature 255:640 (1975).
127. Serfass, R.E., and H.E. Ganther, Life Sci. 19:1139 (1976).
128. Boyne, R., and J.R. Arthur, J. Comp. Pathol 89:151 (1979).
129. Arthur, J.R., R. Boyne, H.A.O. Hill and M.J. Okolow-Zubkowska, FEBS Lett. 135:187 (1981).
130. Van Dorp, D.A., Proc. Nutr. Soc. 34:279 (1975).
131. Christ-Hazelhof, E., and D.H. Nugteren, in Functions of Glutathione in Liver and Kidney (Sies, H., and A. Wendel, eds.), Springer-Verlag, Berlin, 1978, pp. 201.
132. Bryant, R.W., and J.M. Bailey, Biochem. Biophys. Res. Commun. 92:268 (1980).
133. Kien, C.L., and H.E. Ganther, Am. J. Clin. Nutr. 37:319 (1983).
134. Zhu, L., in Trace Element Metabolism in Man and Animals-4 (Howell, J.M., J.M. Gawthorne and C.L. White, eds.), Australian Academy of Science, Canberra, Aust., 1981, pp. 514.
135. Terry, R., Lancet 1:757 (1954).
136. van Rij, A.M., C.D. Thomson, J.M. McKenzie and M.F. Robinson, Am. J. Clin. Nutr. 32:2076 (1979).
137. Lawrence, R.A., and Burk, R.F., Biochem. Biophys. Res. Commun. 71:952 (1976).

[Received June 20, 1984]

URL 05451

Growth Stimulatory Activity of Unsaturated Fatty Acids for Normal and Neoplastic Breast Epithelium

WILLIAM R. KIDWELL and JOHN SHAFFER, Laboratory of Pathophysiology, National Cancer Institute, Bethesda, MD 20205

ABSTRACT

Studies with experimental animals first showed that dietary lipids in excess have a very large stimulatory effect on the development of breast tumors either induced by carcinogens or occurring spontaneously. These observations took on added significance when epidemiologists found a strong positive correlation between breast cancer incidence and the level of dietary fat. Although not unequivocably established, the total observations concerning this phenomenon suggest a cause and effect relationship between high dietary lipids and breast cancer development. In an attempt to understand how the lipids might be acting, we have begun to assess the effects of various fatty acids on the growth and function of breast epithelium from both normal and neoplastic tissue. The results to date suggest that the unsaturated fatty acids are needed for mammary cell division and that they may play roles in this process by serving as substrates for prostaglandin synthesis, as membrane structural elements or possibly as activators of C kinase when they are in the form of diglycerides. Whatever the mechanism of growth stimulation, it appears that the fatty acids are rate limiting for growth and that physiologic mechanisms for recruiting fatty acids from proximal fat cells exist within the mammary gland. It thus appears that the fat cell serves as a physiologic buffer and that exceeding this buffer such as by consuming excessive lipids may override this buffering capacity and thus favor the division of normal or neoplastic breast cells.

INTRODUCTION

Although it has been more than 40 years since the demonstration that carcinogen induced or spontaneously developing mammary tumor incidence in experimental animals was modulatable by dietary fat (1), the numerous reports on other tumor model systems that have confirmed this observation have failed to elucidate the basis for this effect. Dietary lipid effects on hormone levels (2) or on carcinogen metabolism or clearance rates (3) have been suggested, but the results from other laboratories are not consistent with either of these possibilities (4,5). The type of lipids which promote the response of mammary cells to carcinogens also has proven variable. Original reports indicated that unsaturated fatty acids were more efficient than saturated fatty acids and suggested that these compounds might act as promoters (6). More recently it has been found that a minimal amount of unsaturated fatty acid (essential fatty acid) is needed and that over and above this minimal amount an increased tumorigenic response is obtainable with additional amounts of either a saturated or an unsaturated fatty acid (7). This latter result is consistent with recent epidemiological surveys suggesting that the strongest link between dietary fat consumption and human mammary cancer incidence relates most strongly to the total amount of fat in the diet rather than to the amount of unsaturated fatty acid in the diet (8). Unfortunately, retrospective estimates of consumption are notoriously unreliable because of subjects' faulty memories and lack of accuracy concerning the actual amount of fat consumed (for example, of the amount of dietary fat available for consumption, how much is discarded in the food preparation process?). In spite of these problems, there is a strong positive correlation between lipid intake and the incidence of mammary cancer in humans and in the efficiency with which carcinogen induction of mammary cancer takes place in experimental animals.

Because of the lack of a consistent demonstration that dietary lipids affect serum mammatrophic hormone levels such as prolactin, estrogens or progestins, we have considered the possibility that lipids may directly affect the growth of the mammary epithelium and thus increase the possibility of neoplastic conversion by increasing the size of the cell population at risk. What has emerged from these studies is the fact that the growth of both normal and neoplastic mammary epithelium is facilitated by unsaturated fatty acids. Experiments both in vivo and in vitro with cultures of mammary epithelium have led us to propose that there is an integration between the mammary epithelium and mammary fat cells that is linked through mammotrophic hormones and an intermediary cell type, the mast cell. The experiments that have led us to this postulate are reviewed in this report.

RESULTS AND DISCUSSION

The Model

First let us introduce the model we have formulated and then describe briefly the experimental evidence which supports the various aspects of the model. Then we wish to speculate on the implications of the model insofar as mammary cancer and dietary lipids are concerned.

The model is presented in Figure 1, which depicts a cro

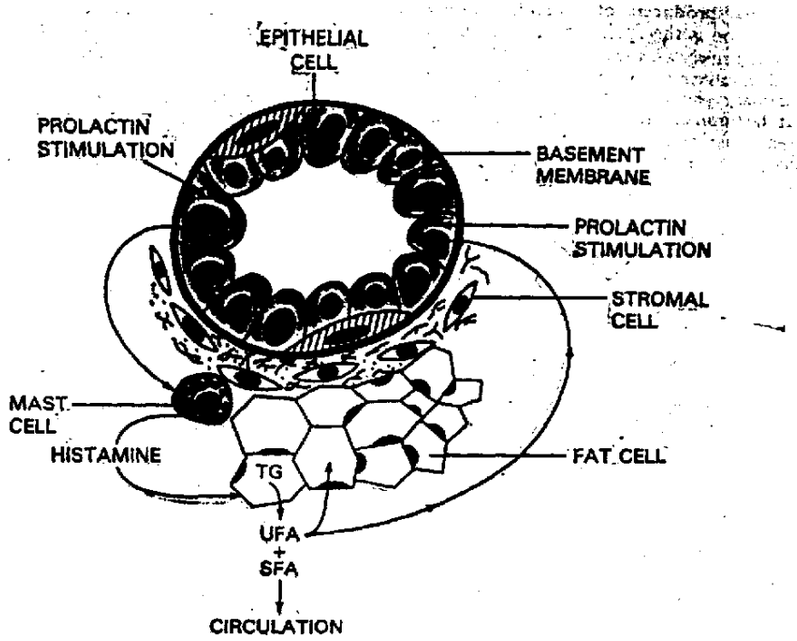


FIG. 1. Model depicting the interrelationships between mammary epithelial cells, mast cells and fat cells in the response to prolactin stimulation. See text for details. TG, triglycerides; UFA, unsaturated fatty acids; SFA, saturated fatty acids.

section of an alveolus of the mammary gland, bounded by a basement membrane which separates the mammary epithelial cells from the surrounding stromal cells, stromal collagen, adipocytes, mast cells, etc. When the glandular epithelium is stimulated to proliferate by a hormone such as prolactin, the epithelium transmits a signal of undefined nature to mast cells in the near vicinity. The mast cells then release histamine which interacts with HI receptors on the adipocytes leading to lipase activation and the release of free fatty acids.

The adipocytes release both unsaturated and saturated fatty acids and these are differentially handled by the gland. Saturated fatty acids are removed from the gland by the venous effluent. Unsaturated fatty acids are either reassimilated into triglycerides in fat cells or are taken up by the prolactin activated epithelium. These fatty acids then supplant the saturated fatty acids in membrane phospholipids and serve as substrates for prostaglandin syntheses (linoleate and arachidonate). The consequence of one or both of the latter is to enhance mammary cell growth.

EXPERIMENTAL SUPPORT FOR THE MODEL

Prolactin Stimulation of Fatty Acid Release from Mammary Adipocytes

Experiments with intact animals as well as with explant cultures of mammary tissue indicate that prolactin activates lipase in mammary adipocytes. Thus the administration of perphenazine to virgin female rats brings about a 5-10-fold elevation of serum prolactin levels (9). It also causes a rapid and massive proliferation of the mammary epithelium (9), decreases the total amount of mammary fat and increases the relative abundance of unsaturated fatty acids in the gland (10). This latter effect is so large that it must involve the mammary adipocytes which contain about 90-95% of the total mammary lipids as triglycerides.

Although perphenazine stimulation also causes an increase in serum hormones other than prolactin, it is most probable that elevated prolactin causes the changes in mammary lipid composition as shown by the effects of prolactin in explant cultures of mammary tissue (Table I). When the mammary explants are incubated with prolactin, there is a release of free fatty acids into the growth medium where they are trapped on bovine serum albumin and subsequently can be quantitated by gas chromatography (11). The prolactin effect is seen with as little as 50 ng/ml concentration (Table I).

How does prolactin affect the free fatty acid release and what cells are their source? The evidence points to the adipocytes because prolactin addition to cultures of isolated mammary epithelium actually enhances fatty acid uptake into these cells rather than their release of free fatty acids. This is especially true for the unsaturated fatty acids (11).

Prolactin Stimulation of Histamine Production

Cultures of rat mammary epithelium release histamine as shown in Table II. This production of histamine was variable from cell preparation to preparation, and histamine production in response to prolactin was seen only if the cultures contained a few contaminating mast cells. When these contaminants were eliminated by subculturing the mammary epithelium on collagen gels, prolactin no longer stimulated histamine production. These results suggested that prolactin stimulated epithelium activates the mast cells

which are the actual producers of histamine. Consistent with this interpretation is the finding that prolactin receptors are present exclusively on the mammary epithelial cells (12). A mast cell involvement in the hormonal responsiveness of the mammary gland is not surprising. It has long been noted that hormonally responsive breast tumors are very rich in mast cells in comparison to hormonally independent mammary tumors (13). Additionally, it has been shown that histamine levels in the mammary gland rise and fall in concert with the DNA synthetic activity of the epithelium and that antihistamines such as Benadryl (Diphenhydramine) block DNA synthesis in the mammary gland (14).

Histamine and FFA Release from the Mammary Gland

Other investigators have reported that histamine can activate lipases in adipocytes of peripheral tissues (15). This apparently is also the case for mammary adipocytes as shown in Figure 2. At concentrations as low as 10 ng/ml (the amount produced/24 hrs per 10^5 mammary cells), histamine causes a doubling in the amount of free fatty acids released from mammary explants. A mast cell involvement in free fatty acid release is also strongly suggested by the effects of a mast cell degranulator, 48/80 on mammary explant cultures as demonstrated in Table III. This compound, which is a calcium ionophore, increases by 5-fold the total amount of free fatty acid released.

Preliminary results suggest that histamine activates adipocytes by interacting through their H1 receptors, since Benadryl reduces the amount of free fatty acids released by the mammary explants in response to prolactin but Cymetidine (SKF 92334) (a H2 blocker) does not. However, we have yet to demonstrate the presence of high affinity histamine receptors in the gland.

Unsaturated Fatty Acids and Mammary Epithelial Cells

Previously we demonstrated that isolated mammary epithelial cells take up free fatty acids when cultured in the presence of prolactin (11). The unsaturated fatty acid linoleate was depleted from the growth medium at 100 times the efficiency of palmitic acid, while oleic acid depletion was 50 times more efficient than the saturated fatty acid. These results are consistent with a requirement of the epithelium for unsaturated fatty acids for proliferation and an inhibitory effect of saturated fatty acids on this process (10). This is true for both normal and neoplastic mammary cells in culture (10).

The Fate of Assimilated Fatty Acids In Mammary Epithelium

The various means by which unsaturated fatty acids promote mammary cell growth have not been elucidated. However it appears that at least in part they enhance growth by altering membrane composition. This conclusion is based on the compositional change in phospholipids derived from the membranes of growing vs resting mammary epithelium. For example, membranes prepared from isolated mammary epithelial cells of perphenazine treated virgin female rats contained 1.3 to 3.4 times as much linoleic acid per unit phospholipid as did the epithelial membranes of untreated animals. This enrichment of unsaturated fatty acid acyl groups of phospholipids also was observed in membranes of epithelium from pregnant vs non-pregnant animals (11).

Consequently, we have suggested that a replacement of saturated fatty acyl groups of phospholipids by unsaturated ones promotes mammary cell proliferation. This might occur because of enhanced membrane receptor accessibility or via enhanced membrane transport capabilities. However, a role of the unsaturated fatty acids, arachidonate and linolenate in prostaglandin production which might also stimulate growth also is a distinct possibility. Knazek's work (16) has demonstrated that arachidonic acid addition to liver cell membranes increases the amount of specific high affinity prolactin receptors. Furthermore, he has found that essential fatty acids are required for the induc-

TABLE I

Prolactin Stimulation of Free Fatty Acid Release From Mammary Tissue in Explant Culture

Prolactin Conc. (ng/ml)	% Stimulation of release			
	18:0	16:0	18:1	18:3
0	0	0	0	0
25	1	1	3	5
50	2	11	19	25
100	3	34	49	67
300	3	24	37	46
1000	3	23	36	42

Prolactin stimulation of free fatty acid release from mammary tissue in explant culture. Rat mammary tissue was isolated, sectioned into about 10 mg pieces and cultured in Eagle's medium containing bovine serum and prolactin (NIH S14) as described in Reference 11. Free fatty acids were recovered from the growth medium and quantitated as follows. Labeled carrier fatty acid was added to the medium after removing the tissue. An antioxidant, BHT, also was added and the lipids extracted into chloroform:methanol. The residue after evaporating the solvent was dissolved in a small amount of solvent and chromatographed on silica gel plates with a developing solvent that contained BHT. The free fatty acid areas were located on the plates by autoradiography and these areas recovered. The free fatty acids were converted to their methyl esters and quantitated on Quadrex capillary columns by gas chromatography. More complete details are given in Reference 11.

TABLE II

Prolactin Stimulation of Histamine Release From Isolated Mammary Epithelial Cells

Prolactin conc. (ng/ml)	Mast cells present	Histamine released (ng/ml)
0	Yes	5
10	Yes	22
50	Yes	31
100	Yes	35
200	Yes	30
100	No	4

Prolactin stimulation of histamine release from isolated mammary epithelial cells. Mammary ducts and alveoli were isolated from virgin female Sprague Dawley rats as described by Wicha et al. (10). The isolated epithelium was incubated in Eagle's medium supplemented with bovine serum albumin (100 μg/ml) and prolactin at the indicated concentration. Histamine released into the growth medium was quantitated by the method of Endo (25) after centrifugation to remove the cells. Briefly the method entailed the addition of trace amounts of labeled histamine, precipitation of proteins in the growth medium with cold 5% PCA, neutralization of the sample with KOH and chromatography on cellulose phosphate columns to isolate the histamine from basic amino acids and polyamines. Histamine was then quantitated on a fluorimeter after derivatization with orthothalaldehyde. Incubation was 24 hr and was performed on bacterial plastic dishes on which cell viability is maintained but neither cell growth nor attachment takes place. Mast cell presence in the isolated epithelium was quantitated by staining the cells with acridine orange and examining with a fluorescence microscope. Mast cell contamination (about 1 mast cell per 10,000 epithelial cells) was eliminated by plating the freshly isolated epithelium on rat tail collagen coated dishes on which mast cells but not epithelial cells attach. After 24 hr the mast cell-free epithelium was recovered and plated on bacterial dishes as described.

URL 05453

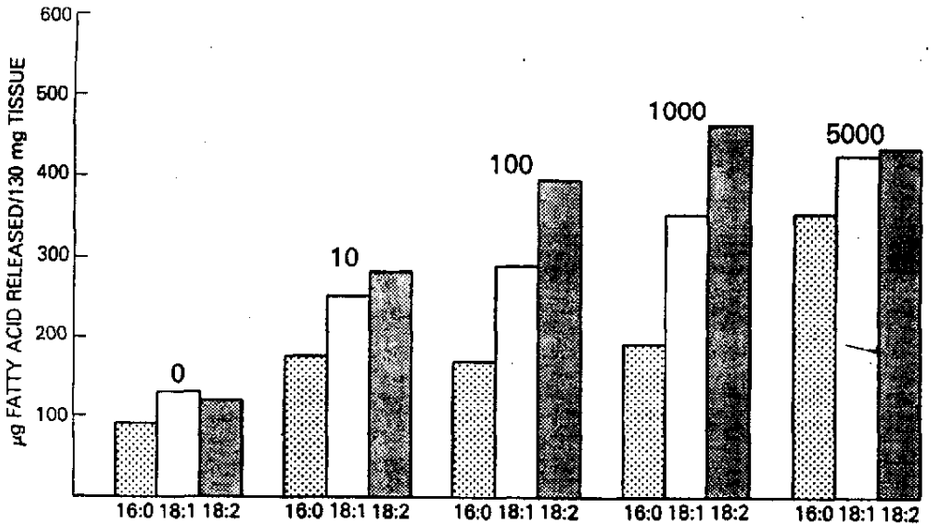


FIG. 2. Histamine stimulated release of free fatty acids from mammary tissue in explant culture. Tissue was cultured and fatty acids released were quantitated as described in Table I. Numbers over the bars on the graph indicate the concentration of histamine present (ng/ml) in the growth medium.

TABLE III

Effect of a Mast Cell Degranulator, 48/80, on Free Fatty Acid Release from Mammary Explants in Culture

Medium supplement	Free fatty acid released (µg/100 mg/24 hr)		
	16:0	18:1	18:3
None	37	49	87
48/80	163	353	276

Effect of a mast cell degranulator, 48/80, on free fatty acid release from mammary explants in culture. Explants were cultivated and the free fatty acids released quantitated as described in Table I.

tion of prolactin receptors in whole animals treated with prolactin (17) and shown that receptor induction is blocked by compounds such as indomethacin which inhibit prostaglandin synthesis (18). The active species in the induction appears to be PGI_2 (19). Other prostaglandins also may play important roles, since we have found that PGE_1 stimulates mammary cell proliferation while PGE_2 and $F_2\alpha$ do not (20). A role of prostaglandins in mammary tumor development also has been suggested by Rao and Abraham (21).

Implications for Dietary Lipid Effects on Mammary Tumorigenesis

All of our findings suggest there is indeed a cause and effect relationship between fat consumption and breast cancer incidence; the link should be more strongly manifest when there is an elevated consumption of unsaturated fatty acids rather than saturated fatty acids. These results need to be considered in relation to the epidemiological studies indicating a higher correlation between total fat consumption and breast cancer incidence than with the consumption of vegetable fat, which is richer in unsaturated fatty acids (8). This latter finding, though subject to many criticisms, is consistent with Carroll's report (7) that essential fatty acids are needed at a certain level for carcinogen-induced tumor development and that over and above this requirement either more saturated or unsaturated fatty acids will enhance tumorigenesis further.

On the face of it the growth stimulating effects of unsaturated fatty acids that we have observed in vivo and in vitro may be a reflection of the basal need of mammary cells, normal or neoplastic, for unsaturated fatty acids. Over and above this need either saturated or unsaturated fatty acids may exert their effects by indirect or direct mechanisms. It is possible to formulate models which could legitimately encompass our results with those of the epidemiological and experimental animal studies. Let us consider the involvement of adipocytes in supplying fatty acids to the mammary epithelium. The uptake and re-release of free fatty acids from adipocytes is a controlled process that normally limits the amount of free fatty acids available to the epithelium. Both the fat cell and the mammary epithelial cell selectively take up unsaturated fatty acids that are available to them. However, saturated fatty acids can compete with unsaturated fatty acids for entry into cells. Our experiments with mammary tumor cells in culture show that linoleic acid uptake into these cells is not dramatically inhibited by saturated fatty acids until the ratio of saturated to unsaturated fatty acids is about 5 to 1. Although similar analyses with mammary adipocytes have not been possible because these cells are extremely difficult to isolate, the relative enrichment of unsaturated vs saturated fatty acids in mammary fat tissue effected by perphenazine treatment in vivo is only about 1.5 to 2 to 1. In other words, a high dietary intake of lipid raises the amount of free fatty acids available to the mammary epithelium because the buffering capacity of fat cells is exceeded. However, the epithelium still can selectively take up the unsaturated fatty acids it requires even in the presence of high amounts of saturated fatty acids. In the case of dietary lipid excess, a mast cell activation for localized increase in free fatty acids would be unnecessary. However, mast cells might be important in this regard with elements other than dietary lipids, such as promoters which enhance cancer development at the progression stage.

URL 05454

Of course, it is also possible that saturated fatty acids inherently and directly promote mammary tumorigenesis by acting on the mammary epithelium. We know that saturated fatty acids inhibit the growth of both normal and neoplastic cells, but this inhibition is a little more dramatic in the case of normal cells (10). This difference may be significant, however, because the presence of normal mammary epithelial cells has been shown to suppress the conversion of preneoplastic to neoplastic mammary cells following transplantation into mammary fat pads (22). A differential inhibition of normal cells thus could favor tumor development. Clearly we are a long way from understanding how dietary lipids might influence breast cancer susceptibility but hopefully our observations and hypotheses will shed some light on the issue, or at least suggest new experimental approaches. Other exciting new ideas already are being brought forth on this subject. For example, Castenaga (23) recently has reported that tumor promoters bind to and activate a calcium and lipid dependent kinase, C kinase. The significance of this observation may be great because C kinase is markedly activated by unsaturated diacylglycerols (24) and thus these compounds may be natural promoters that act as transmembrane signals generated when growth factors interact with membrane receptors and activate phospholipase A_2.

REFERENCES

1. Tannenbaum, A., Cancer Res. 2:49 (1942).
2. Chan, P.C.; F. Didato and L.A. Cohen, Proc. Soc. Exp. Biol. Med. 149:133 (1975).
3. Janss, D.H.; R.C. Moon and C.C. Irving, Cancer Res. 32:254 (1972).
4. Chan, P.C., and T. Dao, Ibid. 41:164 (1981).
5. Welsch, C.W., and C.F. Aylsworth, Symposium of the American Oil Chemists' Society: "Dietary Fats and Health," Dec. 6-11, 1981.
6. Carroll, K.K., Cancer Res. 35:3374 (1975).
7. Hopkins, G.J., and K.K. Carroll, JNCI 62:1009 (1979).
8. Hirayama, T., Preventive Med. 7:173 (1978).
9. Wicha, M.S.; L.A. Liotta, B.K. Vonderhaar and W.R. Kidwell, Develop. Biol. 80:253 (1980).
10. Wicha, M.S.; L.A. Liotta and W.R. Kidwell, Cancer Res. 39: 426 (1979).
11. Kidwell, W.R.; R.A. Knazek, B.K. Vonderhaar and I. Losonczy, in Molecular Interrelations in Nutrition and Cancer, edited by Arnott, M., J. van Eys and T.M. Wang, Raven Press, New York, 1982, p. 219.
12. Bhattacharya, A., and B.K. Vonderhaar, Biochem. Biophys. Res. Commun. 88:1405 (1979).
13. Strum, J.W.; M. Lewko and W.R. Kidwell, Lab Invest. 45:347 (1981).
14. Schrad, P.J., and D.H. Janss, JNCI 65:949 (1980).
15. Fredholm, B.B.; H.C. Meng and S. Rosell, Life Sci. 7:1209 (1968).
16. Dave, J.R.; R.A. Knazek and S.C. Liu, Biochem. Biophys. Res. Commun. 103:727 (1981).
17. Knazek, R.A., and S.C. Liu, Proc. Soc. Exp. Biol. Med. 162: 346 (1979).
18. Knazek, R.A.; S.C. Liu and J.R. Dave, Prostaglandins and Med. 6:403 (1981).
19. Dave, J.R., and R.A. Knazek, Proc. Natl. Acad. Sci. U.S.A. 77:6597 (1980).
20. Kidwell, W.R.; M. Bano and D.S. Salomon, in Barnes, D., and G. Sato, (eds.), Growth of Cells in Hormonally Defined Medium, Alan R. Liss, Inc., New York, Vol. 2, 1984, p. 105.
21. Rao, G.A., and S. Abraham, JNCI 56:431 (1976).
22. Medina, D.; F. Shepherd and J. Gropp, JNCI 60:1121 (1978).
23. Castenaga, M., J. Biol. Chem. 257:7847 (1982).
24. Kuo, J.F., Proc. Natl. Acad. Sci. U.S.A. 77:7039 (1980).
25. Endo, Y., J. Chromatog. 205:155 (1981).

[Received June 20, 1984]

UAL 05455

Lipid Peroxide Catalyzed Chemical Carcinogenesis

PETER J. O'BRIEN, Department of Biochemistry, Memorial University of Newfoundland, St. John's, Newfoundland, Canada, A1B 3X9

ABSTRACT

Peroxides, including lipid peroxides, with heme catalysts cause the binding of C^{14}-acetylaminofluorene to DNA if microsomes are present. This binding was 96% inhibited by paraoxon, a deacetylase inhibitor. It is concluded that peroxide-peroxidase systems rapidly oxidize acetylated arylamines to proximate carcinogens following deacetylation by microsomal deacetylases. The DNA binding observed was greater than that observed with the liver microsomal mixed function oxidase catalyzed activation to N-OH-acetylaminofluorene, which binds to DNA following deacetylation by microsomal deacetylase. Lipid peroxidation or prostaglandin synthesis should therefore enhance carcinogenesis induced by arylamides.

INTRODUCTION

Multiple mechanisms for metabolic activation of chemical carcinogens exist. The activation by different target tissues also may reflect the different activating systems present. It is thought the initial activation usually involves a mixed function oxidase activity of the endoplasmic reticulum, and that this activation involves a two-electron oxidation of the polycyclic aromatic hydrocarbon to an epoxide or of an arylamine to an N-hydroxyarylamine. However, recently an initial one-electron oxidation to free radicals catalyzed by peroxidases or prostaglandin synthetase has been suggested as a first step for the activation of chemical carcinogens (1-4). Most tissues contain all three systems. However, the uterus, thyroid, salivary gland, Zymbal gland or Harderian glands are target tissues, with little cytochrome P450 and highly active peroxidases (5-8). The kidney medulla and bladder are target tissues with active prostaglandin synthetase (2). The liver hepatocyte, on the other hand, has very high levels of the mixed function oxidase activity of cytochrome P450 and little peroxidase or prostaglandin synthetase activity. The liver Kupffer cells contain a peroxidase (9). Even the apparent two-electron oxidation mechanism of mixed function oxidase function may still involve free radicals (10). It is well established that carcinogenesis induced by irradiation or ultraviolet light is free radical mediated.

Dietary fatty acid hydroperoxides can be toxic to the gastrointestinal tract and can be carcinogenic (11). Enhanced in vivo lipid peroxidation is associated with carcinogenesis induced by chlorinated hydrocarbons (12), hydrazines (13) and metals (14). Furthermore, enhanced in vivo lipid peroxidation following choline deficiency is associated with carcinogenesis (Farber, E., personal communication).

The induction of peroxisomes by hypolipidemic drugs also results in in vivo lipid peroxidation and carcinogenesis (15) although the drugs are not activated to mutagens or products which bind to DNA (16). Selenium and most antioxidants protect against chemical carcinogenesis, whereas selenium deficiency potentiates chemical carcinogenesis (17).

It has been known for some time that carcinogenic arylamines, hydrazines and polycyclic aromatic hydrocarbons are effective as antioxidants and free radical scavengers in lipid autoxidation (4). Indeed, some of them were used as such before their carcinogenic properties were discovered. This indicates that these carcinogens are readily oxidized during lipid peroxidation. Our research indicates that this oxidation can result in the carcinogen binding covalently to DNA and thus may be a carcinogenic activation mechanism (3). Lipid peroxides may therefore be able to act as a cocarcinogen or tumor promoter.

Bartsch and Hecker first demonstrated that nitroxyl free radicals were formed when N-OH-acetylaminofluorene (N-OH-AAF) was oxidized by peroxidases and H_2O_2 (18), and that they dismutate to N-acetoxy-AAF and 2-nitrosofluorene. The former readily binds covalently to DNA, tRNA and guanosine (19), whereas nitrosofluorene is one of the most mutagenic compounds known (20) and binds covalently to proteins. Floyd et al. also showed that nitroxyl free radicals were formed when N-OH-AAF was oxidized by linoleic acid hydroperoxide (LAHPO) with methemoglobin (21) or cytochrome P450 and particularly high spin cytochrome P420 (22).

Acetylaminofluorene is not oxidized by peroxidase-H_2O_2 or cytochrome P450-LAHPO systems so that the mixed function oxidase is still required for N-OH-AAF formation. Floyd (19) has suggested recently that mammary gland carcinogenesis induced by AAF may involve a peroxidase or prostaglandin synthetase catalyzed activation of N-OH-AAF.

No studies comparing different activating systems for DNA have been reported. In the following, the irreversible binding of C^{14}-AAF and C^{14}-NOH-AAF to DNA catalyzed by mixed function oxidase or peroxidase-H_2O_2 or methemoglobin-LAHPO systems are compared. It is concluded that a microsomal deacetylase is involved in AAF and NOH-AAF binding. The free radical mediated peroxidase catalyzed oxidation of N-OH-AAF results in much less binding than that with deacetylase/AAF catalyzed by the same oxidizing system. This indicates that a free radical mechanism for AAF activation may not involve an initial N-hydroxylation catalyzed by mixed function oxidase. Furthermore this mechanism, presented for the first time, shows that acetylarylamines can be activated by a free radical mechanism. It is more effective than mixed function oxidase which is normally considered the principal activation mechanism.

EXPERIMENTAL PROCEDURES

Chemicals. The following reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri); calf thymus DNA (type I), Horseradish peroxidase (type VI), porcine carboxy esterase (type I and II) and diethyl-p-nitrophenyl phosphate (paraoxon). Hydrogen peroxide was obtained from British Drug Houses Chemicals (Toronto, Canada). N-(9-^{14}C)-Acetyl-2-aminofluorene (AAF), (specific activity 50 mCi/mmol) was purchased from New England Nuclear (Boston, Massachusetts). (9-^{14}C)-N-hydroxy-acetylaminofluorene (NOH-AAF), (specific activity 25 mCi/mmol) was purchased from ICN Chemical and Radioisotopes (Irvine, California).

DNA binding. The reaction mixture (3.0 ml) for determining peroxidase-H_2O_2 catalyzed N-OH-AAF and AAF binding to DNA contained 5 μM N-(C^{14})-acetylaminofluorene or N-OH-acetylaminofluorene, 0.1 M Tris-HCl (pH 7.4), horseradish peroxidase (10 μg), hydrogen peroxide (0.5 mM) and calf thymus DNA (3 mg). Carboxyesterase II (0.1 mg) or 1 mM diethyl-p-nitrophenyl phosphate was added where indicated. The reaction was started by the addition of hydrogen peroxide and carried out for 30 min at 37 C with shaking. The reaction was stopped by extraction with 2.0 ml ethyl acetate-acetone (2:1), and the organic solvent was removed. The extraction was repeated 3 times. The residual organic solvent in the aqueous layer was then removed by bubbling with nitrogen. Following the removal of the residual organic solvent in the aqueous layer by bubbling with nitrogen gas, sodium dodecyl sulfate solution (10%, 200 μl) and protease (0.5 mg) were added and the mixture allowed to incubate at 37 C for 30 min. After digestion of any possible contaminating protein, the mixture was treated with water-saturated phenol (1 ml) and water-saturated $CHCl_3$ (1 ml) and the mixture was shaken vigorously. After centrifugation, the aqueous phase was transferred to a new test tube. The macromolecules were subsequently precipitated by the addition of NaCl (5 M, 100 μl) and ethanol (6 ml). After centrifugation, the supernatant was discarded. The macromolecules were dissolved in water (1 ml), reprecipitated with NaCl (5 M, 100 μl) and ethanol (2.5 ml), washed with ethanol (1 ml) and ether (1 ml) and dried under nitrogen. The isolated macromolecules were dissolved in water (1.0 ml). An aliquot was used for the determination of macromolecule concentration by UV absorption, and the rest was used for the measurement of the visible absorbance spectra and radioactivity of bound AAF and N-OH-AAF. The radioactivity was measured with a Beckman LS-330 scintillation counter.

Microsomal catalyzed binding. Rat liver microsomes were prepared from 200-250 g, overnight-fasted Sprague-Dawley derived albino rats. A microsomal protein concentration of approximately 1 mg/ml in Tris-HCl buffer pH 7.4 was used in the reaction mixture.

RESULTS

Bartsch and Hecker (18) showed that adducts of tRNA, guanosine and N-acetylmethionine were formed in a peroxidase-H_2O_2-N-OH-acetylaminofluorene system. The adducts were similar to that formed with N-acetoxy-2-acetylaminofluorene, a product of this system. In Table I, it can be seen that such a system also results in DNA binding. About 14% of the radioactive N-OH-acetylaminofluorene was trapped by denatured DNA, and a binding level of 158 p moles N-OH-acetylaminofluorene per mg DNA was found. Double stranded DNA had a binding level of only about one-third of that of the single stranded DNA (thermally denatured DNA). At pH 7.4 the product responsible for the binding was stable as similar binding was observed when DNA was added 2 minutes after starting the reaction. N-acetoxy-2-acetylaminofluorene is believed to be formed by the dismutation of the nitroxy radicals observed following the oxidation of N-OH-acetylaminofluorene. A yield of 17-19% N-acetoxy-2-acetylaminofluorene was observed (18). About 3% of the N-OH-AAF was trapped by tRNA (18) and 8% was trapped by N-acetyl-DL-methionine (19).

In Table I, it can be seen that methemoglobin could catalyze similar adduct formation with H_2O_2, cumene hydroperoxide or linoleic acid hydroperoxide. Floyd et al. (21) have shown that such systems form nitroxy radicals and nitrosofluorene, the other dismutation product (20).

TABLE I

NOH-Acetylaminofluorene Binding to DNA

Peroxide system	DNA binding (p mol/mg DNA) None	+ Paraoxon
None	0.6	0.6
H_2O_2 + HRP	145	140
H_2O_2 + mHb	52	48
CHP + mHb	108	99
LAHPO + mHb	6	6
Microsomes	122	5
NADPH + Microsomes	153	5
CHP + Microsomes	22	4
Esterase	247	6

The reaction mixture (3 ml) contained 3 mg single stranded DNA, 5 μM C^{14}-acetylaminofluorene, 0.1 M Tris-HCl buffer (pH 7.4), 0.5 mM H_2O_2 or cumene hydroperoxide (CHP) or 0.12 mM linoleic acid hydroperoxide (LAHPO) or 0.5 mM NADPH; 10 μg horseradish peroxidase (type VI) (HRP) or 5 μM methemoglobin (mHb) or 1 mg rat liver microsomes. The mixture was incubated for 1 hour at 37 C. Carboxyesterase (0.1 mg) or 1 mM diethyl-p-nitrophenol phosphate (paraoxon) was added where indicated. DNA was isolated as described in "methods."

They were unable to detect N-acetoxy-AAF with cumene hydroperoxide. The major product found, nitrofluorene, may be formed by the further oxidation of nitrosofluorene (34). N-acetoxy-AAF was found with a hematin-linoleic acid hydroperoxide system (22). However, as shown in Table I, cumene hydroperoxide was found to be more effective than linoleic acid hydroperoxide in DNA adduct formation. A comparison with microsomal mixed function oxidase system showed a similar level of DNA adduct formation but, surprisingly, microsomes were effective in the absence of NADPH. The latter binding was inhibited 96% by paraoxon, a microsomal deacetylase inhibitor. No inhibition of the peroxidase systems by paraoxon was found. It is concluded that DNA adduct formation catalyzed by microsomes involves NOH-aminofluorene formed by deacetylation.

C^{14}-Acetylaminofluorene was not bound by peroxidase systems to DNA, and the acetylaminofluorene (as measured by HPLC) was unmetabolized. The acetyl group presumably prevents the one electron oxidation of the amine group. However, the addition of liver microsomes or carboxyesterase (type II) to the peroxidase systems resulted in extensive binding to DNA. Adduct formation with H_2O_2, cumene hydroperoxide or linoleic acid hydroperoxide catalyzed by methemoglobin was also observed in the presence of carboxyesterase or liver microsomes. Up to 9% of the C^{14}-acetylaminofluorene was trapped by DNA to a level of 154 pmol AAF/mg DNA in a peroxidase system. This corresponds to a level of one AAF bound/20,000 nucleotides. The mixed function oxidase system (microsomes/NADPH) was much less effective and was prevented by paraoxon. Liver microsomes from 3-methylcholanthrene injected rats were much more effective. Other investigators have shown a large induction of N hydroxylation of AAF in such microsomes (24). The cytochrome P450 inhibitors, SKF-525A (0.2 mM) or 2-(2,4 dichloro-6-phenyl)-phenoxy-ethylamine (0.5 mM), inhibited the binding. Added carboxyesterase increased the microsomal activity by only a small degree, so it is clear that the microsomal mixed function oxidase mechanism involves an N-hydroxylation followed by deacetylation.

TABLE II

Acetylaminofluorene Binding to DNA

Peroxide system	DNA binding (p mol/mg DNA) None	+ Paraoxon	+ Esterase
H_2O_2 + HRP	0.2	0.2	154.1
H_2O_2 + mHb	0.2	0.2	6.2
CHP + mHb	0.2	0.2	57.1
LAHPO + mHb	0.2	0.2	7.5
H_2O_2 + HRP + Microsomes	107.5	0.9	143.2
H_2O_2 + Microsomes	0.2	0.2	0.2
CHP + Microsomes	3.7	1.2	7.5
NADPH + Microsomes	6.5	1.4	8.9
NADPH + 3MC-Microsomes	5.1	0.4	6.4

Reaction conditions as described in the legend to Table I.

DISCUSSION

N-hydroxylation of AAF by a cytochrome P448 dependent monoxygenase followed by activation by cytosolic sulfotransferases or seryltransferase is believed to form the AAF-DNA adducts that are formed in vivo (25). However, 80% of the adducts formed in vivo are deacetylated and activation by a microsomal-N,O-acetyltransferase has been implicated (25). The experiments reported above suggest that a microsomal deacetylase catalyzes the activation.

As the deacetylase inhibitors paraoxon and toluenesulfonyl-fluoride completely inhibited microsomal mixed function oxidase catalyzed DNA binding by acetylaminofluorene or N-OH-acetylaminofluorene suggests that microsomal deacetylase activates N-OH-AAF and that N-OH-aminofluorene binds to DNA. A nonenzymatic reaction of N-OH-aminofluorene with nuclear DNA was also the explanation given for the DNA binding following the incubation of aminofluorene with rat liver nuclei in the presence of a NADPH generating system (28). The N-OH-aminofluorene formed probably reacts via the nitrenium ion with nucleophilic sites on nucleic acids and proteins at an acidic pH (28). Nuclei also have a paraoxon sensitive deacetylase (29). N-OH-AAF can be converted readily to N-OH-AF by microsomal deacetylases (26,27). Deacetylase inhibitors also decrease the covalent binding of N-OH-AAF to microsomal protein (29,30), its mutagenic activation (32) and its binding to nuclear DNA (31). The O-glucuronide detoxification product of N-OH-AAF is also activated by a microsomal deacetylase to form tRNA adducts (33).

In the absence of a microsomal deacetylase, N-OH-AAF could be activated to DNA reacting species by a free radical mechanism involving H_2O_2-peroxidase or lipid peroxide-methemoglobin systems (Table II). However, in the presence of microsomes the deacetylase mechanism predominated as shown by the inhibition by paraoxon. The activation by a prostaglandin synthetase-arachidonate system using sheep vesicular gland microsomes also was found to be inhibited by paraoxon.

The liver microsomal catalyzed activation of N-OH-AAF was unaffected by NADPH. Cumene hydroperoxide can catalyze cytochrome P450 function when substituted for NADPH (33). However, the liver microsomal catalyzed activation of N-OH-AAF was decreased 90% by cumene hydroperoxide, which may indicate that a 1e oxidation to nitroxy radicals does not occur but rather a competing 2e oxidation to nitrosofluorene occurs followed by further oxidation to nitrofluorene (34). Electron spin resonance studies also indicate no nitroxy radical formation (Nagata, C., personal communication).

By contrast, AAF was poorly oxidized by the above free

URL 05457

radical systems but was readily activated to DNA reacting species in the presence of microsomes or carboxyesterase. The activation was prevented by paraoxon. Interestingly, the free radical systems were much more effective than the mixed function oxidase-cytochrome P450 activity. Although microsomal deacetylases deacetylate AAF more slowly than NOH-AAF (27), they are clearly active enough to participate in the activation of AAF. It is not known which of the aminofluorene oxidation products bind to DNA. Recently, evidence has been presented showing that aminofluorene is oxidized by peroxidase-H_2O_2 or prostaglandin synthetase-arachidonate systems to azofluorene and 2-nitrofluorene (39). The above findings have important implications for arylamine carcinogenesis. The action of deacetylases *in vivo* on acetylated arylamines will result in the formation of excellent substrates for free radical systems. The latter systems include peroxidases and prostaglandin synthetase and are particularly active in nonhepatic tissues, e.g., bladder, mammary gland, Zymbal gland and Harderian gland where mixed function oxidase is very low.

An alternative mechanism for the association of lipid peroxidation and carcinogenesis could be the result of dialdehyde and aldehyde products formed following the decomposition of lipid peroxides. Our previous research demonstrates extensive irreversible binding of C^{14}-arachidonate to DNA following peroxidation catalyzed by lipoxygenase, prostaglandin synthetase or microsomal fractions from various tissues (35). Others have also demonstrated fluorescent Schiff base formation with the nucleic acid bases when malondialdehyde binds to DNA (35). However, malondialdehyde is much less mutagenic than formaldehyde or glutaraldehyde or glyoxal (36). Recently formaldehyde has been shown to be a carcinogen (37). However, the mutagenicity and carcinogenicity of the various aldehydes and dialdehydes formed during lipid peroxide decomposition is unknown. The lipid peroxides may also prove to be highly mutagenic, as cumene hydroperoxide and t-butyl hydroperoxide are more mutagenic than the above aldehydes (37).

ACKNOWLEDGMENTS

Wayne Marshall provided technical assistance. This work was supported by the National Cancer Institute of Canada and the National Research Council of Canada.

REFERENCES

1. Marnett, L.J., P. Wlodawer and B. Samuelsson, J. Biol. Chem. 250:8510 (1975).
2. Zenser, T.V., M.B. Mattammal and B.B. Davis, J. Pharmacol. Exp. Ther. 211:460 (1979).
3. O'Brien, P.J., in "Prostaglandins and Cancer," T. Powles, R.S. Bockman, K.V. Hann and P. Ramwell, eds., p. 155, Alan R. Liss, Inc., New York (1981).
4. O'Brien, P.J., in "Lipid Peroxides in Biology and Medicine," K. Yagi, ed., Academic Press, New York, 1982, p. 317.
5. Strum, J.M., and M.J. Karnovsky, J. Cell Biol. 44:655 (1970).
6. Herzog, V., and H.D. Fahimi, Histochem. 46:273 (1976).
7. Morrison, M., and P.Z. Allen, Science 152:1626 (1966).
8. Osborne, J.C., M. Metzler and H.G. Neumann, Cancer Lett. 8:221 (1980).
9. Fahimi, H.D., J. Cell. Biol. 47:247 (1970).
10. O'Brien, P.J., in "Free Radicals in Biology," Volume VI, W. Pryor, ed., Academic Press, New York, 1984, p. 289.
11. Cutler, M.G., and M.A. Hayward, Nutr. Metab. 16:87 (1974).
12. Stohs, S.J., M.Q. Hassan and W.J. Murray, Biochem. Biophys. Res. Comm. 111:855 (1983).
13. Jain, S.K., and P. Hochstein, Biochim. Biophys. Acta 586: 128 (1978).
14. Ramsoeck, E.R., W.G. Hoekstra and H.E. Ganther, Toxicol. Appl. Pharmacol. 54:251 (1980).
15. Warren, J.R., V.F. Simmon and J.K. Reddy, Cancer Res. 40: 36 (1980).
16. Cohen, A.J., and P. Grasso, Fd. and Cosmetics Toxicol. 19: 585 (1981).
17. Wattenberg, L.W., Adv. in Cancer Res. 28:197 (1979).
18. Bartsch, H., and E. Hecker, Biochim. Biophys. Acta 237:567 (1971).
19. Bartsch, H., J.A. Miller and E.C. Miller, Ibid. 273:40 (1972).
20. Hampton, M.J., R.A. Floyd, J.B. Clark and J.H. Lancaster, Mutation Res. 69:231 (1980).
21. Floyd, R.A., Can. J. Chem. 60:1577 (1982).
22. Floyd, R.A., L.M. Soong and P.L. Culver, Cancer Res. 36:1510 (1976).
23. Floyd, R.A., In "Prostaglandins and Cancer," T. Powles, R.S. Bockman, K.V. Honn and P. Ramwell, eds., p. 167, Alan R. Liss, Inc., New York (1981).
24. Son, O.S., J.W. Fowble, D.D. Miller and D.R. Feller, Toxicol. Appl. Pharmacol. 51:367 (1979).
25. King, C.M., and W.T. Allaben, in "Enzymatic Basis of Detoxification," Vol. 2, p. 187, W. Jakoby, ed., Academic Press, New York (1980).
26. Grantham, P.H., E.K. Weisburger and J.H. Weisburger, Biochim. Biophys. Acta 197:414 (1965).
27. Irving, C.C., Cancer Res. 26:1390 (1966).
28. Frederick, C.B., J.B. Mays, D.M. Ziegler, F.P. Guengerich, and F.F. Kadlubar, Ibid. 42:2671 (1982).
29. Kaderbhai, M.A., T.K. Bradshaw and R.B. Freedman, Chem. Biol. Interacns. 36, 211 (1981).
30. Sakai, S., C.E. Reinhold, P.J. Wirth and S.S. Thorgeirsson, Cancer Res. 36:2058 (1978).
31. Schut, H.A.J., P.J. Wirth and S.S. Thorgeirsson, Molec. Pharmacol. 14:682 (1978).
32. Cardona, R.A., and C.M. King, Biochem. Pharmacol. 25:1051 (1976).
33. O'Brien, P.J., Pharmacol. Ther. Part A, 2:517 (1978).
34. Floyd, R.A., in "Free Radicals in Biology," Vol. IV, W. Pryor, ed., p. 187, Academic Press, New York (1980).
35. Vasdev, S., and P.J. O'Brien, in "Prostaglandins and Cancer," p. 163, T. Powles, R.S. Bockman, K.V. Honn and P. Ramwell, eds., Alan R. Liss, Inc., New York (1981).
36. Reiss, U., and A.L. Tappel, Lipids 8:199 (1973).
37. Levin, D.E., M. Hollstein, M.F. Christman, E.A. Schwiers, and B.N. Ames, Proc. Natl. Acad. Aci. 79:7445 (1982).
38. Swenberg, J.A., W.D. Kerns, R.I. Mitchell, E.J. Gralla, and K.L. Pavkov, Cancer Res. 40:3398 (1980).
39. Boyd, J.A., D.J. Hanvan and T.E. Eling, J. Biol. Chem. 258: 8246 (1983).

[Received June 20, 1984]

URL 05458

Lipid Oxidation: Mechanisms, Products and Biological Significance

E.N. FRANKEL, Northern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Peoria, IL 61604

ABSTRACT

This paper reviews our studies of fatty acid hydroperoxides, their secondary products and mechanisms for their formation in the context of some of their possible biological consequences. The uneven distribution of isomeric hydroperoxides in oxidized linolenate and photosensitized oxidized linoleate is related to the formation of hydroperoxy cyclic peroxides. Interest in the hydroperoxy mono- and bi-cycloendoperoxides from oxidized linolenate stems from their structural relationship to the prostaglandins. However, the biological activity of hydroperoxy cyclic peroxides formed by autoxidation has not yet been reported. Thermal decomposition studies of secondary lipid oxidation products show they are important precursors of volatile compounds. An acid-acetalation decomposition procedure establishes that 5-membered hydroperoxy cyclic peroxides and 1,3-dihydroperoxides are important precursors of malonaldehyde. This approach provides a more specific test than the thiobarbituric acid (TBA) color reaction to evaluate lipid oxidation products as sources of malonaldehyde and its biological effects due to crosslinking. A better understanding is needed of the biological effects of a multitude of lipid oxidation decomposition products other than malonaldehyde.

INTRODUCTION

The biological consequences of oxidized lipids that arise from *in vivo* reactions or from ingested foods long have attracted the attention of biochemists and food scientists. Many researchers are now working worldwide on a wide assortment of biological systems in which lipid peroxides and free radicals are implicated, including cancer, strokes, atheriosclerosis, inflammation and the aging process. Many lipid oxidation products are known to interact with biological materials to cause cellular damage. Bifunctional secondary products of lipid oxidation such as malonaldehyde are powerful crosslinking agents, and react with amino groups of enzymes, proteins and DNA. The resulting conjugated Schiff bases produced by crosslinking are fluorescent. The degree of fluorescence correlates with loss of template activity, which also is related to aging. The biological aspects of lipid oxidation have thus become the subject of a very active area of research, and many reviews have appeared (1-12).

This paper reviews our structural studies of primary and secondary products of lipid oxidation, their volatile and nonvolatile decomposition products, mechanisms for their formation and some of their biological consequences. It must be emphasized at this point that much of the work on biological effects of lipid peroxidation is based on inferential evidence. Much more research is needed to establish a more direct causal relationship.

FREE RADICAL AUTOXIDATION

The reaction of oxygen with unsaturated lipids (LH) involves free radical initiation, propagation and termination processes (13). Initiation takes place by loss of a hydrogen radical in the presence of trace metals, light or heat. The resulting lipid free radicals (L·) react with oxygen to form peroxy radicals (LOO·). In this propagation process, LOO· react with more LH to form lipid hydroperoxides (LOOH), which are the fundamental primary products of autoxidation.

$$\text{LH} \xrightarrow{\text{initiator}} \text{L}\cdot + \text{H}\cdot$$

$$\text{L}\cdot + \text{O}_2 \longrightarrow \text{LOO}\cdot$$

$$\text{LOO}\cdot + \text{LH} \longrightarrow \text{LOOH} + \text{L}\cdot$$

Antioxidants (AH) can break this chain reaction by reacting with LOO· to form stable radicals (A·) which are either too unreactive or form nonradical products.

$$\text{LOO}\cdot + \text{AH} \rightleftharpoons \text{LOOH} + \text{A}\cdot$$

$$\left.\begin{array}{l}\text{A}\cdot + \text{LOO}\cdot \longrightarrow \\ \text{A}\cdot + \text{A}\cdot \longrightarrow\end{array}\right\} \text{Nonradical products}$$

Decomposition of lipid hydroperoxides constitutes a very complicated process and produces a multitude of materials that may have biological effects and cause flavor deterioration in fat-containing foods. This decomposition proceeds by homolytic cleavage of LO-OH to form alkoxy radicals LO·. These radicals undergo carbon-carbon cleavage to form breakdown products including aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones (14,15).

Lipid hydroperoxides can react again with oxygen to form such secondary products as epoxyhydroperoxides, ketohydroperoxides, dihydroperoxides, cyclic peroxides and bicyclic endoperoxides. These secondary products can in turn decompose like monohydroperoxides to form volatile breakdown products. Lipid hydroperoxides also can condense into dimers and polymers that also can break down and produce volatile materials.

$$\text{LOOH} \left\{\begin{array}{l}\nearrow \text{Secondary monomeric products} \\ \searrow \text{Dimers and polymers}\end{array}\right\} \longrightarrow \text{Volatile breakdown products}$$

Finally, lipid hydroperoxides and some of their bifunctional breakdown products can interact with proteins, membranes and enzymes (16-21). These reactions with biological components are of most concern to biochemists because they can affect vital cell functions (1-12). Membrane deterioration caused by free radical mediated reactions contributes to the aging process (6). Age pigments known as lipofuscin are formed by this process and can be retarded by the administration of antioxidants such as vitamin E (10,22-28). The development of fat rancidity in complex food systems is also greatly affected by the interactions of proteins and amino acids with lipid oxidation products. Complex high-molecular-weight interaction products are formed during processing and cooking of foods, and their further degradation into volatile compounds is not well understood (29-32).

PHOTOSENSITIZED OXIDATION

Another important way that unsaturated lipids can be oxidized involves exposure to light and a sensitizer (sens) such as chlorophyll. By this non free radical process, oxygen becomes activated to the singlet state by transfer of

URL 05459

energy from the photosensitizer. The resulting singlet oxygen ($^1O_2$) produced by this process is extremely reactive. Linoleate is reported to react at least 1500 times faster with $^1O_2$ than with normal oxygen in the triplet ground state ($^3O_2$) (33).

$$\text{Sens} \longrightarrow \text{Sens}^* \text{ (excited)}$$
$$\text{Sens}^* + {}^3O_2 \longrightarrow {}^1O_2 + \text{Sens}$$
$$^1O_2 + \text{LH} \longrightarrow \text{LOOH}$$

This hydroperoxidation reaction is so rapid that it has been postulated as a process to initiate free radical autoxidation. Natural quenchers such as carotenoids protect lipids against photosensitized oxidation by interfering with this process (34). Further oxidation and decomposition of lipid hydroperoxides from photosensitized oxidation produce some of the same and some different breakdown products, which may have an impact on biological interactions similar to that of the corresponding hydroperoxides formed by free radical autoxidation.

In vivo LIPID OXIDATION

Much attention has been given to the problems of measuring lipid oxidation in biological systems (7,10,35,36). The formation of fluorescent conjugated Schiff bases by the interaction of amino acids, esters and amines with malonaldehyde has been suggested for a long time as a measure of in vivo lipid oxidation. The biological consequences of their reactions are the same as those of monohydroperoxides. Malonaldehyde has long been used as a model for secondary products of lipid peroxidation. However, as will be discussed later, a multitude of other bifunctional secondary oxidation products are known that can act as potential crosslinkers and may interact with proteins and DNA to cause biological damage.

The analyses of hydrocarbons in the breath of experimental animals has been used extensively as a sensitive index of in vivo lipid oxidation. This noninvasive method has received much attention lately (7,10). It is based on the observed increase in ethane and pentane in animals on a vitamin E-deficient diet, and by the effect of metal catalysts and oxidative agents such as ozone, carbon tetrachloride, ethanol and nitrogen dioxide. Antioxidants and selenium decrease the release of these respiratory hydrocarbons.

The most notable defense mechanisms that the body has against in vivo lipid oxidation include vitamin E and other natural antioxidants, and protective enzymes such as glutathione peroxides and superoxide dismutase. Vitamin E is the most effective in vivo inhibitor of lipid oxidation (23,28,37). In addition to its dual effects as a free radical and $^1O_2$ scavenger, vitamin E may have other cellular effects in protecting the integrity of membranes (25). Glutathione peroxidase catalyzes the reduction of hydroperoxides into innocuous alcohols, which are thus stabilized and no longer decompose into harmful aldehydes and other breakdown products (10). Superoxide dismutase removes superoxide (O_2^-), which is toxic to the cell, by converting it to hydrogen peroxide and normal $^3O_2$.

$$2O_2^- \xrightarrow{2H^+} H_2O_2 + {}^3O_2$$

A metal-catalyzed interaction between O_2^- and H_2O_2 generates a potent oxidant postulated to be $^\bullet OH$, which can cause strand breaks in DNA; superoxide dismutase prevents this damage on DNA (38,39). During aging or under diseased conditions, the lowering of the concentration of protective enzymes may reduce these body defense mechanisms against the damage from free radicals and activated species of oxygen. Whether vitamin E would retard the aging process in higher animals is questionable (6).

HYDROPEROXIDATION OF UNSATURATED FATTY ACIDS

The mechanisms of hydroperoxide formation were reviewed previously for different unsaturated fatty acids (13,40). Further mechanistic studies have been published recently on the stereochemistry of linoleic and arachidonic acid oxidation (41,42). The formation of hydroperoxides by free radical autoxidation and photosensitized oxidation is summarized here to enable us to compare their isomeric distributions and understand the structures of the resulting secondary products.

According to the well-recognized mechanism of oleate autoxidation, hydrogen abstraction from the allylic methylenes on carbon-8 and carbon-11 produces 2 allylic radicals in which electrons are delocalized through 3-carbon systems (Fig. 1). These radicals react with O_2 at the end positions to produce a mixture of 8-, 9-, 10- and 11-hydroperoxide isomers. According to this mechanism, these 4 isomeric hydroperoxides would be formed in equal amounts. However, recent studies based on GC-MS (43,44) and HPLC (45) analyses show that the mechanism for oleate autoxidation is more complicated than that depicted in Figure 1, because the 8- and 11-hydroperoxide isomers are formed in small but consistently higher amounts (27%) than the 9- and 10-hydroperoxide isomers (23%). ^{13}C-NMR studies (44) also show that small amounts of *cis*-9 and *cis*-10-hydroperoxides and large amounts of *trans*-8 and *trans*-11-hydroperoxides are formed in autoxidized oleate. These results suggest a somewhat greater reactivity of carbon-8 and carbon-11 with O_2 and a change in the

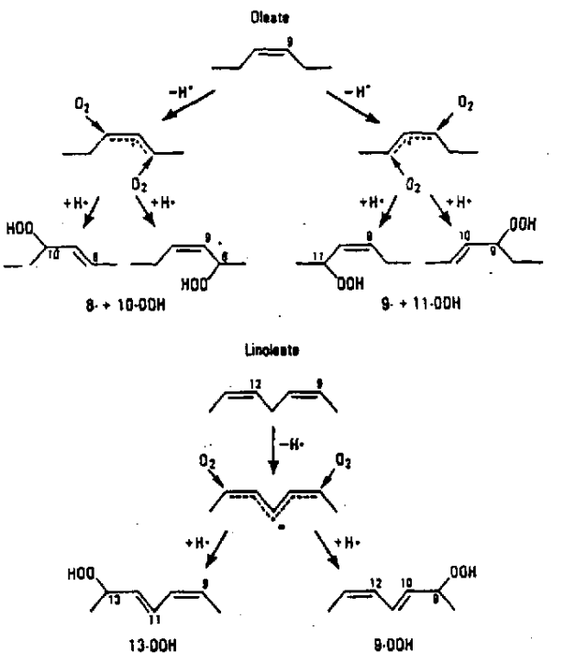


FIG. 1. Mechanism of oleate and linoleate autoxidation (40).

URL 05460

conformation of the allylic radical intermediates (40,46).

The classical mechanism of linoleate autoxidation proceeds by hydrogen abstraction from the doubly allylic methylene on carbon-11 to produce a delocalized pentadienyl radical. Oxygen attack at the end positions produces an equal mixture of conjugated 9- and 13-hydroperoxide isomers with the *trans,cis*-configuration (Fig. 1). Experimentally, a significant proportion of the conjugated hydroperoxides assume the *trans,trans* configuration, which increases with the level and temperature of autoxidation. The mechanisms for this change in hydroperoxide configuration have been discussed previously (40,42).

The mechanism of linolenate autoxidation is based on that of linoleate. Hydrogen abstraction on the doubly allylic methylenes on carbon-11 and carbon-14 produces 2 pentadienyl radicals. O_2 attack at the end positions of these radicals produces a mixture of 9-, 12-, 13- and 16-conjugated diene-triene hydroperoxide isomers (Fig. 2). Our early studies based on chemical cleavage analysis (47) were fully confirmed recently by GC-MS (48) and HPLC

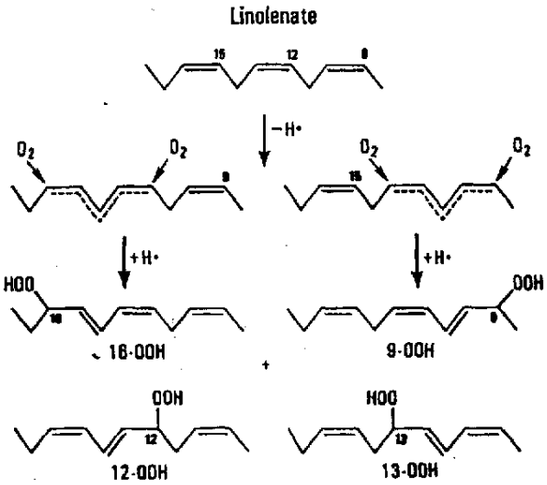


FIG. 2. Mechanism of linolenate autoxidation (47).

(49) studies in showing a significantly higher proportion of the outer 9- and 16-hydroperoxides than of the internal 12- and 13-hydroperoxides. This uneven distribution of isomeric hydroperoxides recently has been shown to be due to the 1,3-cyclization of the internal 12- and 13-hydroperoxide isomers into hydroperoxy cyclic peroxides (50). This cyclization of homoallylic hydroperoxides of linolenate also can be accompanied by a second cyclization to form bicycloendoperoxides structurally related to the prostaglandins (51).

Autoxidation of arachidonate proceeds by the same mechanism as linoleate. Hydrogen abstraction at the three doubly allylic carbons −7, −10 and −13 produces 3 pentadienyl radicals. O_2 attack at the end positions of these radical intermediates produces 6 isomeric hydroperoxides with a conjugated diene system and 2 methylene-interrupted double bonds (Fig. 3). Like in linolenate, the external 5- and 15-hydroperoxide isomers are formed in relatively higher concentrations than the internal 8-, 9-, 11- and 12-hydroperoxide isomers (41), presumably because of their tendency to cyclize.

Photosensitized oxidation of unsaturated fatty acids proceeds by a different nonradical mechanism than autoxidation. There is a direct reaction of $^1O_2$ with the carbon-carbon double bond by a concerted "ene" addition, and hydroperoxides are formed at each unsaturated carbon. Thus, oleate produces 2 isomers: the 9- and 10-hydroperoxides with allylic *trans* double bond. Linoleate produces 4 isomers: 2 conjugated 9- and 13-diene hydroperoxides (as in autoxidation) and 2 unconjugated 10- and 12-diene hydroperoxides (different from autoxidation) (Fig. 4). Similarly, linolenate produces 6 isomers: the 9-, 12-, 13- and 16-isomers are the same as in autoxidation, and the 10- and 15- are different. According to the concerted ene addition mechanism for $^1O_2$, a statistical distribution of isomeric hydroperoxides would be expected in all unsaturated fatty acids. However, our results (52,53), which were confirmed by others (54), show an uneven distribution of hydroperoxide isomers in linoleate and linolenate.

The different distributions of hydroperoxide isomers produced by autoxidation and photosensitized oxidation are summarized in Table I. It is important to note again that the internal isomers of autoxidized linolenate (12- + 13-OOH) and of photosensitized oxidized linoleate (10- + 12-OOH) and linolenate (10- + 12- + 13- + 15-OOH) are

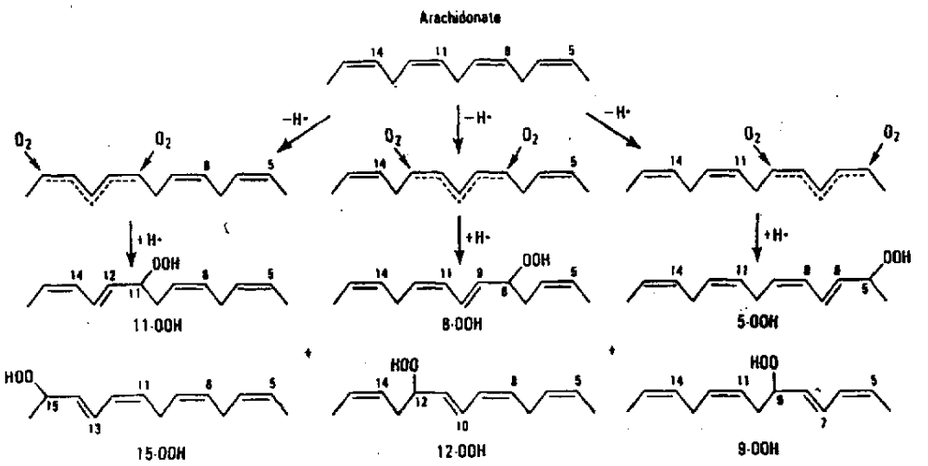


FIG. 3. Mechanism of arachidonate autoxidation.

URL 05461

formed in significantly lower concentrations than the external isomers. The reason for these uneven distributions of hydroperoxides is that the internal isomeric hydroperoxides have a homoallylic structure that permits 1,3-cyclization to form hydroperoxy cyclic peroxides.

SECONDARY OXIDATION PRODUCTS

Because of their structural relationship with the prostaglandins, much attention has been given recently to the cyclic peroxides formed from polyunsaturated fatty acids by autoxidation, enzyme oxidation and photosensitized oxidation (35,48,51,56-65). The precursors of materials

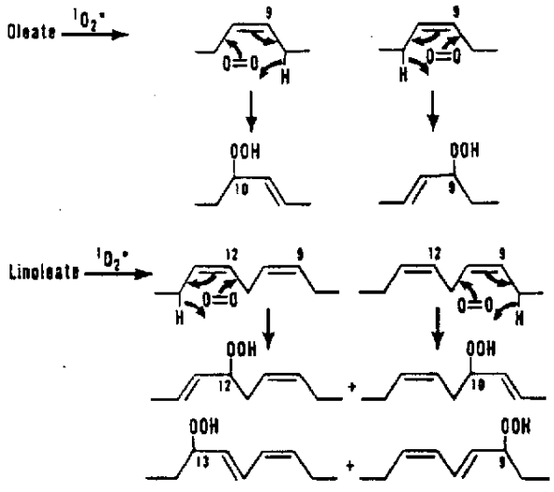


FIG. 4. Mechanism of photosensitized oxidation (13).

that react with thiobarbituric acid (TBA) and prostaglandin E were shown to be mono- and bicycloendoperoxides formed during the autoxidation of linolenate and other fatty acids containing more than 2 double bonds (61). The mechanism first advanced for the formation of these cyclic peroxides involves 1,3-cyclization of the homoallylic hydroperoxide isomers (12- + 13-OOH) of linolenate (Fig. 5). Recent studies have confirmed the formation of monocyclic peroxides from autoxidized methyl linolenate (50,51,66) and from photosensitized oxidized methyl linoleate (65,67) and linolenate (68), and bicycloendoperoxides from autoxidized 13-linolenate hydroperoxides (51) as well as from photosensitized oxidized methyl linolenate (68). Bis-cyclic peroxides also were identified in photosensitized oxidized linolenate (68) and in a free radical-initiated autoxidation of the 15-hydroperoxide isomer of arachidonic acid (69). Six-membered hydroperoxy cyclic peroxides also have been prepared by the photosensitized oxidation of methyl linoleate hydroperoxides (70). Figure 6 summarizes the general structures of different hydroperoxy cyclic peroxides identified in autoxidized linolenate and photosensitized oxidized linoleate and linolenate.

Much interest has been generated in the bicycloendoperoxides identified in oxidized linolenate because of their structural relationship to the prostaglandin endoperoxides formed biosynthetically from arachidonic acid (64) (Fig. 7). Prostaglandins have extremely potent physiological activities. PGH_2 and thromboxane aggregate platelets, whereas PGI_2 inhibit platelet aggregation. These materials thus have been implicated in the inflammatory process in heart attacks, strokes and smooth muscle contraction (64). The bicycloendoperoxides from linolenate were shown to have mainly *cis* substituents (51), in contrast to the *trans* stereochemistry of the prostaglandins derived enzymatically from arachidonic acid (64). The physiological importance of this difference in stereochemistry between the nonenzymatic and enzymatically produced bicyclic peroxides remains to be established.

TABLE 1

Isomeric Distributions of Fatty Acid Hydroperoxides

Fatty acids	Isomeric hydroperoxides,[a] %					
	Free radical autoxidation[b]					
Oleate	8-OOH 27	9-OOH 23	10-OOH 23	11-OOH 27		
Linoleate		9-OOH 50	13-OOH 50			
Linolenate	9-OOH 30	12-OOH 12	13-OOH 12	16-OOH 46		
	Photosensitized oxidation[c]					
Oleate		9-OOH 50	10-OOH 50			
Linoleate		9-OOH 31	10-OOH 18	12-OOH 18	13-OOH 33	
Linolenate	9-OOH 21	10-OOH 13	12-OOH 13	13-OOH 14	15-OOH 13	16-OOH 25

[a]Mean values from GC-MS analyses of samples oxidized to different peroxide values and at different temperatures.
[b]References (43,48,55).
[c]Reference (52).

URL 05462

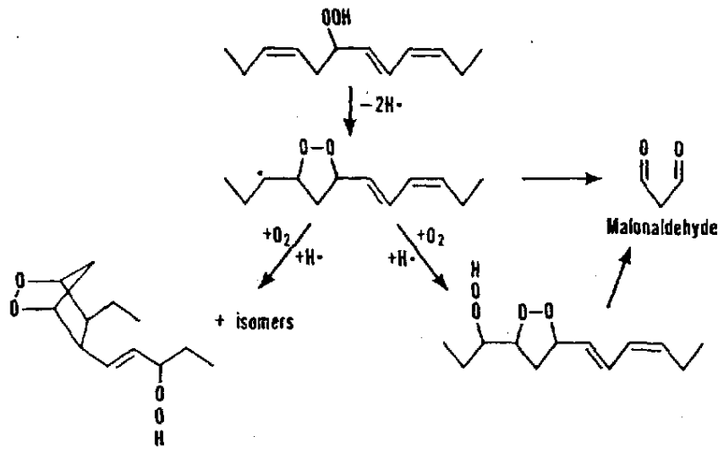


FIG. 5. Mechanism of 1,3-cyclization of 12- and 13-hydroperoxides of linolenate and formation of malonaldehyde (61).

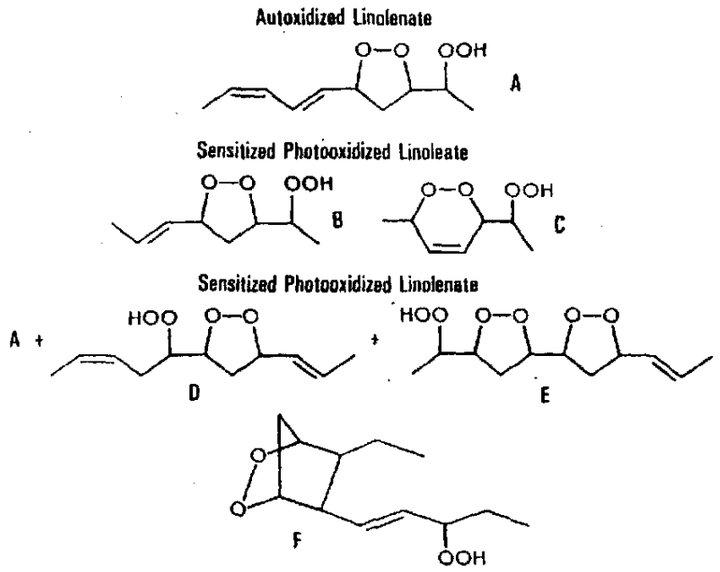


FIG. 6. Structures of hydroperoxy cyclic peroxides.

Other secondary products identified in highly oxidized oleate include saturated epoxy esters, allylic hydroxy- and ketoenes and saturated and monounsaturated dihydroxy esters. From highly oxidized linoleate, a multitude of di- and tri-oxygenated compounds also have been identified, including keto- or hydroxyepoxyene, epoxyenes, diketo- or dihydroxyenes as well as trioxygenated derivatives (71,72) (Fig. 8). The allylic ketoenes and ketodienes from oxidized oleate and linoleate respectively were shown to be particularly active in promoting the induction of nutritional encephalopathy in chicks (73).

In autoxidized methyl linolenate, hydroperoxy cyclic peroxides are formed in the same order of magnitude as the monohydroperoxides (Table II). These secondary products are formed so rapidly that, kinetically, they can be regarded as "primary" products in the sequence of events during autoxidation of linolenate. Dihydroperoxides are the next most important secondary products in autoxidized linolenate. In photosensitized oxidized linoleate and linolenate, the hydroperoxy cyclic peroxides are less important and constitute about 10% of the monohydroperoxides (Table III). Dihydroperoxides are also important secondary products of photosensitized oxidation (67,68). Bicycloendoperoxides and bis-cyclic peroxides are only minor products of linolenate.

Malonaldehyde is claimed to be an important biological

URL 05463

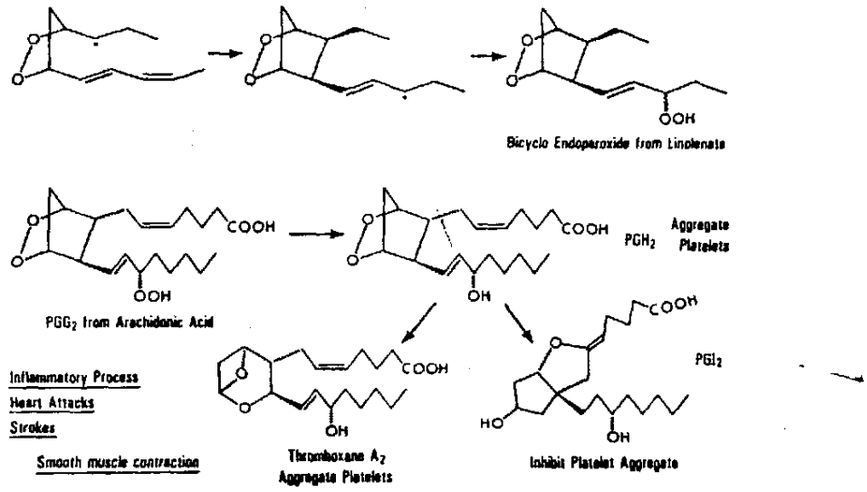


FIG. 7. Relationship between hydroperoxy bicycloendoperoxides and prostaglandins (51,64).

TABLE II

Autoxidation Products of Methyl Linolenate[a]

Compounds	Peroxide values (PV) 904	1286
Unoxidized ester	87.9%	74.8%
Epoxy esters	0.2	0.3
Monohydroperoxides	3.5	8.4
Hydroperoxy cyclic peroxides	3.8	7.7
Epoxyhydroxy dienes	0.1	0.1
Dihydroperoxides	0.9	2.9
Unidentified polar materials	3.7	5.9

[a]Based on weight-per cent composition of fractions isolated by silicic acid column chromatography (50).

TABLE III

Photosensitized Oxidation Products of Methyl Linoleate and Methyl Linolenate[a]

Compounds	Linoleate[b] (PV 1947)	Linolenate[c] (PV 1956)
Unoxidized esters	70.2%	65.5%
Keto/epoxy esters	1.2	1.6
Monohydroperoxides	24.3	25.6
Hydroperoxy cyclic peroxides	2.8	2.2
Hydroperoxy bicycloendoperoxides	—	0.1
Dihydroperoxides	0.9	3.0
Hydroperoxy bis-cyclic peroxides	—	1.0
Unidentified polar materials	0.6	1.0

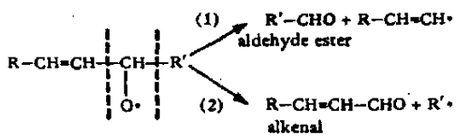
[a]Based on weight-per cent composition of fractions isolated by silicic acid column chromatography.
[b]Reference (67).
[c]Reference (68).

breakdown product expected from 5-membered cyclic peroxides of linoleate and linolenate (60,61) because of its crosslinking ability with amino groups of proteins, enzymes and DNA (10). Higher dialdehydes than malonaldehyde also may be derived from dihydroperoxides, 6-membered cyclic peroxides and other polyfunctional secondary products of linoleate and linolenate. The importance of these secondary oxidation compounds in crosslinking with amino groups and other functional groups of biological materials remains to be established.

VOLATILE DECOMPOSITION PRODUCTS

Hydroperoxide decomposition involves a very complicated set of reaction pathways. The volatile decomposition products have been studied extensively because of their impact on flavors and odors formed during deterioration of lipid-containing foods. More attention has been given to this problem recently by biochemists because the analysis of hydrocarbons in the breath of animals has proved to be a sensitive index of in vivo lipid oxidation. The mechanistic concepts for the formation of volatile lipid oxidation products were reviewed previously (15). Only a few of the fundamentals will be covered here and some of our more recent studies on the volatile decomposition compounds from secondary lipid oxidation products.

A generally accepted and authenticated scheme for the fragmentation of monohydroperoxides involves carbon-carbon cleavage on either side of the alkoxy radical to produce 2 types of aldehydes, an olefin radical and an alkoxy radical.



R' = ester end
R = hydrocarbon end

These radicals can, in turn, react with either •OH or H•. The vinyl alcohol derived from the reaction with •OH is unstable and tautomerizes to a saturated aldehyde.

$$R–CH=CH• + •OH \rightarrow R–CH=CH–OH \rightleftarrows R–CH_2–CHO$$

The product from the reaction with •H is either an α-

UAL 05464

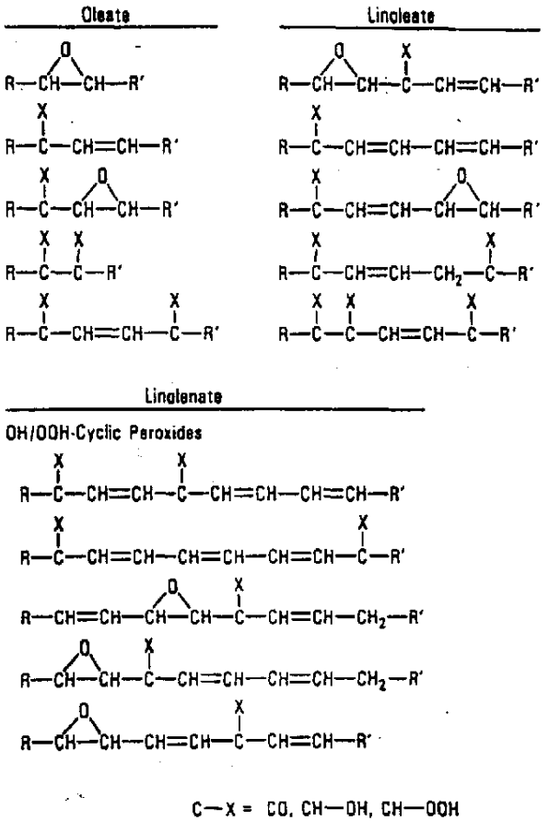


FIG. 8. Structures of secondary autoxidation products of oleate, linoleate and linolenate.

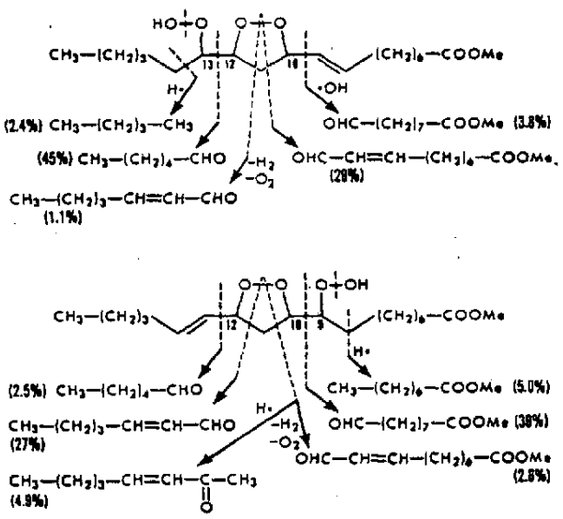


FIG. 9. Thermal decomposition of hydroperoxy cyclic peroxides from linoleate treated with $^1O_2$ (67).

olefin: R—CH=CH• + •H → R—CH=CH_2, or a short chain ester: R′• + •H → R′H. Cleavage reactions (1) and (2) explain most of the volatile products identified from the thermal decomposition of the hydroperoxides of oleate, linoleate and linolenate (15,74). The products include carbonyls, alcohols, esters and hydrocarbons. The formation of substituted furans, epoxy aldehydes, ketones, lactones, alkynes and aromatic compounds is difficult to explain, and the literature is full of speculative mechanisms (14,15).

More recently we have investigated the thermal decomposition of secondary oxidation products to determine their role as precursors of volatile oxidation products. The formation of bifunctional oxidation products of biological importance, such as malonaldehyde, also was studied because of their potential crosslinking reactions with amino acids, proteins and DNA (10). The thermal decomposition of cyclic peroxides from linoleate produced most of the same volatile cleavage products as the corresponding monohydroperoxides (Fig. 9). The most important cleavage between the hydroperoxide group and the cyclic peroxide produced aldehydes and aldehyde esters. Cleavage on the other side of the hydroperoxide group produced hydrocarbons and shorter-chain esters (67,75). Cleavage of the peroxide ring explains the formation of unsaturated aldehydes and aldehyde esters. Unsaturated methyl ketones are among some of the unique products of cyclic peroxides.

The thermal decomposition of hydroperoxy bis-cyclic peroxides from linolenate follows the same fragmentation pattern as the monocyclic peroxides (75) (Fig. 10). The most important cleavage A between the hydroperoxide

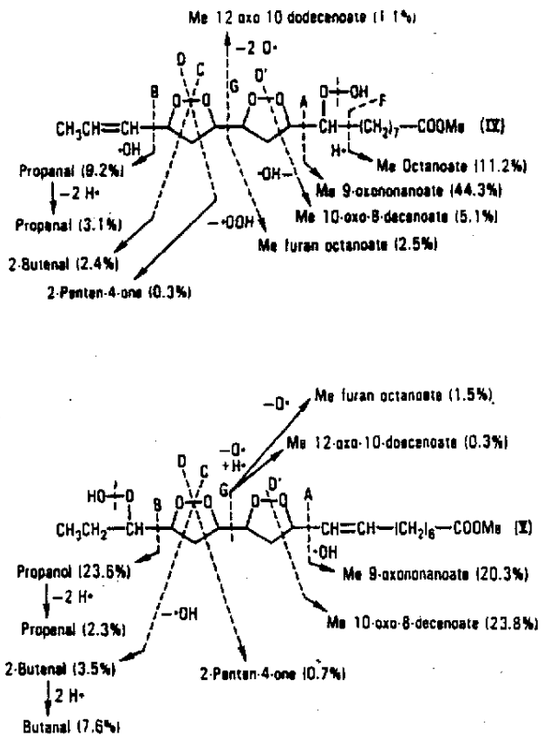


FIG. 10. Thermal decomposition of hydroperoxy bis-cyclic peroxides from linolenate treated with $^1O_2$ (75).

UBL 05465

group and the first peroxide ring produces a C-9 aldehyde ester. Cleavage B on the other side of the second peroxide ring produces propanal. Other cleavages, C and D, produce volatile products similar to those from monohydroperoxides. Methyl furanoctanoate is a unique cleavage product that can be explained from cleavage E between the 2 peroxide rings. The bicycloendoperoxides isolated from photosensitized oxidized methyl linolenate also were thermally decomposed, and carbon-carbon cleavage around the hydroperoxide group was found to be the most important (76). We also have studied the thermal decomposition of dihydroperoxides from linolenate. The volatile products identified are those expected from cleavage on each side of the hydroperoxide group. Under our conditions used for thermal decomposition (gas chromatograph injector port at 200-210 C), in no case have we found evidence for the formation of either malonaldehyde or other dialdehydes expected from both cyclic peroxides and dihydroperoxides. Apparently, these difunctional products were too thermally unstable to be detected by gas chromatography.

Malonaldehyde has received much attention in the biochemical and food science literature, and it was reported to be mutagenic and carcinogenic (77-79). The TBA color reaction generally has been used to determine malonaldehyde in food and biological systems. Unfortunately, the TBA reaction is not specific for malonaldehyde, and many lipid oxidation products and their interaction products with other biological materials give positive reactions (7,10,31, 80-82). To determine malonaldehyde more specifically, we developed a milder procedure to decompose lipid oxidation products under acid conditions instead of using the elevated temperatures of a gas chromatograph. By using a dilute HCl solution in methanol, lipid oxidation products are readily cleaved and converted to stable acetals suitable for gas chromatography. Any malonaldehyde formed is converted to tetramethyl acetal derivatives, which can be determined quantitatively by gas chromatography (83). This acid decomposition-acetalation procedure was applied to different lipid oxidation products. As expected, 5-membered hydroperoxy cyclic peroxides and 1,3-dihydroperoxides were found to be the most important precursors of malonaldehyde (Fig. 11). 1,4-Dihydroperoxides were less important and monohydroperoxides were the least significant

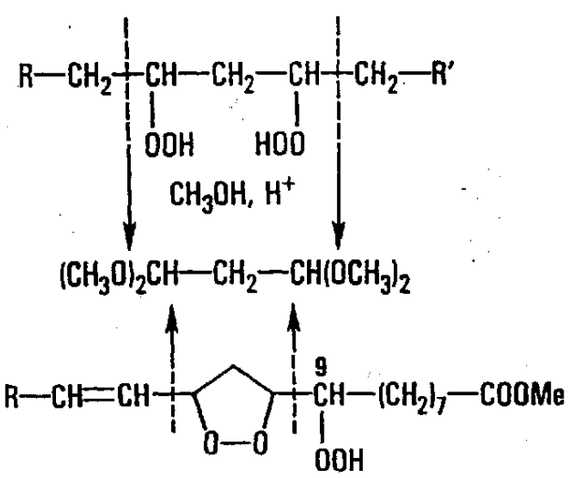


FIG. 11. Acetalation-acid decomposition of hydroperoxy cyclic peroxides and 1,3-dihydroperoxides (83).

precursors of malonaldehyde. In contrast, the TBA test is known to give a positive test with all these lipid oxidation products (84). Therefore, our approach provides a more specific test than the TBA color reaction to evaluate the potential of lipid oxidation products to form malonaldehyde and its biological effects due to crosslinking.

BIOLOGICAL CONSEQUENCES

Many reviews have appeared on the biological effects of lipid oxidation products and their relevance to cancer (7,12,85-88). Lipid oxidation products are implicated in the disruption of biological membranes (1,6,7,9,10), the inactivation of enzymes and damage to proteins (10,16-21, 29), the formation of age pigments in damaged membranes (9,10,25,89), oxidative damage to lungs by atmospheric pollutants (4) and cancer. That free radicals are one of the important factors affecting cancer can be inferred by the beneficial effects of antioxidants such as vitamin E, BHA and BHT (12,86,90-93). These free radical scavengers apparently prevent the oxidation of chemical carcionogenic agents into more active forms. Chemical carcinogenesis may thus result from enzymatic or nonenzymatic oxidation of chemical agents into reactive intermediates formed either from stable free radicals or via singlet oxygen and •OH by metal complex catalysis (6,7,12,94).

Many examples are cited in the literature for the roles of free radicals in carcinogenesis. ESR evidence is reported for the formation of nitroxyl radicals as an intermediate from N-hydroxyacetyl-aminofluorene (N-OH-AAF) in its conversion to the more active carcinogenic species N-acetoxy-AAF and 2-nitrosofluorene (95-97). This conversion occurs also in the presence of 13-linoleate hydroperoxide and methemoglobin or hematin (98). A lipid hydroxy derivative was assumed to be important in this reaction. In view of the multitude of decomposition products expected to be formed by metal catalysis, it would be important to determine what particular functionality activates a carcinogen.

Benzo(a)pyrene [B(a)P] is another important chemical carcinogen that is converted to oxy radical either by photoirradiation or enzymatically by incubation with liver microsomes (87,99). The precursor of 6-oxy-B(a)P, 6-hydroxy-B(a)P, binds covalently with DNA in vitro, and the free radical of the bound complex is demonstrated directly by ESR studies. From these reports of free radical involvement in cancer, it can be readily deduced that any agent known to promote (e.g., metals and their active complexes) or inhibit (e.g., reducing agents and antioxidants) free radicals, would have a great impact on cancer formation. In view of the activity of polyaromatic hydrocarbons such as B(a)P as photosensitizers (34), the possible involvement of $^1O_2$ in activation of carcinogen also should be seriously considered (87).

Besides antixodants, what are some of the other biological defense mechanisms against lipid peroxidation? Enzymes such as peroxidase, catalase and superoxide dismutase remove different species of activated oxygen that promote lipid peroxidation (38). Any pathological or degenerative conditions such as aging may decrease the concentration of these protective enzymes, with consequent damage from the toxic effects of activated oxygen. Cell membrane integrity is another mechanism that the body has to separate biological catalysts and oxygen from lipid unsaturation and its resulting oxidation. Any factors that affect this structural separation will result in damaging lipid peroxidation. Finally, the relatively low intracellular concentration of oxygen is another defense mechanism against lipid oxidation (25,100).

REFERENCES

1. Mead, J.F., Free Radicals in Biology, edited by W.A. Pryor, Vol. I, Academic Press, New York, 1976, p. 51.
2. Kerr, J.A., J.G. Calvert and K.L. Demerjion, Free Radicals in Biology, edited by W.A. Pryor, Vol. II, Academic Press, New York, 1976, p. 159.
3. Mudd, J.B., Free Radicals in Biology, edited by W.A. Pryor, Vol. II, Academic Press, New York, 1976, p. 263.
4. Menzel, D.B., Free Radicals in Biology, edited by W.A. Pryor, Vol. II, Academic Press, New York, 1976, p. 181.
5. Wilson, R.B., CRC Crit. Rev. Food Sci. Nutr. 7:325 (1976).
6. Packer, L., and J. Walton, Chem. Tech. 276 (1977).
7. Pryor, W.A., Photochem. Photobiol. 28:787 (1978).
8. Korycka-Dahl, M.B., and T. Richardson, CRC Crit. Rev. Food Sci. Nutr. 11:209 (1978).
9. Simic, M.G., and M. Karel, editors, Autoxidation in Food and Biological Systems, Plenum Press, New York, 1980.
10. Tappel, A.L., Free Radicals in Biology, edited by W.A. Pryor, Vol. IV, Academic Press, New York, 1980, p. 1.
11. Rodgers, M.A.J., and E.L. Powers, editors, Oxygen and Oxy-Radicals in Chemistry and Biology, Academic Press, New York, 1981.
12. Floyd, R.A., editor, Free Radicals and Cancer, Marcel Dekker, New York, 1982.
13. Frankel, E.N., Prog. Lipid Res. 19:1 (1980).
14. Forss, D.A., Ibid. 13:177 (1972).
15. Frankel, E.N., Ibid. 22:1 (1983).
16. Chio, K.S., and A.L. Tappel, Biochemistry 8:2827 (1969).
17. Gamage, P.T., and S. Matsushita, Agric. Biol. Chem. 34:817 (1970).
18. Matsushita, S., J. Agric. Food Chem. 23:150 (1975).
19. Matsushita, S., and M. Kobayashi, Agric. Biol. Chem. 34:825 (1970).
20. Matsushita, S., M. Kobayashi and Y. Nitta, Agric. Biol. Chem. 34:817 (1970).
21. Mukai, F.H., and B.D. Goldstein, Science 191:868 (1976).
22. Bender, A.D., C.G. Kormendy and R. Powell, Exp. Gerontol. 5:97 (1970).
23. deDuve, C., and O. Hayaishi, editors, Tocopherol, Oxygen and Biomembranes, Elsevier, Amsterdam, 1978.
24. Desai, I.D., B.L. Fletcher and A.L. Tappel, Lipids 10:307 (1975).
25. Miquel, J.; J. Oro, K.G. Bensch and J.E. Johnson, Free Radicals in Biology, Vol. III, Academic Press, New York, 1927, p. 133.
26. Munkres, K.D., Age Pigments, edited by R.S. Sohal, Elsevier/North-Holland Biochemical Press, Amsterdam, 1981, p. 83.
27. Porta, E.A., and W.S. Hartroff, Pigments in Pathology, edited by M. Wolman, Academic Press, New York, 1969, p. 19.
28. Scott, M.L., The Fat Soluble Vitamins, edited by H.F. deLuca, Plenum Press, New York, 1978, p. 133.
29. Karel, M., K. Schaich and R.B. Roy, J. Agric. Food Chem. 23:159 (1975).
30. Matsushita, S., Ibid. 23:150 (1975).
31. Gardner, H.W., Ibid. 27:220 (1979).
32. Pokorny, J., Rev. Franc. Corps Gras 28:151 (1981).
33. Rawls, H.R., and P.J. van Santen, Ann. N.Y. Acad. Sci. 171:135 (1970); JAOCS 47:121 (1970).
34. Foote, C.S., Free Radicals in Biology, edited by W.A. Pryor, Vol. II, Academic Press, New York, 1976, p. 85.
35. Pryor, W.A., Molecular Basis of Environmental Toxicity, edited by R.S. Bhatnagar, Ann Arbor Science, Ann Arbor, 1980, p. 3.
36. Tappel, A.L., Fed. Proc. Fed. Am. Soc. Exp. Biol. 32:1870 (1973).
37. Witting, L.A., Free Radicals in Biology, edited by W.A. Pryor, Vol. IV, Academic Press, New York, 1980, p. 295.
38. Fridovitch, I., Free Radicals in Biology, edited by W.A. Pryor, Vol. I, Academic Press, New York, 1976, p. 239.
39. Brown, K., and I. Fridovitch, Autoxidation in Foods and Biological Systems, edited by M.G. Simic and M. Karel, Plenum Press, New York, 1980, p. 429.
40. Frankel, E.N., Fatty Acids, edited by E.H. Pryde, AOCS Monograph 7, American Oil Chemists' Society, Champaign, IL, 1979, p. 353.
41. Porter, N.A.; L.S. Lehman, B.A. Weber and K.J. Smith, J. Am. Chem. Soc. 103-6447 (1981).
42. Porter, N.A.; B.A. Weber; H. Weenen and J.A. Khan, Ibid. 102:5597 (1980).
43. Frankel, E.N.; W.E. Neff; W.K. Rohwedder; B.P.S. Khambay; R.F. Garwood and B.C.L. Weedon, Lipids 12:901 (1977).
44. Garwood, R.F.; B.P.S. Khambray; B.C.L. Weedon and E.N. Frankel, J. Chem. Soc. Chem. Commun. 364 (1977).
45. Chan, H.W.-S., and G. Levett, Chem. Ind. (London) 692 (1977).
46. Frankel, E.N.; R.F. Garwood; J.R. Vinson and B.C.L. Weedon, J. Chem. Soc. Perkin Trans. I, 2715 (1982).
47. Frankel, E.N.; C.D. Evans; D.G. McConnell; E. Selke and H.J. Dutton, J. Org. Chem. 26:4663 (1961).
48. Frankel, E.N.; W.E. Neff; W.K. Rohwedder; B.P.S. Khambay; R.F. Garwood and B.C.L. Weedon, Lipids 12:1055 (1977).
49. Chan, H.W.-S., and G. Levett, Ibid. 12:837 (1977).
50. Neff, W.E.; E.N. Frankel and D. Weisleder, Ibid. 16:439 (1981).
51. O'Connor, D.E.; E.D. Mihelich and M.C. Coleman, J. Am. Chem. Soc. 103:223 (1981).
52. Frankel, E.N.; W.E. Neff and T.R. Bessler, Lipids 14:961 (1979).
53. Neff, W.E., and E.N. Frankel, Ibid. 15:587 (1980).
54. Matsushita, S., and J. Terao, Autoxidation in Food and Biological Systems, edited by M.G. Simic and M. Karel, Plenum Press, New York, 1980, p. 27.
55. Frankel, E.N.; W.E. Neff; W.K. Rohwedder; B.P.S. Khambay; R.F. Garwood and B.C.L. Weedon, Lipids 12:908 (1977).
56. Nugteren, D.H.; R.K. Beerthuis and D.A. Van Dorp, Rec. Trav. Chim. 85:405 (1966).
57. Haverkamp-Begeman, P.; W.J. Woestenburg and S. Leer, J. Agric. Food Chem. 16:679 (1968).
58. Samuelson, B., Lipid Metabolism, edited by S. Wakil, Academic Press, New York, 1970, p. 107.
59. Porter, N.A., and M.O. Funk, J. Org. Chem. 40:3614 (1975).
60. Pryor, W.A., and J.P. Stanley, Ibid. 40:3015 (1975).
61. Pryor, W.A.; J.P. Stanley and E. Blair, Lipids 11:370 (1976).
62. Porter, N.A.; M.O. Funk; G. Gilmore; R. Isaac and J. Nixon, J. Am. Chem. Soc. 98:6000 (1976).
63. Roza, M., and A. Franck, Biochim. Biophys. Acta 528:119 (1978).
64. Porter, N.A., Free Radicals in Biology, edited by W.A. Pryor, Vol. IV, Academic Press, New York, 1980, p. 261.
65. Mihelich, E., J. Am. Chem. Soc. 102:7141 (1980).
66. Coxon, D.T.; K.R. Price and H.W.-S. Chan, Chem. Phys. Lipids 28:365 (1981).
67. Frankel, E.N.; W.E. Neff; E. Selke and D. Weisleder, Lipids 17:11 (1982).
68. Neff, W.E.; E.N. Frankel and D. Weisleder, Ibid. 17:780 (1982).
69. Khan, J.A., and N.A. Porter, Angew. Chem. Suppl. 513 (1982).
70. Neff, W.E.; E.N. Frankel, E. Selke and D. Weisleder, Lipids 18:868 (1983).
71. Neff, W.E.; E.N. Frankel; C.R. Scholfield and D. Weisleder, Lipids 13:415 (1978).
72. Terao, J., and S. Matsushita, Agric. Biol. Chem. 39:2027 (1975).
73. Budowski, P.; L. Bartor; Y. Dror and E.N. Frankel, Lipids 14:768 (1979).
74. Frankel, E.N.; W.E. Neff and E. Selke, Ibid. 16:279 (1981).
75. Frankel, E.N.; W.E. Neff and E. Selke, Ibid. 18:353 (1983).
76. Frankel, E.N.; W.E. Neff and E. Selke, JAOCS 60:686 (1983) (Abstract 33).
77. Shamberger, R.J.; T.L. Andreone and C.E. Willis, J. Natl. Cancer Inst. U.S. 53:1771 (1974).
78. Mukai, F.H., and B.D. Goldstein, Science 191:868 (1976).
79. Shamberger, R.J., Autoxidation in Food and Biological Systems, edited by M.G. Simic and M. Karel, Plenum Press, New York, 1980, p. 639.
80. Patton, S., and G.W. Kurtz, J. Dairy Sci. 34:669 (1951).
81. Shamberger, R.J.; B.A. Shamberger and C.E. Willis, J. Nutr. 107:1404 (1977).
82. Uchiyama, M., and M. Mihara, Anal. Biochem. 86:271 (1978).
83. Frankel, E.N., and W.E. Neff, Biochim. Biophys. Acta 754:264 (1983).
84. Porter, W.A.; J. Nixon and I. Ramdas, Biochim. Biophys. Acta 441:506 (1976).
85. Ts'o, O.P.; W.J. Caspary and R.J. Lorentzen, Free Radicals in Biology, edited by W.A. Pryor, Vol. III, Academic Press, New York, 1977, p. 251.
86. Floyd, R.A., Free Radicals in Biology, edited by W.A. Pryor, Vol. IV, Academic Press, New York, 1980, p. 187.
87. Nagata, C.; M. Kodama; Y. Ioki and T. Kimura, Free Radicals and Cancer, edited by R.A. Floyd, Marcel Dekker, New York, 1982, p. 1.
88. Hopkins, G.J., and C.E. West, Life Sci. 19:1103 (1976).
89. Koster, J.F., and R.G. Slee, Biochim. Biophys. Acta 620:489 (1980).
90. Shamberger, R., J. Natl. Cancer Inst. U.S. 44:931 (1970); 48:1497 (1972).
91. Wattenberg, L.W., Ibid. 48:1425 (1972); 50:1541 (1973).
92. Wattenberg, L.W.; W.D. Laub; L.K. Lam and J.L. Speier, Fed. Proc. 35:1327 (1976).
93. Sporn, M.B.; R.A. Squire; C.C. Brown; J.M. Smith; M.L.

URL 05467

Wenk and S. Springer, Science 195:487 (1977).
94. O'Brien, P.J., Autoxidation in Food and Biological Systems, edited by M.G. Simic and M. Karel, Plenum Press, New York, 1980, p. 563.
95. Floyd, R.A.; L.M. Soong; R.N. Walker and M. Stuart, Cancer Res. 36:2761 (1976).
96. Bartsch, H., and E. Hecker, Biochim. Biophys. Acta 237:567 (1971).
97. Bartsch, H.; M. Traut and E. Hecker, Ibid. 237:556 (1971).
98. Floyd, R.A., Free Radicals and Cancer, edited by R.A. Floyd, Marcel Dekker, New York, 1982, p. 361.
99. Lorentzen, R.J.; W.J. Caspary; S. Lesko and P.O.P. Ts'o, Biochemistry 14:3978 (1975).
100. Recknagel, R.O.; E.A. Glende, Jr. and A.M. Hruszkewycz, Free Radicals in Biology, edited by W.A. Pryor, Vol. III, Academic Press, New York, 1977, p. 97.

[Received February 13, 1984]

Sources and Consumption of Antioxidants in the Diet

JOHN G. BIERI*, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20205

ABSTRACT

Vitamin E is the most important tissue antioxidant in preventing or controlling non-specific reactions from various oxidizing species produced in normal metabolism. Through this action, the vitamin protects polyunsaturated fatty acid loss from phospholipids and consequent membrane damage. The U.S. diet has an abundance of vitamin E and normal individuals accumulate effective amounts in their tissues, which is consistent with the latest recommended dietary allowances for vitamin E as indicated by the National Research Council.

INTRODUCTION

One may reasonably ask why there should be an interest in antioxidants in our food supply. Are they beneficial or harmful? We know from biochemical studies that in the body's tissues there are chemical reactions which under some circumstances could lead to metabolic problems and tissue damage. These reactions produce free radicals or oxidizing species that may react readily with cell components. Such systems as the microsomal mixed function oxidases, the xanthine-xanthine oxidase system, cyclooxygenase and various other enzymes that produce hydrogen peroxide, superoxide or singlet oxygen, all may contribute to potentially damaging conditions in vivo. Fortunately, tissues also contain a varied system of defense against oxidant damage, and primary components of this system are antioxidants.

Foremost is vitamin E, since a deficiency of this vitamin leads to many cellular changes readily explained by its antioxidant action. Other nutrients which also can demonstrate an antioxidant effect, via metal scavenging and under limited conditions, are ascorbic acid, cystine, histidine, tryptophan and intact proteins. Numerous enzymes in tissues also will destroy oxidizing species: catalase, glutathione reductase, glutathione peroxidase (both selenium containing and non-selenium containing), and superoxide dismutase. It should be mentioned that the trace element, selenium, is not an antioxidant, but when incorporated into glutathione peroxidase it readily destroys peroxides.

Two types of antioxidants in the diet will be considered, the natural antioxidant vitamin E, and the synthetic antioxidants added in manufacturing of a multitude of food products. Of the many synthetic antioxidants available, only 2 were approved by the Food and Drug Administration for use in human food in the past. These are BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole). These are permitted in fats and oils at a concentration of 0.02%, and also are added to packaging materials. However, BHT is no longer considered acceptable and its use has been stopped in most lipid-containing foods in the U.S. and other countries. In extensive animal testing 20 to 30 years ago, these compounds generally were found to be much less active than vitamin E in preventing the classical signs of vitamin E deficiency. The amounts required in the diet were 20-100 times that of vitamin E, and often bordered on a toxic level. Studies of the metabolism of BHT and BHA in man, using isotopic labeling, revealed that the compounds were rapidly excreted from the body, 80-90% in the urine within 7 days and the remainder in the feces. Furthermore, they are oxidized to 5 or more metabolites. In terms of the amounts that may be ingested daily by man from the U.S. food supply, probably only a few milligrams, it cannot be considered that these two antioxidants make a significant contribution to the body's overall antioxidant defense system. In preventing certain experimentally produced cancers in laboratory animals, these compounds must be in the diet at relatively high levels, 0.5% or more. This would be the equivalent of about 2.5-3 g per day for man.

Vitamin E is the most important dietary component contributing to antioxidant defenses in tissue. Vitamin E is a collective term comprising 8 compounds synthesized by plants. These fall into 2 classes, the tocols having a saturated side chain, and the tocotrienols having an unsaturated side chain. Within each class there are 4 "vitamers," designated alpha, beta, gamma and delta, which vary in the number of methyl groups on the chroman ring. Of these 8 compounds, only 4 have nutritional significance: alpha, beta, and gamma-tocopherols and alpha tocotrienol. When tested in animals for their vitamin E activity, the relative activities are: alpha tocopherol 100, beta tocopherol 30, gamma tocopherol 10, and delta tocopherol 1. In the tocotrienol series the activities are alpha 30, beta 5, and gamma and delta, < 1 (1).

According to their relative abundance in the diet and their relative biological activities, it can be estimated that of the total vitamin E activity in the U.S. food supply 75% comes from alpha tocopherol, 20% from gamma tocopherol, and 5% from beta tocopherol and alpha tocotrienol. Even though gamma tocopherol has only one-tenth the biological activity of alpha tocopherol, it is present in our diet at twice the amount of alpha tocopherol and thus makes a significant contribution. The U.S. diet may be unique in this regard compared with other western countries because of our relatively high consumption of soybean and corn oils, in which gamma tocopherol exceeds alpha tocopherol

*To whom correspondence should be addressed at Bldg. 6, Rm. B1-06, National Institutes of Health, Bethesda, MD 20205.

URL 05468

three- to five-fold (2). Once γ-tocopherol is incorporated into cell membranes its antioxidant activity can be as high as 37% that of alpha tocopherol (3). γ-tocopherol is slightly more poorly absorbed than alpha and also exhibits a faster turnover in the tissues.

The richest sources of vitamin E in the U.S. diet are vegetable oils (Table I). In the U.S., soybean oil accounts for about 70% of edible vegetable oils. During processing there are small losses of the tocopherols, but even the hydrogenated shortenings retain relatively high levels. Probably vitamin E is the most widely distributed vitamin in foods. The content in other foods is shown in Table II. Analyses of composite meals by several investigators (4) have shown that U.S. diets contain about 5-13 mg alpha tocopherol equivalent (7.5-19 IU). The range depends primarily on the vegetable fat content of the diet and total calories. Generally, low fat diets will have less vitamin E than high fat diets. Because of the relatively high content of fat in the U.S. diet, and our wide use of vegetable oils, the U.S. food supply is probably the richest in the world in vitamin E, and published values support this (4).

The amount and type of fat in the U.S. food supply has changed markedly since 1950, with a shift away from animal fat to vegetable fat. This change led some nutritionists to question the vitamin E adequacy of our diet, since experiments in animals had shown that increased amounts of polyunsaturated fat increase vitamin E requirements (5). These fears were unfounded, however, because it was not considered that the dietary sources of the polyunsaturated fat, vegetable oils, also are the richest sources of vitamin E. As shown in Table III, from 1950 to 1970, while the amount of oils increased from 21.2 lbs per person per yr to 36.4 lbs, the alpha tocopherol increased from 2.63 g per person per yr to 3.35 g. When the contribution of gamma tocopherol is included, the ratio of mg vitamin E activity:g linoleic acid was 0.61 in 1950 and 0.57 in 1970, not a significant change. Thus, there has not been any deterioration in vitamin E nutrition in the U.S. as a result of changes in type and amount of fat; surveys of human vitamin E status have not shown any change over this period.

One consequence of the change in U.S. dietary fat from animal to vegetable sources has been a marked increase in the intake of gamma tocopherol (Table IV). Analyses of human tissues in 1958 and in 1975 showed an increased content of gamma tocopherol, consistent with the increased dietary content of the gamma "vitamer" over this time period (6). Of interest is the wide variation in the ratio of gamma:alpha tocopherols in different tissues (Table III). It would appear that gamma tocopherol has much more significance for some tissues than for others, but the variation between individuals prevents a generalization.

How do these intakes of tocopherols compare with the Recommended Dietary Allowance for vitamin E as specified by the National Research Council? In the latest recommendations of 1980, the allowance for adult women is 8 mg alpha tocopherol equivalents and for men, 10 mg. These intakes are achieved easily with most diets in this country. It should be noted, however, that low-fat diets generally will have less vitamin E than high-fat diets. At the same time, the need for the vitamin will change as the amount of fat changes, primarily the linoleic acid content.

TABLE I

Tocopherol Content of U.S. Fats and Oils

Fat	Alpha *tocopherol* mg/100 g	Gamma *tocopherol* mg/100 g
Butter	2	0
Lard	1	0
Soybean oil	10-15	75-100
Corn oil	10-20	50-80
Cottonseed oil	40-50	30-40
Safflower oil	25-35	5

TABLE II

Vitamin E Content of Foods as Alpha Tocopherol Equivalents

Food	mg/100 g
Salad oils	15-55
Margarine	10-15
Vegetable shortening	10-15
Peanuts	7
Whole wheat	1
Vegetables	0.1-1
Fruits	0.1-0.3
Meat, fish	0.2-0.5
Eggs	0.5

TABLE III

Change in Dietary Fats and Tocopherols, 1950-1970 (Per person per yr)

	Pounds of fat		Mg alpha-tocopherol	
	1950	1970	1950	1970
Butter	8.7	4.3	119	59
Lard	13.5	7.1	123	64
Soybean oil	9.5	28.5	733	2200
Cottonseed oil	9.5	4.8	1725	872
Corn oil	1.5	2.0	129	173
Peanut oil	0.7	0.7	41	41
Safflower oil	0.0	0.4	0	60
Totals	43.4	47.8	2870	3469

TABLE IV

Change in Tocopherol Intake from U.S. Food Fats, 1950-1970 (Per person per day)

	1950	1970
Gamma tocopherol, mg	11.8	35.5
Alpha tocopherol, mg	7.9	9.5
Total vitamin E activity, (mg alpha tocopherol equivalent)	9.1	13.1

REFERENCES

1. Bieri, J.G., and P.M. Farrell, Vits. Horm. 34:31 (1976).
2. Bieri, J.G., and R.P. Evarts, Am. J. Clin. Nutr. 27:980 (1974).
3. Bieri, J.G.; R.P. Evarts and J.J. Gart, J. Nutr. 106:124 (1976).
4. Bieri, J.G., Nutr. Rev. 33:161 (1975).
5. Bieri, J.G., and R.P. Evarts, J. Am. Diet. Assn. 66:134 (1975).
6. Bieri, J.G., and R.P. Evarts, Am. J. Clin. Nutr. 28:717 (1975).

URL 05469