Homogenized Milk and Atherosclerotic Disease: A Review

H. C. DEETH
Otto Madsen Dairy Research Laboratory
Department of Primary Industries
Hamilton, Queensland, 4007
Australia

ABSTRACT

The theory that consumption of homogenized milk containing active xanthine oxidase is a causative factor in development of atherosclerosis is reviewed. Biologically available xanthine oxidase in consumed milk products may be absorbed in the small intestine and enter the blood stream. However, there appears to be no unequivocal evidence that the absorbed enzyme has any pathological effects that may contribute to development of atherosclerotic heart disease.

INTRODUCTION

The suggestion that consumption of homogenized milk may be a causative factor in development of atherosclerosis was made first by Oster in 1971 (81). He postulated that xanthine oxidase (XO), an enzyme in milk, could enter the circulatory system of milk consumers and cause damage to the arterial wall and heart tissue and initiate atherosclerosis.

Oster and coworkers have published several papers on the subject and recently reported results that in their opinion provide strong evidence for the veracity of the hypothesis (107). Oster strongly believes that consumption of homogenized milk is one cause of atherosclerosis, and he, therefore, advocates three courses of action: 1) that the dairy industry discontinue homogenization of milk, 2) that the dairy industry ensure that market milk products are free from biologically available XO, or 3) that the dairy industry place warning labels to the effect that the product contains biologically available XO, which may be damaging to health, on all packages of homogenized milk (4, 82). Before any such actions can be contemplated by the dairy industry, all aspects of and all data relating to the hypothesis must be examined critically. This review seeks to do this by collating information on the subject and identifying areas in which there is conflicting or insufficient information. Tenability of the hypothesis is assessed from information available.

THE HYPOTHESIS

Statement

The hypothesis advanced by Oster in 1971 (81) and developed in several subsequent articles (82–92, 96, 97, 105–107) can be stated by the following points:

1. Bovine milk contains substantial amounts of the enzyme XO. Much of this enzyme is associated with the milk fat globule membrane (MFGM) (17, 18, 70).
2. Xanthine oxidase is not inactivated completely during pasteurization of milk (42).
3. Homogenization of milk breaks up the fat globules from an average diameter of 3.5 \( \mu m \) to an average of about 1 \( \mu m \) and increases the number of globules about 100 times. Biological availability of XO is thereby at least 3.5 times as great as that in unprocessed milk (81, 82). Furthermore, homogenization enhances the absorptive potential of XO by creating XO-containing micronized fat globules (81, 107).
4. These fat globules or liposomes (81) can pass through the intestinal barrier and by way of the lymph stream, subclavian vein, right heart, pulmonary circulation, and left heart can enter the aorta and general circulation. In this way XO bypasses the liver and enters the arterial system before being deposited in arterial walls and heart muscle (82, 84).
5. The deposited XO causes depletion of phospholipid plasmalogen by oxidizing the...
aldehyde moiety (95), plasmal, released from this lipid by vinyl etherase (82, 106, 131). This breakdown of plasmalogen by vinyl etherase is normally continuous and reversible, but when XO removes the plasmal, plasmalogen is depleted (80).

6. Because plasmalogen is an important constituent of arterial walls and heart muscle (up to 30% of the phospholipid in myocardial cell membranes) (81, 88), its destruction alters the phospholipid balance and produces changes of the structural integrity of the membranes.

7. Defective cell membranes cause failure in the active transmembrane transport system, which in turn results in defective cell volume regulation, eventual cell death, and irreversible tissue injury (59, 86). In heart tissue this may be manifested as arhythmia (86) or eventually myocardial infarction (80).

8. Cholesterol ester, other lipids, calcium, etc., may accumulate at the site of injury as a “compensatory repair mechanism” and initiate atherosclerotic plaque formation in arterial walls (81, 84, 88).

**Background**

Xanthine oxidase was implicated first in heart disease when Oster in 1968 (80) reported successful reduction of symptoms of nine angina pectoris cases with allopurinol, a well-known XO inhibitor frequently used for preventing uric acid production in gout patients. Oster's biochemical rationale for this therapy is shown diagrammatically in Figure 1. He reasoned that plasmalogen in heart tissue was hydrolyzed to plasmal (long-chain, saturated fatty aldehydes such as palmitaldehyde (C16) and stearaldehyde (C18) by a phospholipase A-like enzyme, and under normal conditions this process was continual and reversible. However, if an imbalance occurred in the process through removal of plasmal, plasmalogen could disappear from the infected area causing cell membrane damage, cell death, and eventual myocardial infarction.

That XO may be involved in disappearance of plasmalogen was indicated by the work of Oster and Mulinos (95) in which they demonstrated that bovine milk XO could oxidize plasmals released from plasmalogens by treatment with acid. Furthermore, tissues such as liver and intestinal mucosa, which normally contain XO, were almost devoid of plasmalogens whereas other tissues such as heart tissues, which normally do not contain XO, were rich in plasmalogens (79).

Plasmalogen depletion from atherosclerotic plaques and from myocardial tissue affected by infarction had been observed (19, 72, 94). This led Oster to suspect an exogenous XO, and in 1971 he published his paper “Plasmalogen Diseases: A New Concept of the Etiology of the Atherosclerotic Process” (81) in which he suggested that milk, the only food containing significant amounts of XO, was responsible for depletion of plasmalogen in arterial tissue and heart muscle. He specifically implicated homogenized milk, because he believed that micronization of fat globules during homogenization would produce XO-containing particles that could pass unchallenged through the natural barrier of the intestinal wall in a manner analogous to that for microcrystals (102). Some evidence that XO may be absorbed into the human body was provided by the discovery of antibodies to milk proteins, including XO, in the blood of male patients with ischemic heart disease (25, 27).

Oster (81, 89) used epidemiological data to support his contention that the origin of the XO was homogenized milk. He interpreted the data (Table 1) as indicating a strong link between death rate from heart disease and consumption of homogenized milk. He also concluded that the mortality rate from heart disease was lowest in those countries where milk was given sufficient heat treatment to
inactivate XO. For some countries, notably Australia and Canada, Oster could not explain the high death rates by his data on homogenized milk consumption.

Oster also used the case of the Masai tribesmen in Kenya to support his hypothesis (81, 89). These people drink copious quantities of unhomogenized, raw, fermented milk but rarely develop coronary heart disease. Oster maintained that fermentation of their milk caused production of large curd particles, which rendered XO biologically unavailable and permitted it to be digested rather than absorbed (81, 84).

\[ \text{XANTHINE OXIDASE} \]

\[ \text{PROPERTIES, REACTIONS} \]

Bovine milk is a rich source of XO. Milk of other ruminants such as sheep and goats contains much lesser amounts. Milk of nonruminants such as rats and donkeys has high XO activity whereas that of others such as dogs, cats, mares, and humans has moderate to low activity (139, 141). Much of the XO in bovine milk is associated with milk fat globule membrane, constituting about 8 to 10% of the total membrane protein (18, 70). Although XO is concentrated in the cream phase, in fresh, raw milk, the total amount in the skim phase is about 6 to 10% of the total membrane protein. Milk of other species such as pigs and goats contains lesser amounts. Milk of nonruminants such as rats and goats contains no detectable membrane constituent with milk fat globule membrane characteristics.

The enzyme can be released from the cream phase into the aqueous phase by certain conditions. These conditions include cooling, freezing, mild heating, storage, agitation, and turbulent flow. Such conditions also release XO from the raw, unhomogenized milk, where it can be digested rather than absorbed. The enzyme is relatively heat-stable, requiring a heat treatment of 81 to 84°C for 3 to 5 s for complete inactivation (87). Pasteurization at 74°C for 15 s reduces activity by about 60% (41, 50). Commercial pasteurization of milk varies in severity with different processors, and hence, XO activity in pasteurized milk varies accordingly. Zikakis et al. (142) report that the enzyme is not inactivated in pasteurized milk.

**TABLE 1. Relationship between consumption of milk and mortality rate from coronary heart disease (CHD) in 13 countries.** Adapted from Oster (81).
and Wooters (142) concluded from a study of processed (homogenized, pasteurized) whole milks that processing destroyed at least 82% of XO activity whereas Cerbulis and Farrell (22) found average reduction about 69%. Considerable variation of XO in processed milks was observed in both of these surveys. In present context Zikakis and Wooters (142) found that almost all XO activity in processed milk was in an available form because addition of detergent Triton X–100 produced only a slight increase of measurable activity.

Bovine milk XO has been isolated, purified, and studied extensively. Its molecular properties have been described in several reviews (11, 43, 46, 114, 143). It is a molybdenum- and iron-containing protein with two moles of flavin adenine dinucleotide (FAD) per mole of protein. The FAD imparts reddish color to the enzyme and is largely responsible for the pinkish color of buttermilk. Xanthine oxidase has a molecular weight (M_r) of about 300,000, which can be reduced to subunits of 150,000 in guanidine and acid (74) or sodium dodecyl sulphate-urea (133). Although neither Nelson and Handler (74) nor Waud et al. (133) could reduce the native enzyme to smaller units, Biasotto and Zikakis (14) reported isolation from bovine milk of an active XO with M_r < 75,000. Waud et al. (133) showed that proteolysis cleaved off a segment leaving an active oxidase with M_r about 260,000.

Xanthine oxidase catalyzes oxidation of purine bases, xanthine, and hypoxanthine to uric acid. It also catalyzes oxidation of aldehydes including long-chain fatty aldehydes (plasmals), although its affinity for these aldehydes is much lower than for xanthine (50). Xanthine oxidase can be converted reversibly into a NAD^+-dependent dehydrogenase by treatment with dithiothreitol or dihydrolipoic acid. However, treatment with proteolytic enzymes irreversibly converts it into a form with oxidase activity only (13). Briley and Eisenthal (17) found that the ratio of xanthine oxidase activity to NADH oxidase activity (ratio x/n) was dependent on the association state of the enzyme. When bound to MFGM the x/n was more than twice as high as x/n for the free form.

Xanthine oxidase has been implicated in oxidation of lipids and production of “oxidized flavor” of milk (9, 10, 48). Other workers (119) have been unable to confirm these findings. In fact, some authors have considered that XO may have antioxidative properties (7, 30).

Considerable work recently has been on active oxygen species produced by XO. It now appears that XO produces superoxide anion that can undergo spontaneous, chemical, or enzymic (by superoxide dismutase) dismutation to produce singlet oxygen and peroxide radical (8, 100). Singlet oxygen is considered to be the active species of lipid peroxidation. Aurand et al. (8) showed that singlet oxygen, especially in the presence of hypoxanthine, was responsible for XO-promoted peroxidation of milk lipids. Although superoxide per se cannot initiate the free-radical chain reactions in lipid peroxidations, it can enhance oxidation by decomposing hydroperoxides to form chain-initiating radicals (64). This can be illustrated by the following equation for linoleate hydroperoxide (LOOH)

\[
\text{LOOH} + \text{O}_2^- \rightarrow \text{LO}^\cdot + \text{O}_2 + \text{HO}^-
\]

The superoxide anion produced by XO also can act as a strong nucleophile. It can hydrolyze ester linkages of triglycerides and phospholipids by attacking the carbonyl carbon and displacing a peroxide radical (75). The significance of this type of reaction remains to be determined.

**ABSORPTION OF ACTIVE XANTHINE OXIDASE**

The credibility of Oster’s hypothesis depends largely on whether XO can be absorbed through the intestinal wall and remain in an active state. A discussion of this question must consider both the effect of gastric juices on the enzyme and the ability of the intestinal mucosa to absorb it.

**Gastric Passage**

Several workers have addressed themselves to the question of whether the enzyme can survive acid conditions of the stomach and digestive juices of the small intestine. It generally is agreed that the enzyme is inactivated completely at pH less than 3.2. Inactivation may be irreversible (49, 69, 131) or partially reversible (141). Gastric juice has pH < 2, and with a high ratio volume to volume of this to milk, XO is
inactivated completely. However, at ratios of less than about 1:1 pH is not reduced sufficiently to cause complete inactivation. At a ratio of 1:2 of simulated gastric juice to milk, Zikakis et al. (141) found pH was reduced to only 5.16, and 14.2% of XO activity remained; at a ratio of 1:3 pH was 5.56, and 23.8% activity remained. Addition of pancreatin to the 1:2 mixture caused further reduction of activity to 6.1% after 7.5 h. Ho et al. (52) reported that 27% of total and 13% of membrane-bound activity remained after treatment simulating the passage of pasteurized, homogenized milk through the human stomach and small intestine. Thus, it appears that active XO can reach the intestinal wall.

Intestinal Absorption

The question of whether XO can be absorbed intact across the intestinal wall is contentious. From consideration of intestinal absorption of other substances, authors have reached different conclusions regarding absorption of XO. Ho and Clifford (50) calculated that only about .00008% of XO with a $M_r$ of 300,000 would be absorbed. They based their calculation on relative absorptions of horseradish peroxidase ($M_r = 40,000$), horse spleen ferritin ($M_r = 650,000$) and adenovirus particles ($M_r = > 1 \times 10^6$) for a linear relationship between molecular size and absorption. This assumption for size and absorbability was criticized by Ross and Oster (105), who contended that liposomally entrapped enzymes and other large particles could be absorbed from the intestine by persorption. Clifford and Ho (24) responded by indicating that persorption deals only with solid, hard, insoluble particles and that, in the absence of evidence to the contrary, their assumption of a linear relationship between molecular size and absorbability was reasonable. Zikakis and coworkers (140, 141) considered that the intestinal absorption of XO was more probable than Ho and Clifford (50) had indicated because of the reported absorption of egg proteins, botulinal toxin, and starch particles. The probability of xanthine oxidase absorption may be even greater if it can exist in the form with low molecular weight (< 75,000) isolated by Biasotto and Zikakis (14) as there is ample evidence from animal studies of the intestinal absorption of intact proteins of $M_r < 80,000$ (132).

Oster (81) considered that the small XO-containing fat globules (< 1 μm) in homogenized milk could pass through the intestinal wall in a manner analogous to known absorption of microcrystals. Ross et al. (107) likened the XO-containing particles in homogenized milk to liposomes, which are absorbed from the intestine by “persorption” (129) and are useful for transporting therapeutic substances across the intestinal barrier (42). Hence, they considered that XO could be absorbed readily in this form.

In what form is XO in the intestine? Ho and Clifford (50) concluded that it was mostly in free form rather than membrane bound. After treatment of XO with gastric juice and pancreatin, they found that the molecular weight of XO eluted from Sephadex G–200 was about 300,000. This is in contrast to the report by Ross et al. (107) in which they claimed XO in homogenized milk both before and after gastric digestion was associated with lipoproteins in liposomal form. They prepared liposomes from a soluble sodium deoxycholate (DOC) extract of pasteurized, homogenized milk by the procedure in Figure 2. This procedure was based on that used by Hayashi and Smith (47) to prepare deoxycholate-soluble membrane material from washed (unhomogenized) cream and buttermilk. Ross et al. (107) found that similar treatment of raw (unhomogenized) milk produced only minute amounts of liposomes.

The evidence of Ross et al. (107) for liposomes in homogenized milk would be more convincing if the detergent DOC had not been used in their preparation, because it is impossible to ascertain whether the liposomes existed prior to DOC treatment. Furthermore, these authors reported that the liposomes they obtained from homogenized milk contained almost all of the XO and that this XO activity was expressed only after treatment with surfactants such as Triton X–100. These results contradict the work of Zikakis and Wooters (142) that showed little increase in measured XO activity in processed milk after treatment with Triton X–100. Further work in this area is indicated by these conflicting data.

Two empirical approaches have been used to resolve the question of absorption. These are the use of sensitive techniques for assaying XO activity and detection of antibodies to XO in human blood and tissues.
Figure 2. Procedure for isolation and purification of liposomes from milk. Adapted from Ross et al. (107). (Note: XO percentages given in brackets are reported (107) recoveries of XO when pasteurized, homogenized milk was used as the liposome source. tr. = trace.)

By sensitive radioactive assay methods for XO neither McCarthy and Long (68) using pigs nor Volp and Lage (130) using everted rat intestine could demonstrate any passage of the enzyme across intestinal wall. McCarthy and Long (68), however, did find significant activity in blood sera from 25 human volunteers, indicating a possible passage of active enzyme from intestine to blood. The amount of activity did not correlate with milk consumption. Clark et al. (23) observed a significant increase of XO activity in blood sera of rats after administration of "half-and-half" (cream:milk, 1:1) with or without added enzyme but could not determine whether the increase was due to absorption of exogenous XO or to stimulation of endogenous XO. They observed that XO administered in saline solution did not cause any increase of serum activity. They attributed this observation either to absence of fat, which they speculated could facilitate absorption of the enzyme, or to lack of buffering capacity of the saline compared with milk components of half-and-half. Ho et al. (53) examined the possibility that ingested fat may enhance endogenous XO. They found that corn oil alone administered orally to rats increased serum activity of XO, thus indicating that the rise of XO observed by Clark et al. (23) after feeding half-and-half was not due to absorbed milk XO.

Gandhi and Ahuja (36) reported in vivo absorption of XO from ingested milk fat globules in rats and of orally administered commercial XO in 12-h-old rabbits. They also demonstrated in vitro passage of purified XO through the walls of small loops of duodenum from 12-h-old rabbit cubs. This latter finding is inconsistent with that of Volp and Lage (130) for rat intestine. Contradictions may be accounted for by the differences of methodology for measuring XO and the different behavior of animal models. In addition, everted rat intestine may be methodologically unsuited for demonstrating persorption.1

Ross et al. (107) reported results of preliminary experiments in which XO activity was detected in blood leucocytes (but not blood sera) of human volunteers 2 h after ingestion of 1 liter of homogenized, pasteurized milk. This was interpreted as indicating intestinal absorption of XO-containing liposomes. Few details of this work were given by Ross et al. (107).

There is considerable endogenous XO in the human body that cannot be distinguished readily from the milk enzyme even by immunological techniques (140). It is in liver and intestinal tissues (50), in human milk (115), and in blood in certain pathological conditions, e.g., vitamin E deficiency (68), breast cancer (2), and liver disease, particularly viral hepatitis where extremely high concentrations (up to 1,000 U/liter) occur (115). The blood of some normal healthy people (3 of 20 in one trial (115) and 10 of 25 in another trial (101)) have low XO (up to .5 U/liter) (115). Any XO in the blood stream could, according to Oster's theory, be deposited in vascular and heart tissue. Therefore, it cannot be assumed that XO detected in human blood or tissue originates from bovine milk.

The second approach, of measuring the titer of antibodies to XO, was used by Oster et al. (96) and Rzucidlo and Zikakis (108). The latter authors found these antibodies in the blood of 73 out of 94 human subjects and reported a significant correlation between antibody titers

1 The author acknowledges a reviewer for this suggestion.
and consumption of milk by the subjects. Oster et al. (96) demonstrated antibodies in 72 of 75 patients and found significantly higher titers in those with clinically evident atherosclerosis. Bierman and Shank (15) expressed doubts about the methodology and questioned the scientific validity of the work. They maintained that the antigen used was a partially purified XO, that there was no assessment of the sensitivity and specificity of the reaction, and that the measure of antibody potency was crude. They also indicated that the two groups of patients were not comparable for age or sex distribution and that the control group included patients who could have had some atherosclerotic involvement. Ho et al. (53) using a microcomplement fixation test could find no evidence of absorbed milk XO in the lymph of rats fed half-and-half fortified with XO. The microcomplement fixation test is much less sensitive than the tanned erythrocyte hemagglutination tests used by Oster et al. (96) and Rzucidlo and Zikakis (108).

In earlier work Ho and Clifford (51) using immunodiffusion and immunofluorescent techniques had been unable to observe an increase of XO in hearts and aorta of rabbits administered XO intravenously at 4-day intervals over 13 wk. These findings cast doubt on the validity of Oster's scheme for absorption of XO from the intestine via the lymph stream with eventual deposition in the heart and aorta (82).

Both the active and inactive forms of the milk XO elicit a similar antigenic response (127, 140). Furthermore, formation of the immunocomplex with active enzyme considerably reduces (up to 70%) its biological activity (127). Hence, a high antibody titer does not indicate necessarily a high concentration of biologically active XO but may indicate persorption.

As mentioned, Oster found support for his hypothesis in immunological studies of Davies and coworkers (25, 26, 27, 28) in which they implicated milk proteins in pathogenesis of atherosclerosis. They reported significantly higher titers of antibodies to milk proteins for patients who had suffered myocardial infarction than for other patients and suggested a causative mechanism by which localized accumulation of immunocomplexes could cause tissue damage by cytolysis. Such damage could lead to formation of atherosclerotic plaque in a manner similar to that proposed by Oster in the hypothesis under consideration in this paper. Other groups of workers (38, 110, 126) have been unable to confirm findings of Davies and coworkers (25, 26, 27, 28) and Oster et al. (96) of an increased frequency or higher titers of antibodies to milk proteins for patients with myocardial infarction.

Further work with human volunteers in which XO activity, originating specifically from bovine milk, can be monitored during absorption from the intestine will be necessary before definitive proof of the absorption of XO activity from ingested milk in humans can be obtained. However, the evidence indicates that XO may be absorbed in the intestine albeit at a low rate.

**EPIDEMIOLOGICAL CONSIDERATIONS**

The epidemiological data in Table 1 were used by Oster (81) to support his theory. However, his interpretation of these data, particularly for consumption of homogenized milk, is open to question. In Australia homogenization was not practiced before 1960, and, hence, it is difficult to imagine that the 1967 mortality rates could in any way reflect this innovation. Oster (91) has himself considered a lead time of “approximately 20 years from the inception of atherosclerosis to its clinical manifestation and eventual death,” so any effect of homogenization should not have been noticed in this country until about now. In Australia, as in some other countries, the age-adjusted death rates from coronary heart disease (CHD) have been declining (32). A leveling off of the CHD death rate occurred in the late 1960's after a marked increase during the previous three decades. In the present context the leveling off coincided with widespread introduction of homogenization of milk. A similar trend of CHD incidence occurred in the US (104), where homogenization was introduced in 1938 (81). Hence, given a lead time of 20 yr (91), the US should have noticed a marked rise of atherosclerosis in the late 1950's and 1960's. This did not occur.

Oster (81, 82, 84, 89) cited atherosclerosis-free Masai tribesmen in Kenya whose diet consisted largely of fermented raw milk to support his argument. He maintained that the physical nature of the milk curd in their diet prevented absorption of active XO from the intestine, and, hence, the amount of active XO
absorbed was small. There appears to be a lack of empirical evidence to support this contention. Many other workers have considered the Masai, and several explanations have been advanced for their low incidence of atherosclerosis. These include their low and variable calorie intake (37), their inherited resistance (49), their physical fitness (71), hypocholesterolemic factors in the fermented milk they drink (29, 103), and the bile salt-deconjugative ability of lactobacilli used to ferment their milk (122). The validity of these explanations versus those of Oster remains to be determined.

CLINICAL TRIALS BASED ON INHIBITION OF XANTHINE OXIDASE

In formulating his hypothesis, Oster (81) drew on results of clinical trials in which the known XO inhibitor, allopurinol, was used successfully to treat nine angina pectoris patients (80). In subsequent trials, Oster prescribed folic acid, another XO inhibitor (60), rather than allopurinol because of the potential toxicity of the latter with prolonged use (84). He reported successful treatment of 40 patients with "severe atherosclerotic changes" by daily folic acid doses of 40 to 80 mg (86). Because folic acid had no harmful effects, he suggested that folic acid may become the "penicillin equivalent" for treatment of atherosclerotic clinical manifestations. In 1976, Oster described clinical trials on 60 patients given daily doses of folic acid and ascorbic acid (90). Results of a 9-yr study of efficacy of treating 198 atherosclerosis sufferers with folic acid were reported by Oster (92). He claimed that folic acid had prevented recurrence of myocardial infarction in 25 cases, reduced nitroglycerin needs in 31 cases, prevented gangrene in peripheral atherosclerosis in 13 cases, and alleviated angina pectoris.

According to Oster's study (92) high folic acid in serum was required for the vitamin to be effective. This accords with the results of Kaplan's studies (61) on inhibition of milk XO by folate analogs and derivatives in which folic acid was unlikely to be an effective clinical agent for inhibition of XO unless high intracellular concentrations of the vitamin or its breakdown products pterin aldehyde and hydroxymethylpterin could be achieved. (Folic acid was a much poorer inhibitor of XO than these breakdown products.) However, Ho and Clifford (50) in examining the effect of folic acid in vivo found that large doses of dietary folic acid did not inhibit liver or intestinal XO in the rat. They argued that because 2 mg of folic acid taken every other day is sufficient to maintain saturation in human tissue, the beneficial effect ascribed to large doses of folic acid was unlikely to result from inhibition of XO. However, it is possible that dietary folic acid could inhibit ectopically placed XO but have little effect on liver and intestinal XO.

In a letter to the editor of the Journal of Nutrition criticizing Ho and Clifford's paper (50), Ross and Oster (105) offered a second rationale for the value of folic acid — its role as a coenzyme in biosynthesis of plasmalogen. In support of this explanation, they referred to work of Tietz et al. (125) in which butyl alcohol, a long chain glyceryl ether, was oxidized to fatty acid and glycerol by an enzyme system in liver. Pteridines such as tetrahydrofolate were effective coenzymes. Tietz et al. considered the possibility that oxidation occurred via formation of a vinyl ether (as in plasmalogens) intermediate but concluded that a far more likely intermediate was a hemiacetal (125). They did not show folic acid as a coenzyme in the biosynthesis of plasmalogen as claimed by Ross and Oster (105). Povoa et al. (99) observed increased plasmalogen in arterial but not myocardial tissue of rats after oral administration of folic acid.

Further work is necessary to test the efficacy of treatment by folic acid of atherosclerosis sufferers. If Oster's observations are confirmed, a detailed examination of its mode of action will be imperative.

BIOCHEMICAL ASPECTS

An important aspect that appears to have received little attention is the nature of the chemical action of XO at the site of deposition if, in fact, it does reach the tissue in active form.

Enzymic Hydrolysis of Plasmalogens

The work of Oster and Mulinos (95) is often cited as demonstrating "the depleting action of bovine milk XO on tissue plasmalogens" (e.g., 107). However, Oster and Mulinos stated that "the enzyme was shown to have no effect on
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The plasmalogen concentration (95). The plasmal has to be released from the plasmalogen before it can be acted on by XO. In vitro the plasmal can be released at low pH (ca. 1), but this breakdown in vivo is highly unlikely (40). However, as Oster (81) suggested, the breakdown could be enzymic. A plasmalogenase which hydrolyzes the vinyl ether bond of plasmalogen to produce lysophospholipid and plasmal (fatty aldehyde) has been described in brain tissue (5, 55), and a similar enzyme which hydrolyzes the vinyl ether bond in lysoplasmalogen has been observed in liver (131, 137). There do not appear to be any reports of a vinyl etherase in heart tissue.

Oster (81) envisaged the presence of a phospholipase A2 that would act on plasmalogen to produce lysoplasmalogen, which in turn would be hydrolyzed via the action of a vinyl etherase to produce the plasmal. There is ample evidence of phospholipase A2 in heart tissue (34, 58, 134), and it appears that activation of this enzyme accompanies myocardial ischemia (121). Oster and Ross (97) concluded from studies using phospholipase A2 of bee venom that the phospholipase A2 of heart muscle may be activated by catecholamines. Sobel et al. (121) found an accumulation of lysophospholipids in ischemic rabbit myocardium but observed no significant hydrolysis of plasmalogens to lysoplasmalogens. In contrast to the findings of Oster and Hope-Ross (94) and others (19, 72) with human atherosclerotic heart and aortic tissue, Sobel et al. (121) observed similar plasmalogen concentrations in control and ischemic hearts. Owens et al. (98), however, observed hydrolysis of plasmalogen of dog myocardial membrane fractions treated with exogenous phospholipase A2, although it appears from their data that the phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) were hydrolyzed faster than the corresponding phosphatidyl compounds (plasmalogens). These authors found no evidence of endogenous plasmalogenase activity in the tissue fractions.

Figure 3. Schematic representation of intervention role of xanthine oxidase (XO) in the plasmalogen degradation-resynthesis cycle envisaged by Oster (82).

This assumes that plasmalogen is synthesized from plasmal. However, it is doubtful that this occurs. The ether bond is formed by replacement of the acyl group on sn 1-acyldihydroxyacetone phosphate by an alkyl group from a fatty alcohol (35), and after acylation of position sn-2, dehydrogenation occurs to form the vinyl ether linkage at position sn-1. This appears to be a general biosynthetic pathway for all mammalian tissue (136).

Little is known about the degradation and biosynthesis cycle of plasmalogens within membranes, but it appears that fatty aldehydes per se are not used for synthesis of these phospholipids in vivo. It is possible, however, that the aldehydes are reduced enzymically to fatty alcohols that then can enter a pool and serve as plasmalogen precursors.

Reactions of Xanthine Oxidase

There is no direct evidence that exogenous XO deposited in the aorta or heart tissues affects the integrity of the membrane either directly or indirectly. The presence of XO together with lowered plasmalogen in some atherosclerotic tissues offers only circumstantial evidence for a relationship of cause and effect.

Gandhi and Ahuja (36) attempted to resolve this question by assaying XO and lipids in various organs of three rabbits administered XO intravenously for 12 days and of three rats fed "raw cow milk fat globules" (apparently
unhomogenized) for 10 wk. A selection of their data is shown in Table 2. They found substantial amounts of XO in hearts in both trials. Heart tissue of control animals exhibited no XO activity. Aortas of both control and trial animals showed no XO activity. Differences between XO activity in liver, kidneys, and skeletal muscle of control and trial animals were small.

From Table 2, plasmalogen content of heart tissue decreased in both rabbits and rats receiving XO; the 75% decrease for the rabbit was most marked. There were also substantial decreases (up to 25%) of total lipid content of both heart and aorta tissue, decreases of phospholipid percentages (of total lipid) in rabbit heart and aorta and rat aorta, as well as decreases of phosphatidyl ethanolamine and phosphatidyl choline in rat aorta of XO-fed animals relative to controls. No explanation was advanced for these last changes. Small numbers of experimental animals and lack of statistical analysis of data make interpretation of this work difficult, and further experimentation of this nature is indicated.

Despite XO deposition in heart tissue of XO-fed animals in the trials described, no histopathological changes were observed. The authors attributed this to the short time of the experiment. In the work of Ho and Clifford (51), no arterial plaque formation occurred in the rabbits fed XO intravenously over 13 wk. However, in contrast to findings of Gandhi and Ahuja (36), no significant reduction of plasmalogen content of heart or aorta of XO-fed rabbits was observed by Ho and Clifford (51). Because both of these trials (36, 51) involved small numbers of animals, the findings should be viewed with caution.

One line of reasoning from which the depleting action of XO on plasmalogens was adduced partly was the lack of these phospholipids in tissue containing XO. Bovine milk fat globule membrane, which is a particularly rich source of XO, also has significant quantities of plasmalogens (up to 4% of total phospholipids) (73).

A further interesting point in relation to this hypothesis is the finding that milk and some milk products, particularly yogurt, have a hypocholesterolemic effect and by implication (because hypercholesterolemia is widely considered a major risk factor) may reduce the risk of heart disease (100). Although the effect has attracted several explanations, it is relevant in the present context that Howard and Marks (57) suggested that a factor in the milk fat globule membrane (the milk component rich in

| TABLE 2. Effects of xanthine oxidase (XO) fed intravenously in purified form to rabbits and XO fed as milk fat globules to rats on the XO activity and lipids of heart and aorta. Adapted from Gandhi and Ahuja (36). |
|-------------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
|                               | Heart             | Aorta             | Heart             | Aorta             |
|                               | C                  | XO                | C                  | XO                |
| XO activity (mU/g tissue)     | 0                  | 115               | 0                  | 0                 |
| Total lipid                   | 5.1                | 4.2               | 8.9                | 7.5               |
| Total phospholipid (% of total lipids) | 68.1 | 57.2 | 89.2 | 79.7 | 69.1 | 69.4 | 72.6 | 55.2 |
| Free cholesterol              | 2.9                | 2.8               | 1.9                | 1.9               |
| Cholesterol esters            | .64                | .67               | .60                | .60               |
| Phosphatidyl choline          | 19.8               | 17.3              | 26.3               | 25.1              |
| Phosphatidyl ethanolamine     | 14.3               | 12.5              | 24.6               | 25.1              |
| Sphingomyelin                 | 3.4                | 4.0               | 1.8                | 3.7               |
| Phosphatidyl serine           | 2.2                | 3.6               | 6.7                | 5.7               |
| Phosphatidyl inositol         | 2.5                | 2.3               | 3.8                | 3.0               |
| Plasmalogens                  | 10.2               | 2.5               | b                  | b                 |

aC = Control; XO = xanthine oxidase fed.
bCould not be estimated because of small sample.

HOMOGENIZED MILK AND ATHEROSCLEROSIS

XO) may be hypocholesterolemic. Antila et al. (6) reported evidence supporting this contention.

Oster indicated that he now considers the superoxide anion produced by XO to be the cause of the initial damage to the plasma membrane (93). As discussed, superoxide and its dismutation product, singlet oxygen, are capable of effecting oxidation and other reactions in lipids. Milk XO can catalyze oxidation of membrane lipids in milk. The reaction proceeded via the superoxide anion as addition of superoxide dismutase inhibited oxidation (48). Hill (48) suggested that milk should be pasteurized at a higher temperature (80°C) to reduce oxidation of lipids and ascorbic acid through greater inactivation of XO. Oster (84) advocated a similar course (heating to 82 to 84°C) to inactivate XO completely for the reasons described in this paper.

Oxidation of polyunsaturated membrane lipids is damaging to tissues, including those of heart. Xanthine oxidase is a more effective lipid oxidant in the presence of a substrate such as xanthine or an aldehyde (48). Thus, if XO together with an XO substrate were in heart tissue, it is conceivable that oxidation of membrane lipids could occur. Such oxidations proceed via hydroperoxy derivatives and can produce an array of end products including short-chain aldehydes and malondialdehyde (48).

Atherosclerotic plaques contain hydroperoxy fatty acids that inhibit synthesis of prostacyclin. This latter compound is a normal metabolite of arachidonic acid in the heart and is a potent inhibitor of platelet aggregation (118). Thus, oxidation of the fatty acid moieties of lipids in the heart and associated vasculature could make the vessels more susceptible to thrombosis, the major lethal complication of atherosclerosis.

An additional mechanism for involvement of lipid oxidation in atherogenesis was demonstrated by Fogelman et al. (33). They showed that low density lipoproteins (LDL) modified by malondialdehyde, a product of lipid oxidation, are taken up by macrophages, cells involved in plaque formation, much faster than unmodified LDL — thus facilitating plaque formation. The extent to which such a pathway may operate is not known.

If exogenous XO is deposited in heart tissue and causes tissue damage, then the mechanisms described would appear more credible explanations of its action than that suggested by Oster involving plasmalogen depletion (81).

Role of Phospholipase A

An element of Oster’s theory was the role of phospholipase A of heart tissue in causing the pain of angina pectoris. Oster (82) considered that the pain was caused when this enzyme was deprived of one of its substrates, plasmalogen, depleted through the action of XO. However, he subsequently (97) offered a more credible explanation that products of phospholipase A reaction, fatty acids and lysophospholipids, display pharmacological qualities that may have adverse effects on the heart. More recently, Oster (93) suggested that phospholipase A may cause pain by its action on phospholipids of nerve fibers.

Phospholipase A2 produces lysophospholipid and polyunsaturated fatty acids including arachidonic acid. Lysophospholipids have powerful cytolytic and membrane-perturbing properties (135) and are thought to be responsible for early changes of membrane permeability that have been demonstrated after a myocardial infarction and that lead to irreversible injury to cells in a relatively short time (<2 h) after the event (54, 59, 121). Specific release of arachidonic acid from the sn-2 position of phospholipids by phospholipases A2 of both vascular tissue and myocardial cells also may be significant (58). This acid can be converted rapidly to prostaglandins, and these can cause inflammatory responses of affected tissue. This effect, together with effects of free fatty acids and lysophospholipids, indicates an important active role of phospholipase A in heart and vascular disease.

CONTENTIOUS ASPECTS AND UNRESOLVED QUESTIONS

Form and Availability of Xanthine Oxidase in Gut

There is a need to investigate the molecular size of the enzyme after gastric digestion. Is the low $M_f$ form of XO reported by Biasotto and Zikakis (14) of significance? Can low $M_f$ active fragments be formed in vivo or does all XO
have a $M_r$ of 300,000 (50)? Is XO from pasteurized, homogenized milk after gastric digestion in the form of liposomes (107) or in free, available form (142)? Is the enzyme from fermented, raw milk (such as the Masai drink) in an unavailable form even after gastric passage (81)?

Absorption of Xanthine Oxidase from Gut

Investigations with experimental animals have produced conflicting results. With humans, immunological results showed a significant correlation between milk intake and antibody titers of blood serum, but XO activity measured in blood did not correlate with amount of milk consumed. Better results for the latter correlation may be obtained on blood or blood leukocytes soon after milk ingestion (107). Unequivocal data could be obtainable if milk containing labeled XO is fed and progress of the label is monitored.

If milk XO is absorbed from the gut, does absorption occur more readily from homogenized than from unhomogenized milk? This question could be answered readily from animal feeding trials.

Xanthine Oxidase in Heart Tissue

Abnormal concentrations of XO have been found in heart tissue of experimental animals and in atherosclerotic plaques of human aorta and heart tissue. What is its origin, and does it have any detrimental effect on the tissue? Oster maintains it does, but Gandhi and Ajuha (36), in a short experiment, found no histologic changes of heart tissue of rats and rabbits containing high XO. Can XO have a depleting effect on membrane plasmalogens? Can XO catalyze oxidation of membrane lipids? Perhaps these questions could be answered by laboratory studies using heart perfusion techniques.

Plasmalogen in Heart Tissue

What is the function of plasmalogens in heart tissue, and what is the nature of their normal metabolic cycle within the tissue? Are they biosynthesized from dihydroxyacetone phosphate by the pathway already elucidated (120), or can they be synthesized from plasmals (36, 80)? Are the plasmalogens normally catabolized by vinyl etherases or oxidases?

Answers to these questions must await detailed metabolic experiments.

Folic Acid Effect

If folic acid is effective in alleviating atherosclerotic disease symptoms, does it act by inhibiting XO (86), or does it have an effect unrelated to XO (50)?

Epidemiological Data

Interpretation of data used by Oster (81, 82, 83) is questionable, and data for some countries do not add support to the theory. In Australia, for instance, homogenization of milk was introduced only after rapid rise of CHD mortality rate and just before a decline in this rate occurred. Further detailed dietary information from carefully designed investigations will be required before epidemiological data can be reliably used in relation to this theory. Account must be taken of the decline of CHD mortality in the US (104) and other countries (32) in recent years.

HOMOGENIZED MILK IN RELATION TO OTHER RISK FACTORS OF ATHEROSCLEROSIS

It is important to put Oster's hypothesis into perspective. Many theories implicating a wide range of factors have been postulated. These factors, known as risk factors, are of genetic, nutritional, and environmental origin (116). The major recognized risk factors are heredity, hypercholesterolemia, hypertension, cigarette smoking, obesity, lack of exercise, diabetes mellitus, psychosocial factors such as stress and personality type (39, 124), and low serum concentrations of high density lipoprotein (77). Several other factors remain to be identified because risk factor analysis still fails to identify about half of those who suffer heart attacks (45).

Nutritional factors are important as they influence, either directly or indirectly, many of the risk factors. Furthermore, they are amenable to manipulation. However, the genetic influence is also important, and, consequently, the disease cannot be controlled totally by dietary measures (78).

Of the several nutritional factors that have been implicated, fats, particularly those with
high cholesterol and saturated fatty acids, are the most widely accepted dietary risk factor for atherosclerosis (1, 76). They have been the subject of a vast amount of research over the last 30 yr. Other dietary factors that have been implicated include sucrose (138), animal protein (21), soft water (117), deficiency of undigestible fiber (65), imbalance of certain minerals such as zinc and copper (63) and calcium and magnesium (111, 128), excessive vitamin D, and trans-unsaturated fatty acids (67).

Milk and dairy products have received considerable attention and have been implicated for reasons unrelated to those given by Oster. The fat of milk, being relatively rich in saturated fatty acids and containing a substantial amount of cholesterol, has been shown in numerous studies to be hypercholesterolemic and atherogenic. The protein of heated milk has been proposed as a primary factor in atherogenesis (3). Segall (112, 113) concluded that milk consumption was associated with CHD after studying consumption data for several foods and correlating these with CHD mortality data of several countries. He suggested that lactose may be the harmful factor in milk (113). In contrast to Segall, others have suggested that milk, particularly if fermented, may mitigate against CHD by being hypocholesterolemic (29, 66, 103). Interestingly, lactose, inter alia, has been suggested as a possible hypocholesterolemic factor (56).

Current research into the effect of diet on composition and metabolism of blood lipoproteins and on formation of compounds such as thromboxane and prostacyclin, which regulate blood clotting, in the near future may provide solutions to the many unanswered questions on the relationship between certain dietary factors and CHD. In the meantime a large number of dietary risk factors, both proven and suspected, remain (109). The claim (82) that there would be reduction of CHD of at least 50% if the US dairy industry produced homogenized milk free of biologically active XO is difficult to believe. It appears unnecessary at this time for the dairy industry to change its processing conditions for homogenized milk or for milk consumers to change their dietary habits on the basis of Oster’s theory.

ASSESSMENT OF HYPOTHESIS

Oster’s hypothesis that milk XO is a major causative factor of CHD has not gained wide acceptance. This is probably because it is largely based on circumstantial evidence, and several aspects of it, biochemical, physiological, and epidemiological, are unsatisfactorily explained.

Previous examinations of the hypothesis have found the evidence inconclusive and insufficient (15, 16, 20). In a major review, Carr et al. (20) concluded that there was considerable doubt about the significance of milk XO as a causal factor of atherogenesis and advocated additional research in certain critical areas.

Recent work has provided evidence that indicates some XO may be absorbed from the intestine after ingestion of milk. However, action of the enzyme, once absorbed, still appears to be highly speculative. There is circumstantial evidence that ectopically-placed XO is associated with plasmalogen depletion of heart and aortic tissue, but until evidence is presented that proves that absorbed milk XO causes tissue damage directly related to atherogenesis, the hypothesis is unlikely to be accepted generally.

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