A REVIEW OF THE SIGNIFICANCE
OF BOVINE MILK XANTHINE OXIDASE
IN THE ETIOLOGY OF ATHEROSCLEROSIS

DECEMBER 1975

Prepared for
DIVISION OF NUTRITION
BUREAU OF FOODS
FOOD AND DRUG ADMINISTRATION
WASHINGTON, D.C. 20204

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by

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The Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology (FASEB) provides scientific assessments of topics in the biomedical sciences. Reports are based upon comprehensive literature reviews and the scientific opinions of knowledgeable investigators engaged in work in specific areas of biology and medicine.

This technical report was prepared for the Division of Nutrition, Bureau of Foods, Food and Drug Administration (FDA), by the staff of the LSRO, FASEB, in accordance with the provisions of Contract No. 223-75-2090.

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The authors acknowledge the contributions of all the consultants who have assisted in this review of the literature and current research. A judicious attempt has been made to incorporate the different viewpoints and opinions in the report. Because there were major differences of scientific opinion, it was not possible to reconcile the divergent viewpoints into a consensus. Therefore, the final report reflects all viewpoints expressed by the consultants. The report has been reviewed by these consultants; however, the authors accept responsibility for the contents of the report. The listing of the Consultants' names in Section VIII does not imply that they endorse the study.

The report has been reviewed and approved by the LSRO Advisory Committee (which consists of representatives of each constituent society of FASEB) under authority delegated by the Executive Committee of the Federation Board. Upon completion of these review procedures the report has been approved and transmitted to FDA by the Executive Director, FASEB.

While this is a report of the Federation of American Societies for Experimental Biology, it does not necessarily reflect the opinion of all of the individual members of its constituent societies.

C. Jelleff Carr, Ph.D.
Director
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SUMMARY

This report reviews the hypothesis that xanthine oxidase in homogenized bovine milk is a significant factor in the etiology of atherosclerosis. The concept holds that the enzyme is absorbed from the intestinal tract along with the small particles of homogenized milk fat, circulates and is deposited in the arterial and myocardial cells where it reacts with the plasmal moiety of cell membranes. The resulting lesions represent the first damage to the arterial and myocardial cells; cholesterol and fibrin infiltration in the arterial wall and scarring of the myocardium are thought to occur subsequently. The evaluation of the hypothesis includes a discussion of the nature of xanthine oxidase, its fate after ingestion in homogenized milk, factors influencing enzyme absorption from the gastrointestinal tract, bioavailability of exogenous xanthine oxidase, its endogenous occurrence in the body, and the possible significance of xanthine oxidase in arterial pathophysiology. The report concludes that the evidence supporting the hypothesis is inconclusive. Most consultants in this study expressed doubt that xanthine oxidase in bovine milk could be significant as a causal or risk factor in atherogenesis. The report includes suggestions for certain critical experiments that need to be performed to answer questions raised by the hypothesis.
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I. INTRODUCTION

A. BACKGROUND

The Office of the Associate Director for Nutrition and Consumer Sciences, Bureau of Foods, Food and Drug Administration (FDA) has a continuing interest in the quality of foods in the American diet. The agency is responsible for evaluating and monitoring the safety of foods, establishing regulations, and providing nutrition information to consumers. Food processing developments that might modify the nutritional quality or introduce health hazards for the consumer are of continuing concern to this agency.

The Life Sciences Research Office was requested by the FDA to review the possible significance of the enzyme xanthine oxidase in cow's milk in the etiology of atherosclerosis. The need for a study of this subject stems from information supplied to FDA by Dr. Kurt A. Oster that implicates the enzyme as ingested in homogenized bovine milk, as a causative factor in the development of atheromatous vascular diseases. This review was undertaken to ascertain whether or not this hypothesis was supported by scientific data and worthy of further evaluation.

The public health importance of atheromatous cardiovascular diseases lends a sense of urgency to test any hypothesis that might elucidate their etiology and therapy. For this reason and because the etiology of the diseases may be influenced by dietary factors, the question posed by the FDA is worthy of careful scrutiny. While prevailing concepts of atherogenesis include multiple etiologic factors, and while there seems to be no question about the association of high levels of blood cholesterol and certain hyperlipidemias with atherosclerosis, direct cause-effect roles have not been established. Indeed, these and other concepts may not be mutually exclusive.

B. SCOPE

This review has included a search for relevant scientific publications and a solicitation of the opinions of experts in the fields of dairy chemistry, nutrition, biochemistry, enzymology, and clinical research on the etiology of atherosclerosis. The opinions were obtained during conferences convened for this purpose and by letters requesting review and critique of reprints of pertinent literature supplied by LSRO.
A comprehensive search was conducted through the National Library of Medicine MEDLINE service, and other current publications for literature dealing with factors influencing inactivation, absorption and enzymatic digestion in general as well as the pathophysiology of plasmalogen and xanthine oxidase interactions. Current research studies including those to be published in the near future were discussed with the consultants.

This report includes a presentation of the Oster hypothesis on the significance of homogenized bovine milk xanthine oxidase in the etiology of atherosclerosis, a discussion of the nature of xanthine oxidases, and an evaluation of his concept. From this evaluation additional research needs are identified.
II. THE HYPOTHESIS: XANTHINE OXIDASE IN ATHEROSCLEROSIS

Oster (1968) advanced the hypothesis of a possible etiological mechanism for human atherosclerosis. Because he has proposed and expanded his hypothesis in several publications since that time, this section of the report provides a synopsis without comment of the Oster hypothesis as it is currently held. Discussion of the major points of the hypothesis is presented in Section IV.

In developing the hypothesis, Oster (1968) coined a new term, "plasmalogen disease," based on his concept of a deficiency of plasmalogens in the arterial intima and myocardial cells. He postulated that ectopic xanthine oxidase from bovine milk destroys the aldehydes liberated from the cell membrane-based plasmalogens as they are metabolically turned over. This results in the initial damage to the cell membranes of the arterial intima and the myocardium. The resulting histochemical injury evokes repair processes manifested by cell proliferation and scar formation in the affected myocardium, local deposition of cholesteryl esters, and ultimate development of typical atherosclerotic lesions in the arteries.

According to this concept, bovine milk xanthine oxidase not only survives passage through the human stomach, but is absorbed enzymatically intact from the gastrointestinal tract, entering the general circulation via the intestinal lymphatics and thoracic duct. Because homogenization of milk markedly increases the biological availability of its xanthine oxidase, consumption of this milk is a predisposing factor in the atherosclerotic process.

Oster has derived support for his hypothesis from his clinical practice with patients suffering from effects of atherosclerosis. He has observed that treatment with folic acid, a xanthine oxidase inhibitor, combined with ascorbic acid and avoidance of homogenized milk and milk products containing the active enzyme resulted in marked symptomatic improvement among patients with angina pectoris, intermittent claudication and other clinical manifestations of atherosclerosis.

Further support for the hypothesis comes from observations that plasmalogens are widely distributed in the human body, the highest amounts occurring in the skeletal muscle, myocardium, arterial intima and myelin sheaths (Oster, 1974a). According to Oster, during plasmalogen turnover the enzymes phospholipase A and vinyl etherase split plasmalogens into lysolecithin and the free fatty aldehydes palmitial and stearal. These aldehydes can then be oxidized by xanthine oxidase to fatty acids, gradually
depleting the cell membranes of their plasmalogen content, creating the initial biological injury. Oster's concept of plasmalogen metabolism is presented in Figure 1. Based on current concepts of superoxide radical formation, Oster has suggested that simultaneous xanthine oxidase-generated superoxide radicals may also play some role in the initial injury.

Oster and Hope-Ross (1966) observed the absence of plasmalogen in freshly infarcted human myocardium. Because frozen histologic sections treated with mercuric chloride and fuchsin-sulfurous acid failed to show the presence of plasmal aldehydes, they concluded that the plasmalogen had disappeared. Unfixed sections stained with hematoxylin and eosin from the infarcted area showed no structural difference from sections taken from the adjacent noninfarcted myocardium. The early disappearance of plasmalogens from the infarct in this case was interpreted to mean that in the early stages of myocardial infarction, vinyl-etherase is liberated and has the ability to split the plasmalogen, allowing the resulting plasmal aldehydes to diffuse and be oxidized (Oster and Hope-Ross, 1966). Several studies have shown that plasmalogens are depleted in atherosclerotic areas of the aorta and in otherwise normal aortas in older individuals (Buddeke and Andresen, 1959; Miller et al., 1964; Oster, 1971).

Ectopically deposited xanthine oxidase was demonstrated enzymatically in atherosclerotic and myocardial lesions but it was not present in normal myocardium and arterial tissues from the same subject (Ross et al., 1973). Oster and his colleagues concluded that the most likely source of this xanthine oxidase was dietary and that because cow's milk and certain dairy products contain the active enzyme in abundance, this was probably the source.

To detect antibodies to bovine milk xanthine oxidase in persons with atherosclerosis, Oster et al. (1974) using the hemagglutination method of Boyd (1951), measured xanthine oxidase antibodies in the blood sera of 75 patients, some of whom had manifest atherosclerosis. The titers of specific antibodies to the bovine xanthine oxidase varied, but were for the most part higher in those patients with clinically evident atherosclerosis. The investigators concluded that the bovine xanthine oxidase must have been absorbed through the intestinal wall because the measured antibodies were specific to the enzyme and that the antibodies are the body's defense mechanism against persistent ectopically-deposited bovine xanthine oxidase. They considered that their findings invalidate the argument that the ectopically-deposited xanthine oxidase originates from the liver or the intestine. They concluded that ectopic xanthine oxidase came from bovine milk because they found specific antibodies to the enzyme in human sera.

Oster et al. (1974) reasoned that milk fat globules containing xanthine oxidase can readily pass the intestinal barrier, enter the bloodstream via the mesenteric lymphatics and thoracic duct, and that the xanthine oxidase
Pathways of plasmalogen metabolism and effect of xanthine oxidase on plasmal as proposed by Oster (with permission from Oster, 1974)
then becomes ectopically deposited in the arterial intima and myocardial cells by a process termed insudation (opposite of exudation).

The concept of persistent, ectopically-deposited xanthine oxidase led to a search for a suitable inhibitor that might serve as a therapeutic agent against xanthine oxidase-induced atherosclerosis. Oster used the xanthine oxidase inhibitor allopurinol in patients suffering from severe angina pectoris (Oster, 1968). The response was a reduction in the frequency and severity of the anginal attacks. The rationale for this treatment arose from the assumption that the ectopic xanthine oxidase acts as a constant oxidizer of plasmal liberated from plasmalogen, causing it to disappear completely.

Because of undesirable side effects of prolonged use of allopurinol, folic acid in divided doses of up to 80 mg per day was administered based upon the knowledge that pteridines are known xanthine oxidase inhibitors (Oster, 1973).

In a five-year study, Oster is currently evaluating combined folic acid-ascorbic acid therapy in patients with symptomatic forms of atherosclerosis. The ascorbic acid is combined with folic acid as a protective antioxidant and as a cofactor for folic acid reductases. The results are described as gratifying in relieving symptoms and diminishing the progress of atherosclerosis (Oster, 1974a, 1974b).

In support of his hypothesis, Oster (1971) emphasizes certain international statistical information. Comparison of levels of consumption of milk and milk products containing biologically active xanthine oxidase with published death rates from arteriosclerotic and degenerative heart disease suggests that the largest consumers of milk and milk products have the highest mortality rates, provided that xanthine oxidase is biologically available in the consumed products. He has pointed out that the Masai of East Africa are consumers of enormous quantities of milk but have a low prevalence of atherosclerosis. According to Oster, this is explained on the basis that the Masai's milk is consumed in large curd, yogurt form, which reduces the biological availability of the xanthine oxidase. In addition, he has noted that in those countries in which milk is traditionally heated or boiled rather than pasteurized, there is a lower incidence of deaths from cardiovascular diseases.

Oster has also pointed out that a group of Europeans with the same risk-factor profile as Americans had 50 percent fewer heart attacks (Tibblin et al., 1971). The higher incidence in Americans suggested to the investigators the need to search for some other factor which was uniform in all the American subjects in the study. Oster has suggested that xanthine oxidase in homogenized milk could conceivably be this unknown factor.
III. RESUME OF THE SUBJECT

A. CONCEPTS OF DIET-RELATED CAUSES OF ATHEROSCLEROSIS

Although the specific mechanisms are unknown, it is generally believed that diet has an important influence on the development of atherosclerosis. There are a number of hypotheses that suggest dietary factors are involved in atherogenesis. This brief synopsis of concepts of the formation of atheromatous lesions causally related or associated with diet provides background information for considering the Oster hypothesis and its relation to other prevailing etiopathogenic theories.

Epidemiologic studies of the prevalence of atherosclerotic diseases gave rise to the notion of "risk factors" that reflect personal characteristics associated with a higher than average risk of clinical disease in addition to the recognized consequences of aging (Stamler, 1973). Risk factors include possible etiologic agents such as certain dietary fats, abnormal serum lipoprotein levels, or possibly undiscovered factors. Sucrose ingestion has also been claimed to be a factor in the development of atherosclerosis (Yudkin, 1966). However, this has been strongly criticized by Grande (1975). According to the risk factor concept, removing the etiologic agent would reduce the incidence or severity of the disease. These issues are a continuing source of controversy among investigators and clinicians. The Oster hypothesis includes eliminating an alleged causal or risk factor (bovine milk xanthine oxidase) from the diet.

Over the years impressive evidence has accumulated showing that populations differing in habitual intake of saturated fats and dietary cholesterol may vary significantly in their serum cholesterol levels and the prevalence of coronary heart disease (Stamler, 1973). Postmortem findings in different populations support the relationship between the intake of fat and the degree of atherosclerosis of the aorta and coronary arteries. Many investigators have reported significant correlations between fat intake, serum cholesterol levels and the severity of coronary atherosclerosis (Keys, 1970; McGill, 1968; Stamler, 1973); however, other variables are known to influence atherogenesis.

The most consistently found index associated with atherosclerosis is Type II hyperlipoproteinemia (Fredrickson et al., 1967a, 1967b). This form of hyperlipidemia may be genetically determined, but is usually not familial and is due to heterogeneous causes some of which are diet-related. A variety of clinical studies also have demonstrated that dietary modification can be effectively utilized to reduce previously elevated serum lipids.
(Bierenbaum et al., 1963; Kinsell et al., 1952; Mattson et al., 1972). What has not been conclusively established is that lowering plasma lipids in man will reduce the risk of coronary artery disease, although evidence of such an effect has been reported (Miettinen et al., 1972). While the statistical associations between hyperlipidemia and premature coronary disease have been established, the relationship still requires verification of cause and effect in man.

On the one hand, compelling epidemiologic evidence indicates that some types of hyperlipoproteinemia are risk factors in coronary heart disease. However, conclusive proof is lacking that lowering plasma lipids in man will lower the risk of coronary artery disease. The widely-held assumption that "treatment of hyperlipoproteinemia" will diminish the risk of coronary heart disease and delay the development of atherosclerosis requires experimental validation. Therefore the etiology and pathogenesis of atherosclerosis remain as matters of considerable dispute. Above all, it is probable that multiple factors are involved and atherosclerosis is, in fact, heterogeneous in origin.

Recognizing that the etiology and pathogenesis of atherosclerosis are not firmly established, several authoritative U.S. scientific bodies, i.e., the Food and Nutrition Board (NRC) and the Council on Food and Nutrition (AMA), on review of the literature, have advised the public that it is desirable and in the interests of public health to modify the fat content of the diet if serum lipids are abnormally elevated.

A clear notion of the etiology and pathogenesis of atherosclerosis requires that the role of the arterial wall, hemodynamic factors, and blood components other than lipids in atherogenesis be elucidated (Haust and More, 1972). For example, the aortic smooth muscle cell is a connective tissue cell in the growing animal with the ability to synthesize elastic fiber microfibrils. This suggests a potential de novo role for these cells in the metabolism of the arterial wall (Ross, 1972). For years investigators have speculated about the nature of the "initial lesion" or local injury and reaction in the arterial wall that predisposes to the lipid accumulation characteristic of the atheromatous plaque or fatty streaks. The lipids are chiefly cholesterol and cholesteryl esters. Plasma lipoprotein is suspected as a primary source of the accumulating lipid. However, the mechanisms by which lipid is transported into the vessel wall and the metabolic fate of the lipids at the cellular level are largely unknown (Geer et al., 1972).

Elaborate investigations into the finer details of the factors that control an abnormal influx of lipids into the cells of the artery wall have revealed that the mechanism is not simply one of passive diffusion (Bloch, 1975). Some inciting factor or factors likely involving changes in the metabolism of these cells initiate the process. Analyses have been made of the
composition of the fatty streaks as precursors of atheromatous plaques to obtain clues to the initiating events. For example, the cerebroside and plasmalogen composition of arteries have been studied (Foote and Coles, 1968). The proportion of the total phospholipid content represented by cerebrosides is greater in fatty streaks than in normal intimal tissue or plaques. On the other hand, plasmalogen (aldehydegenic phospholipids) in human aorta decrease in concentration in advanced lesions of atherosclerosis (Miller et al., 1964). As discussed by Oster (1971), the significance of the decrease in plasmalogen may be directly ascribable to the degradation of the palmitaldehyde (plasmal) pool used in the synthesis of membrane plasmalogen. The attendant plasmalogen depletion generates "holes" in the phospholipid bilayer, affecting cell permeability.

Another hypothesis that suggests a possible role of milk proteins in the etiology of coronary atherosclerosis has been proposed by Davies (1971). Based partially on his observations of abnormally high serum levels of antibodies to heat-dried cow's milk protein in patients with coronary heart disease, he concluded that the thrombogenic and filtration hypotheses could be modified into one immunological concept of atherogenesis. He suggested that the milk antibodies activate arterial endothelium, resulting in the local deposition of platelets and fibrin and aggregation of platelets to form thrombi, phenomena that have been observed experimentally with other circulating immune complexes. Changes in vascular permeability, hence in the character of lipoprotein filtration through the arterial walls, may result from the release of histamine and serotonin by mast cells which have been activated by immune complexes (Davies, 1971).

More recently, Ross and Glomset (1973) have proposed that the arterial smooth muscle cells play a fundamental role in atherosclerosis. They suggest that local injury to the endothelium increases plasma protein concentrations in the vicinity of the medial smooth muscle cells and subsequently some of these cells migrate into the intima and proliferate. Further changes in architecture of the arterial wall may result from several factors such as plasma lipoprotein concentration changes, hormonal imbalances or hypertension.

B. NATURE OF XANTHINE OXIDASES

1. Occurrence and Properties

Xanthine oxidase (xanthine: O₂ oxidoreductase, E.C. 1.2.3.2.) is an ubiquitous enzyme that is widely distributed in animals, plants and microorganisms where it has a role in purine metabolism. In primates the enzyme is found in the liver although it has been reported to be present in
the kidney, blood and intestinal mucosa (Li and Vallee, 1973). It catalyzes uric acid production from hypoxanthine and xanthine in terminal purine catabolism. Isotopic studies have established that uric acid may be derived from both exogenous and endogenous nucleic acids (Lehninger, 1975). Despite the identification of the primary pathway of uric acid production, the metabolic importance of xanthine oxidase in this and other catabolic reactions is not fully understood.

Since its discovery in 1902, xanthine oxidase has been the subject of many research studies, primarily because of its complexity and catalytic versatility rather than its metabolic importance. The enzyme has a low specificity for both substrates and electron acceptors. It is capable of catalyzing oxidation of a wide variety of different purines, aldehydes, pteridines and other heterocyclic compounds by a variety of electron acceptors, including oxygen, nicotinamide adenine dinucleotide, nitrate and nitro compounds, various quinones, dyes, ferricyanide and cytochrome c (Avis et al., 1956; Corran et al., 1959; Duggan and Titus, 1959; Fridovich, 1962; Murray et al., 1966; Scott and Brown, 1962; Williams and Lansford, 1967).

The oxidation of hypoxanthine to xanthine and to uric acid, and the oxidation of aldehydes to carboxylic acids are chemically hydroxylation reactions (Williams and Lansford, 1967). Studies with isotopic water have shown that oxygen is introduced into the substrate from the oxygen of water. Oxidation catalyzed by xanthine oxidase is in reality the removal of an electron pair and a hydrogen nucleus, followed by their replacement by an hydroxyl ion from the solvent. When removed from the substrate the electron pair passes to the enzyme and is transferred to the final electron acceptor. If this is oxygen, it is reduced to hydrogen peroxide directly (Olson et al., 1974a) or via superoxide production (Fridovich, 1975). Recent studies using electron spin resonance spectroscopy have shown that electrons from the substrate are equilibrated among the various redox constituents of the enzyme (molybdenum, iron-sulfur center, and FAD) depending on their relative redox potentials. FAD has been identified as the site of reaction of the reduced enzyme with oxygen (Komai et al., 1969). Thus, xanthine oxidase contains an electron transport chain which may be analogous to the terminal oxidation chain in the mitochondria. This capacity is consistent with observations of the spectrum of metabolic activities that characterize xanthine oxidase. For further details of the interaction of xanthine oxidase with molecular oxygen, see Komai et al., 1969; and Olson et al., 1974a, 1974b.

In addition to liver aldehyde oxidase, four enzymes that can catalyze the oxidation of hypoxanthine and xanthine to uric acid have been characterized in some detail. One of these is the xanthine oxidase found in bovine and other vertebrate animal mammary gland secretions. The second is xanthine dehydrogenase found in avian liver (Murison, 1969; Remy et al., 1955), and the third is mammalian liver oxidase (Li and Vallee, 1973). The fourth is
reported to be copper-containing xanthine oxidase from bovine intestine (Roussos and Morrow, 1966). The molybdenum:FAD:iron ratio of the avian liver enzyme is 2:2:8 per mole while mammalian liver xanthine oxidase contains one g atom of molybdenum and four g atoms of iron per mole of FAD (Goodhart and Shils, 1973; Kanda and Rajogopalan, 1972; Murray et al., 1966). Highly purified preparations of milk xanthine oxidase contain eight g atoms of iron and two g atoms of molybdenum per two moles of FAD. The milk, avian liver and mammalian liver enzymes have a molecular weight of 280,000 to 300,000. Bergmann and Dikstein (1956) reported that bovine milk xanthine oxidase and human liver xanthine oxidase had similar substrate specificity.

Roussos and Morrow (1966) reported the isolation of a xanthine oxidase from bovine intestine. It contained iron, copper and FAD, but no molybdenum; however, this has not been confirmed. Most enzymologists agree xanthine oxidase activity requires that the enzyme contain molybdenum. The molecular weight of this enzyme was approximately 240,000. In addition, Roussos and Dillon (1969) isolated four different types of xanthine oxidase from bovine spleen. These enzyme preparations were apparently less active than intestinal and milk xanthine oxidase preparations. Further comparative studies of these xanthine oxidases and the enzymes from milk and liver are needed.

Al-Khalidi and Chaglassian (1965) reported xanthine oxidase in the blood and/or serum of 19 out of 22 species of vertebrates studied. The enzyme was absent or low in human, pig, cat, camel and goat sera; the sera of cow, dog, donkey, guinea pig, rabbit, rat and mouse showed high activity.

Except for dogs, the values for xanthine oxidase activity in the sera correlated well with the values for xanthine oxidase activity in the milk of these animal species. Examination of the various tissues from four species (cat, dog, sheep and cow) indicated that xanthine oxidase is widely distributed in 20 different tissues with high activities in the liver, lungs and small intestine (Al-Khalidi and Chaglassian, 1965). These results are in general agreement with those reported by Morgan (1926).

Xanthine oxidase is also present in several strains of bacteria found in the human intestine (Dikstein et al., 1957; Villela et al., 1955). It is possible that some reported xanthine oxidase activity in milk may come from bacterial contamination.

2. The Milk Fat-Globule Membrane

Brunner (1974) has provided a detailed discussion of the chemical and physical characteristics of the components of the lipid phase of bovine milk. The milk fat-globule has a proteinaceous surface that
interfaces with the milk plasma phase on the exterior and the globule lipids on the interior. It is called the fat-globule membrane, but is not biologically analogous to a cell membrane. It is composed of proteins and lipids including a unique, heterogeneous protein system with glycoproteins, lipoproteins and numerous enzymes, and phospholipids, neutral triglycerides, mono- and diglycerides, free fatty acids, cholesterol, cholesteryl esters, glycolipids, carotenoids and a squalene-like component. While many of these have been thoroughly characterized, several discrete components remain to be clarified, especially the exact composition of the membrane proteins.

The molecular organization of the fat-globule membrane is incompletely understood despite a great deal of investigation. Of the models that have been proposed, generally accepted features include a relatively continuous bimolecular basic structure to which lipoprotein particles are apparently adsorbed. Whether the lipoprotein particles are on the inner or outer surface of the basic membrane structure has not been established. Electron micrographs of segments of membranes reveal typical tripartite structures about 9 nm thick; however, other segments show a less ordered arrangement featuring a layer of spherical particles presumed to be lipoproteins or aggregates of cytoplasmic material from the milk secretory cells (Brunner, 1974).

The enzymes, phospholipids and other components are evidently associated with the membrane with varying degrees of affinity so that they may separate from it during laboratory preparation of membranes or as a result of milk processing techniques such as homogenization. The enzymes associated with the membrane material are apparently mammary cell-derived microsomal constituents existing as lipoprotein complexes.

The original fat-globule membrane undergoes marked physical and chemical changes starting immediately after secretion into the mammary alveolar lumen. In the presence of the milk-plasma phase enzymes, the membrane appears to disintegrate. High temperature pasteurization alters the nature of the fat-globule membrane, and homogenization produces a marked change in its chemical and physical characteristics including a "resurfacing" with casein micelles. Thus, the fat globules formed during homogenization are no longer completely coated with the original membrane material, but instead, with a mixture of proteins from the milk-plasma phase (Brunner, 1974).

3. **Milk Xanthine Oxidase**

Xanthine oxidase is present in the milk of cows (Ball, 1939; Gudnason and Shipe, 1962; Zikakis and Treece, 1971), goats and sheep (Shahani, 1966), but it has been reported to be absent from pig, horse and

-20-
human milk (Modi et al., 1959). However, other studies suggest human milk does contain the enzyme (see p33). Bovine milk is the best known source of xanthine oxidase and the content ranges between 120-180 mg per liter (Corran et al., 1939; Greenbank, 1954). In addition, bovine milk and derived dairy products are the major sources of active xanthine oxidase in the human diet even though pasteurization inactivates about 42-62 percent of the enzyme activity present (Greenbank and Pallansch, 1962). In 1939, Ball described the isolation of a xanthine oxidase preparation from fresh bovine milk, and Avis et al. (1955, 1956) have purified and crystallized the enzyme from this source.

The ability of xanthine oxidase to catalyze diverse oxidation-reduction reactions led early investigators to attribute the aldehyde and purine oxidizing activities of whole milk to a series of separate but related enzymes. However, according to Shahani et al. (1973), bovine milk contains neither xanthine dehydrogenase nor aldehyde oxidase even though it does contain a number of other active enzymes. The metabolic basis underlying the occurrence of xanthine oxidase in mammary secretions of the cow and other mammals has never been explained adequately.

In all cases, including bovine milk, the enzyme is closely associated with the surface of the milk fat-globule membrane. In general, the enzyme is more abundant in butterfat than in skim milk (Massey et al., 1969), also in colostrum and in milk from mastitic udders (Nilsson, 1954; Zikakis and Treece, 1971). Milk xanthine oxidase activity is affected by changes in temperature (Ball, 1939; Gudnason and Shipe, 1962), homogenization (Doan, 1953), presence of ionic and nonionic detergents (Zikakis and Treece, 1970), and by the action of proteases and lipases (Avis et al., 1955; Battelli et al., 1973; Carey et al., 1961). Such treatments presumably release xanthine oxidase from the fat-globule membrane and/or from complexing compounds in milk.

The enzyme occurs in two forms in milk; either bound to the membrane of the fat globule or in the free form. For example, Briley and Eisenthal (1974) have recently found that approximately one-half of xanthine oxidase present in buttermilk is associated with a particulate fraction containing alkaline phosphatase. In further studies of the particulate fraction, they observed that xanthine oxidase activity is unchanged after proteolytic release of the enzyme from the membrane. The other half of the xanthine oxidase activity occurred in a free state unassociated with the fat-globule membrane complex. Briley and Eisenthal (1974) separated the two enzymes on the basis of their relative capacity to oxidize xanthine and reduced nicotinamide adenine dinucleotide (NADH). In addition, Greenbank and Pallansch (1962) reported that xanthine oxidase is reactivated upon storage of condensed milk; presumably, this is the result of the release of bound enzyme to the free form.
Battelli et al. (1973) have reported that bovine milk xanthine oxidase appears as an oxidase and can be converted into an NAD$^+$ dependent dehydrogenase by treatment with dithioerythritol or dihydrolipoic acid, but only to a small extent by other thiols. The dehydrogenase form is inhibited by NADH. The kinetic constants of the two forms of the enzyme are similar to those of the corresponding forms of rat liver xanthine oxidase. Milk xanthine oxidase is converted into an irreversible oxidase form by pretreatment with chymotrypsin, papain or subtilisin (Battelli et al., 1973).

It is apparent that the enzyme(s) that is designated xanthine oxidase is a versatile protein capable of catalyzing the oxidation of numerous purine derivatives and aldehydes by a variety of electron acceptors. As might be expected for an enzyme of such low substrate specificity many inhibitors have been found. It may be simplistic to refer to this biochemically significant enzyme as "xanthine oxidase" implying a degree of specificity that is not warranted. In this connection, it is of interest to note that Junge and Krisch (1975) have reviewed the available information on pig liver esterase and concluded that highly-purified preparations exhibit heterogeneity at the molecular level. The enzyme has at least two active sites and different preparations can be critically identified only by such techniques as isoelectric focusing or gel electrophoresis in polyacrylamide gels of low concentration (e.g., 4 to 5 percent). Reexamination of the several xanthine oxidase enzymes with similar techniques would provide further information on the basis of the apparent molecular heterogeneity of these enzymes.

In the remaining sections of this document, the term, "xanthine oxidase," refers specifically to bovine milk xanthine oxidase. The liver enzyme, endogenous human xanthine oxidase or enzymes from other sources are separately identified.
A. FATE OF THE ENZYME

One of the prevalent criticisms of the Oster hypothesis holds that all xanthine oxidase ingested will be irreversibly denatured in the stomach. This is based upon the knowledge that the acidity of human gastric juice would destroy the enzyme. Furthermore, if some of the enzyme should remain active during its passage from the stomach to the duodenum, it most probably would be hydrolyzed and inactivated by the proteolytic enzymes of the small intestine.

Recent evidence shows that in rats some ingested bovine milk xanthine oxidase survives the gastric environment and probably that of the small intestine as well. Zikakis et al. (1975) studied the activity of the enzyme in cow's milk intubated into the stomachs of rats by examining curds at autopsy. They have reported relative enzyme activities in the curds of 62 percent after 30 minutes and 45 percent after 90 minutes. The found typical gastric transit times for ingested milk to be about 30 minutes for the rat. However, measurable enzyme activity was evident up to 7.5 hours in some of the experimental animals.

These data are not too surprising in view of the fact that milk is an effective buffer of gastric juice; but the final pH would vary with the ratio of milk to gastric juice. For example, ingestion of 250 ml (approximately 8 oz) whole milk altered the pH of the empty human stomach to about 5.4 and despite continued gastric juice secretion, the pH did not drop further (Zikakis et al., 1975). Based on these observations a prepared mixture of two parts milk and one part simulated gastric juice (hydrochloric acid, pepsin and sodium chloride) was maintained at 37°C and stirred continuously; the lowest pH observed after 24 hours' digestion was 5.28, and xanthine oxidase activity was present. It is generally held that if the pH of stomach contents drops to 5.0 or below, most of the enzyme in pasteurized homogenized milk is inactivated (Gotto and Jackson, 1974). However, the pH of gastric contents varies with the size and nature of a meal and the length of time food remains in the stomach. It is logical to conclude that the pH could drop below 4.0 depending upon the above factors, but most particularly upon the ratio of gastric juice to stomach contents. In vitro measurement of mixtures of one part gastric juice with one part milk had a pH of about 4.0 and no xanthine oxidase activity was detected by the polarographic method (Zikakis et al., 1975).
In another investigation of the fate of ingested milk known to contain xanthine oxidase, Clifford* has estimated the influence of hydrochloric acid as well as gastric juice on the enzyme in vitro. Fresh raw milk was incubated at 37°C at several pH values in a citrate-phosphate buffer and xanthine oxidase activity was measured polarographically. At pH 2.8, activity disappeared in less than 10 minutes while at pH 3.35, all activity disappeared at about 30 minutes; at pH 3.8, approximately 55 percent of the initial activity persisted beyond 30 minutes; at pH 7.2, activity persisted for more than 30 minutes. The in vitro effect of hydrochloric acid alone on xanthine oxidase activity of raw milk was measured polarographically at several pH levels. Concentrations of 0.1N HCl (pH 2.9) and higher caused rapid inactivation of the enzyme and with 0.05N HCl (pH 4.94), 75 percent of the activity remained for 60 minutes. In another experiment, he mixed raw whole milk and human gastric juice in different ratios, incubated the mixtures for 30 minutes, measured the xanthine oxidase activity polarographically, and observed that 65 percent of the original activity remained after 30 minutes in a mixture of equal parts of gastric juice and milk. With more milk there was less enzyme inactivation and with more gastric juice, there was more inactivation.

The presence of active enzyme in the acidic contents of the stomach suggests that about 25 percent of the xanthine oxidase in bovine milk could pass through the stomach in some protected form. It is logical to suggest that the enzyme is essentially bound to the fat-globule membrane. The hypothesis holds that the enzyme passes through the stomach and is absorbed in the intestine while bound to the fat globules. However, Clifford* has reported that after incubation with pancreatin for one hour in vitro, some of the enzyme in a bovine milk-gastric juice mixture is converted to a free form of xanthine oxidase, unbound to the fat globule. This experiment did not include attempts to estimate the quantitative recovery of the free xanthine oxidase following pancreatin treatment.

It is possible that some loss of xanthine oxidase could result from proteolytic enzyme activity. It is also possible that the free form of the enzyme could reassociate with fat globules in the small intestine prior to absorption. There is no direct evidence for either possibility, but the latter could be studied by the techniques developed by Briley and Eisenthal (1974). However, in view of the generally held concept that molecules of

*Unpublished data; presented by Dr. A. J. Clifford, Davis Campus, University of California, at a conference held on July 11, 1975 at the Federation of American Societies for Experimental Biology, Bethesda, Md.
the order of 85,000 daltons appear to be the largest that may be absorbed from the small intestine, it is unlikely that the 300,000 dalton xanthine oxidase molecule would be absorbed.

The smallest active site subunit of xanthine oxidase generally recognized among enzymologists is 150,000 - 180,000 daltons (Massey, 1973). However, there is some evidence that an active subunit of xanthine oxidase in the 75,000-85,000 molecular weight range may exist (Biasotto and Zikakis, 1975; Nathans and Hade, 1975). Questions concerning the methods of enzyme purification and molecular size estimation suggest that confirmatory studies using alternate methods are needed.

These recent studies indicate that some active xanthine oxidase in ingested bovine milk in rats, and in \textit{in vitro} preparations designed to simulate exposure to the human stomach, retains its activity, and that exposure to pancreatin liberates some of the enzyme from its bound position on the fat globule in milk. Based on the data from these experiments and estimates of human gastric transit time (Costill and Saltin, 1974), it can be estimated that between 15 and 65 percent of the xanthine oxidase activity present in ingested whole fresh bovine milk could remain active during 30 minutes' digestion and subsequently pass into the duodenum. Similar experiments using pasteurized homogenized milk should be performed, and, if feasible, quantitative estimates of the fate of the enzyme from milk in the small intestine should be made. While these recent experiments indicate that xanthine oxidase may survive passage through the stomach, they did not address, except indirectly, the influence on the enzyme of acid curdling of milk within the stomach. It has been noted that because homogenized and nonhomogenized milk both curdle in an acid medium, it is probable that there would be no difference in xanthine oxidase availability from these two sources. Therefore, the effects of curdling on the fat-globule membrane composition and particle size need to be determined with respect to the bioavailability of xanthine oxidase.

\section*{B. ABSORPTION}

A crucial question to be answered in assessing the possibility of xanthine oxidase absorption from the gastrointestinal tract is whether or not a large molecular-weight protein can pass intact through the normal gastrointestinal tract wall. There are investigations that report absorption of relatively large particles of undigested materials such as liquid petrolatum and uncooked starch from the intestines in human subjects and in experimental animals (Stryker, 1941; Volkheimer and Schulz, 1968; Volkheimer \textit{et al.}, 1968a, 1968b). In addition, emulsified olive oil particles of approximately 0.5 \( \mu \) diameter have been detected in the intestinal wall cells of rats by histological examination (Frazer \textit{et al.}, 1942).
In a series of experiments with animals and human subjects, Volkheimer and Schulz (1968) and Volkheimer et al. (1968a, 1968b) have demonstrated that raw starch granules administered as a suspension to rats, dogs or to young, healthy adult men are transported through the intestinal wall by a process of "persorption." In this process, large, solid particles are kneaded with constant regularity from the intestinal lumen into the subepithelial region by a mechanical process. The particles apparently pass through the intercellular spaces and can be demonstrated in the venous blood a few minutes later. The phenomenon of persorption is described as different from absorption because the epithelial cells do not exert an absorptive function in this process. On the basis of the histologic examination of the tissues of rats fed a suspension of raw potato starch in cream, it was observed that starch granules were found between the epithelial cells of the intestinal mucosa, in the subepithelial region of the mucosa, and in the lumen of lymph and blood vessels of the intestinal wall and also the heart, brain, kidney and lungs. After oral administration of 200 g (2.4 x 10^9 starch granules) potato starch to human subjects, peak concentrations (70 granules/10 ml) were found within 10 to 90 minutes in the venous blood. After 3 days it was practically impossible to find starch granules in their venous blood. Volkheimer et al. (1968b) fed dogs 250 g of potato starch suspended in milk and found that the portal vein blood contained more starch granules than arterial blood. This difference was regarded as proof of a primary portal transport in addition to transport of persorbed starch granules via the chylus.

In reviewing this work, Volkheimer (1972) noted that these raw starch particles of approximately 40 μ diameter detected in the blood by a polarizing microscope or after staining with iodine, represented a relatively small amount of the total dose administered. However, by calculation of the blood volume in human subjects, he estimated that as many as 18,000 starch granules might be in circulation during the experimental period. Persorption of starch granules is considered by supporters of the plasmalogen theory as evidence that xanthine oxidase is absorbed from the intestine; but there is no evidence that persorption of 40 μ diameter particles is directly related to possible persorption of protein macromolecules such as free or bound xanthine oxidase. However these data contradict the accepted idea that the normal adult gastrointestinal tract is an impermeable and impenetrable barrier to the uptake and transport of particles such as starch granules.

Walker and Isselbacher (1974) have reviewed the evidence for the uptake and transport of large molecules such as endotoxins, proteolytic and hydrolytic enzymes and ingested antigens that may be important in the pathogenesis of intestinal or systemic diseases. They noted that, in general, the gastrointestinal tract is considered impermeable to these substances because the normal digestive process efficiently breaks down large molecular weight compounds, and presumably physiological mechanisms for the transfer of particles of this size do not exist.
Drawing upon knowledge of the transport of antibodies in the neonate, Walker and Isselbacher (1974) proposed that an analogous uptake mechanism representing an extension of the primitive absorption process in the infant is most likely to be found in the mature adult intestine. The evidence to support this concept is reviewed and reference is made to findings that the neonatal intestine is permeable to various macromolecules including bovine serum albumin and milk antigens that are taken up by a nonselective pinocytotic process. The uptake of gamma globulin in certain species, including man, is selective and dependent on several factors including a specific receptor site on intestinal cells. They concluded that in any event, the quantity of these substances absorbed in short term absorption experiments is small in comparison with the major nutritional role of absorption in the gastrointestinal tract.

Proteins, including undigested egg proteins (Wilson and Walzer, 1935) and milk (Davies et al., 1969; 1974) as demonstrated by immunologic tests, can cross the mucosal barrier in adults. The lethality of botulinus toxin demonstrates the capacity of this large protein molecule (molecular weight approximately 894,000) to be absorbed and transported through the gastrointestinal wall intact (Lamanna, 1960; Lamanna and Carr, 1967). The fact that a high molecular weight substance such as crystalline Type A botulinus toxin can pass through the intestinal wall to the lymphatics conflicts with the view that only small-sized particles can be absorbed. Admittedly, because the potency of the toxin is great, it has been estimated that only approximately $20 \times 10^9$ molecules need be absorbed from the intestine of the mouse to be lethal.

Absorption of fat micelles by some physicochemical process is also known. Johnston (1968), in a review of mechanisms of fat absorption, has pointed out that fat micelles (diameter approximately 0.005 μ) have been observed between, on and within the microvilli of mucosal cells following fat ingestion. This mechanism of fat absorption is thought to be nonenzymatic.

Any derangement of the integrity of the natural intraluminal and mucosal defenses such as inflammation or ulceration has been shown to increase the uptake of these large molecular weight substances and their transport out of the cells into the systemic circulation. Even in the abnormal gastrointestinal tract, the absorption would have little nutritional significance; however, the quantities could be antigenic and biologically active (Walker and Isselbacher, 1974).

Investigators concerned with the issue of transport of large molecules through the gastrointestinal barrier have stressed the difference between normal physiologic absorption and the intracellular uptake that involves a fundamentally different process. Walker and Isselbacher (1974)
have proposed that the latter phenomenon involves endocytosis by the microvillus membrane, transportation as small vesicles and phagosomes within the cell and the eventual formation of large vacuoles where intracellular digestion occurs. However, they point out that little is known about the entire process although it is modeled after the mechanisms of macromolecular absorption by the neonatal mammalian intestine.

The available evidence indicates that the enzyme xanthine oxidase in milk is associated with the fat globule that undergoes significant changes during homogenization. Despite the small size of milk fat globules, their digestion likely resembles that of other food fat particles. It is generally agreed that the products of fat digestion, primarily free fatty acids and \( \beta \)-monoglycerides, enter the microvilli and apical portion of mucosal epithelial cells by diffusion (Orten and Neuhaus, 1975; White et al., 1973). Triglyceride resynthesis, an endergonic process, occurs in the epithelial cells. Once resynthesized, triglycerides are transported out of the cells as chylomicrons to the systemic circulation via the lymphatics and portal blood. There is no evidence that an enzyme unbound or bound to a fat globule could enter and emerge from the mucosal cells into the systemic circulation by this mechanism and still retain enzymatic activity. There is no experimental evidence that the sequence of events pertaining to the absorption of xanthine oxidase could follow such a pathway.

Notwithstanding these conclusions, the evidence for uptake and transport of macromolecules by the intestine suggests that some other mechanism could exist for absorption and transport of enzymes such as xanthine oxidase.

Immunologic studies suggest additional evidence for movement of macromolecules from the gastrointestinal lumen to the blood. For example, Davies et al., (1969) have reported that the serum level of antibodies to reconstituted heat-dried cow's milk protein is elevated in patients with coronary heart disease when compared with an age-matched control group. Oster et al. (1974) estimated bovine xanthine oxidase antibody levels as well as milk protein antibodies in the blood sera of 75 patients chosen at random from an internal medicine office practice. Thirty-four of the patients had clinical disorders associated with atherosclerosis such as angina pectoris, myocardial infarction, cardiac arrhythmias, intermittent claudication, peripheral vascular disease and chronic brain syndrome. Higher xanthine oxidase antibody titers were found among these patients than in the 41 who had no clinical manifestation of atherosclerosis. The investigators concluded: "Bovine xanthine oxidase must have been absorbed through the intestinal wall because specific antibodies to the enzyme were demonstrated in human sera" (Oster et al., 1974). The reported data did not include individual histories of quantities and types of milk and milk products consumed. However, they have stated that all patients involved
drank homogenized milk except those specified in Table 1 of their report (Oster et al., 1974).

This study of Oster et al. (1974) is open to criticism on several counts. While selected at random, the patients in the test and control groups were not matched by sex, age, ethnic background or state of health. The antigenic purity of the commercial xanthine oxidase used in the agglutination tests was not established. In addition, the test method relied upon an agglutination reaction which, by itself, does not identify the actual source of the proteinaceous antigenic substances but only the presence of an antigen-antibody reaction. From the data presented in this study, it is not possible to determine if the antigen-antibody reaction was elicited by enzymatically active or inactive xanthine oxidase. Finally, while all sera of patients in the test group did exhibit agglutination when diluted, no data on endpoints in these serial dilutions were given. The fact that 6 of 41 "control" sera gave "4 or 5++" agglutination responses versus 12 of 34 in the atherosclerotic group suggests that there is not a specific association between positive agglutination responses and clinically evident atherosclerosis. The two groups overlapped in an area between 2+ and 4+ agglutination. Oster and his associates explain the overlap on the basis that this probably indicated the presence in the test group of a number of patients with undetected atherosclerosis.

Several consultants have suggested that the agglutination technique is insufficiently precise immunologically to distinguish circulating exogenous xanthine oxidase antibodies from related circulating proteins. This criticism is based upon lack of specificity of the technique, not on the idea that production of such antibodies may be induced by endogenous apoenzymes. However, it should be noted that Ultmann and Feigelson (1963) have reported that purified rabbit antibodies against both native and thermally denatured bovine cream xanthine oxidase cross-reacted with xanthine oxidase from human liver. Without further evidence for the specific origin of the observed serum antibodies, the exact identity of the circulating antigen remains in doubt. Immunologic techniques are now available to identify these proteins more precisely.

It is generally accepted that antibodies are specific for the antigen eliciting the response and that the amino acid sequence and three dimensional structure of the antibody molecule are the bases of the immune reaction (Richards et al., 1975). However, current immunologic concepts also include the idea that antibodies may or may not bind to antigens that are closely related to the eliciting antigen. Furthermore, the humoral immune response is typically heterogeneous and involves production of several types of immunoglobulins or individual immunoglobulins with multiple binding sites in response to an antigen (Richards et al., 1975).

There is a need for investigations such as immunologic cross-reactivity studies which could detect absolute similarity or differences of even
one amino acid in the serum antibodies to xanthine oxidase. Exact identification of the antigens which induce formation of antibodies to bovine milk xanthine oxidase requires more critical immunologic investigation using double-label isotopic and immunofluorescent antibody or hapten identification techniques. These sophisticated immunochemical studies would demonstrate the similarities and differences in circulating antibodies to each of the several xanthine oxidase apoenzymes, could identify precisely the origin of antigens initiating synthesis of circulating antibodies, and could be useful in exact definition of possible areas of deposition within the cardiovascular system.

Because Oster postulates that the bovine milk xanthine oxidase is absorbed via the chylomicrons and because the catalytic properties of the enzyme are influenced by its physical state, it is important to understand the effects of human digestion on the state of the enzyme. It is believed that in undigested cow's milk, the xanthine oxidase is essentially all bound to the fat-globule membrane protein and that it remains in this state during gastric digestion. However, when a mixture of cow's milk and gastric juice was incubated with pancreatin, the xanthine oxidase was converted to free enzyme.* The extent of xanthine oxidase binding may be estimated by comparing the enzyme activity in terms of the oxygen consumption of the two substrates, xanthine and reduced nicotinamide adenine dinucleotide (Briley and Eisenthal, 1974). Whether or not xanthine oxidase liberated from the fat-globule membrane remains in the free state in the intestine and is absorbed as such is unknown. In addition, there is no convincing evidence that xanthine oxidase bound to the fat-globule membrane is absorbed. The blood sera of volunteer students given cow's milk to drink showed no significant differences in xanthine oxidase content compared to levels prior to milk ingestion.* The significance of such a "loading experiment" in relation to the Oster hypothesis of slow, long-term absorption and accumulation of xanthine oxidase remains to be established.

C. BIOAVAILABILITY

In this report, bioavailability refers to the presence of exogenous, active xanthine oxidase in the small intestine in a physicochemical state that might permit its passage into the hepatic portal system or into the general circulation via the mesenteric lymphatic system. Oster (1971) emphasized the importance of milk homogenization as a key factor in enhancing the biological availability of xanthine oxidase. Homogenization of milk creates fat globules with an average diameter of 1 μ as compared with the 3.6 μ average

*Unpublished data; presented by Dr. A.J. Clifford, Davis Campus, University of California, at a conference held on July 11, 1975 at the Federation of American Societies for Experimental Biology, Bethesda, Md.
of unhomogenized milk fat-globules, resulting in an approximate 100-fold increase in the number of fat-globules and a 6-7-fold increase of their surface area (Doan, 1953). The Oster hypothesis holds that the increased surface area enhances the adsorption of xanthine oxidase on the globule membranes and, based on the principle that the rate of absorption from the intestine varies inversely with the particle size, the absorptive potential and hence the biological availability of the enzyme are markedly increased by homogenization.

On the other hand, the xanthine oxidase activity that can be measured in fresh, raw, untreated and unrefrigerated milk is low; however, the activity slowly increases the longer the milk stands, and it increases rapidly upon refrigeration as well as by heating (Robert and Polonovski, 1956). It is noteworthy that Zikakis** found no xanthine oxidase activity in over a dozen different cheeses except one sample of Danish cheddar.

Oster (1971) concluded that world statistics on death rates from arteriosclerotic and degenerative heart diseases compared with the consumption of "biologically active" xanthine oxidase-containing milk, butter and cheese support the notion of enhanced bioavailability of xanthine oxidase as a result of homogenization. In addition, he noted that in certain western European countries, with relatively lower death rates from heart disease, it is customary to boil the milk before consumption, thus inactivating the milk enzymes. Oster (1971) referred to the low level of atherosclerosis in the Masai of East Africa and contended that the reduced prevalence was related to the consumption of a curdled, yogurt-like form of milk which he considered to have a low xanthine oxidase activity. However, there are no data on the xanthine oxidase activity in this milk product. Furthermore, Mann and Spoerry (1974) and Mann and his colleagues (1964; 1972) have studied cardiovascular function and disease in the Masai extensively and found that males of this tribe have little clinically-evident coronary heart disease and low levels of serum cholesterol. But, Mann et al. (1972) reported that postmortem examination of 50 Masai males showed extensive aortic atherosclerosis with lipid infiltration and fibrous changes, but few complicated lesions. The coronary arteries showed atherosclerotic thickening "equal to that of old U.S. men." The authors noted that the coronary vessels of the Masai enlarge with age to more than compensate for this disease. They suggested that the Masai are protected from their atherosclerosis by a sustained state of physical fitness which results in capacious coronary vessels.

Obviously, the question of bioavailability of xanthine oxidase is concerned with several other questions such as absorption and the fate of the

**Unpublished data; presented by Dr. J.P. Zikakis, University of Delaware, at a conference held on June 30, 1975 at the Federation of American Societies for Experimental Biology, Bethesda, Md.
enzyme if it enters the general circulation following passage via the mesenteric lymphatic system, the hepatic portal system, or possibly, both. No studies of xanthine oxidase directly applicable to these questions have been reported.

If one accepts the concept of bovine xanthine oxidase circulating in the human blood stream either bound to the microscopic fat globules or free, the question of its availability at the postulated target organs remains unanswered, i.e., is the enzyme capable of interacting with cell membranes and catalyzing the oxidation of plasmals in the intimal and myocardial cells? Rather than selective deposition in the arterial walls and myocardial cells, it is more logical to anticipate that generalized deposition in a series of organs including the liver, spleen, lymph nodes, lung, muscle, kidney and fat depots would be involved. It is clear that answers to such questions require specifically designed experiments which must be done if the basis of the Oster hypothesis is to be clarified.

D. ENDOGENOUS OCCURRENCE IN THE BODY

To help place the Oster hypothesis concerning the role of exogenous bovine xanthine oxidase in perspective, it is desirable to understand the natural and pathological occurrence of xanthine oxidase in the human being. The scientific information in the literature on this subject is limited and contradictory.

In view of the role of xanthine oxidase in purine degradation and the fact that uric acid is the nitrogenous excretory end product in mammals, it seems logical to conclude that most human tissues would contain the xanthine oxidase enzyme system. The enzyme has been found primarily in the liver of many animal species and is known to be present in kidney, blood and intestinal mucosa of certain species (Williams and Lansford, 1967). In man, xanthine oxidase has been reported to be present in significant amounts in the liver and jejunum (Al-Khalidi and Chaglassian, 1965; Morgan, 1926).

Al-Khalidi and Chaglassian (1965), using a sensitive radioassay method, reported no xanthine oxidase in the blood or blood sera of 40 normal human subjects although the sera of a number of other mammalian species were rich in the enzyme. However, Ramboer and coworkers (1972), using a highly sensitive method of measuring uric acid production from xanthine after gel filtration of plasma proteins, found measurable but low xanthine oxidase levels ranging from 1 to 9 millienzyme units*** (mEU)/1 of serum in 17 of

***One millienzyme unit is the amount of enzyme which will catalyze the conversion of one nanomole of xanthine to uric acid in one minute at 25°C and pH 8.35 and at a saturated xanthine concentration.
36 normal subjects. Shamma'a et al. (1965) found low levels of xanthine oxidase in the sera of 20 normal human subjects (17 were below 0.1; one had 0.2; and two had 0.5 international milliunits (ImU)/l. The ImU is close in value to the meEU used by Ramboer et al. (1972). Shamma'a et al. (1973) observed elevated serum xanthine oxidase levels in patients with certain types of acute liver disease and suggested serum analysis as a procedure for diagnosis of viral hepatitis and other liver diseases.

The occurrence of xanthine oxidase in human milk remains to be proved conclusively. While present in milk of many animals as noted previously (see p20), most investigators have reported human milk is devoid of the enzyme. For example, Modi and his colleagues (1959) cited their previously reported studies which confirmed that xanthine oxidase does not occur in human milk. On the other hand, Rodkey and Ball (1946) reported xanthine oxidase was present, albeit at low levels, in 3 of 26 human milk samples. Several texts and secondary references indicate that xanthine oxidase is present in human milk (Williams and Lansford, 1967; Wyngaarden, 1972), but the cited references do not support this statement (e.g., Morgan, 1926).

Because of these differing reports, Zikakis (see footnote p 31) has reinvestigated the occurrence of xanthine oxidase in human milk using the polarographic method. They reported that the enzyme is present in varying concentrations in human milk for several months postpartum. The concentrations found are relatively low; this investigation is being extended at the present time.

There is little question that serum xanthine oxidase levels are elevated with acute liver disease or damage. Significant increases in serum xanthine oxidase levels have been observed in patients with diffuse liver disease such as viral hepatitis (Ramboer et al., 1972; Shamma'a et al., 1973). In children afflicted with malnutrition or chronic infections, liver xanthine oxidase levels were an order of magnitude higher than in children with cirrhosis, hepatitis or nephrotic syndrome (Bhide et al., 1974). Apparently, at least in adults, the elevated serum xanthine oxidase levels reflect liver parenchymal damage. It is possible that the serum xanthine oxidase level will prove to be a valuable test for certain types of hepatitis provided a practical clinical laboratory methodology can be developed.

In rare individuals, xanthine oxidase may be absent or markedly reduced in all tissues. For example, xanthinuria, an hereditary disorder, results from a homozygous deficiency of xanthine oxidase, which leads to decreased oxidation of hypoxanthine to xanthine and xanthine to uric acid. The clinical features have been described (Wyngaarden and Kelley, 1972). However very little is known about the nature of purine metabolism in persons with this genetic disorder (Kelley and Wyngaarden, 1974). It would be of interest.
to investigate the occurrence of atherosclerosis in these individuals, but the disorder is very rare.

Taken together, the available evidence suggests that xanthine oxidase is probably present in man in many tissues as a normal component of purine metabolism. In certain acute liver disorders, increased synthesis or cellular excretion leads to elevated serum xanthine oxidase levels. Recently developed sensitive analytical techniques have revealed the presence of the enzyme in human milk. The origin and role of endogenous xanthine oxidase in human milk are unknown.

E. SIGNIFICANCE OF XANTHINE OXIDASE IN ARTERIAL PATHOPHYSIOLOGY

1. Plasmalogens

According to the Oster concept, after entering the general circulation, bovine xanthine oxidase becomes ectopically deposited in the cell membranes of the target organs (myocardium and arterial intima) where it causes the initial atherogenic injury (disturbance of the structural integrity of the cell membranes as a result of plasmalogen depletion). The ectopic xanthine oxidase oxidizes free tissue aldehydes (plasmals, e.g., palmitaldehyde) when they are liberated from the membrane plasmalogens by the hydrolytic action of phospholipase A and vinyl etherase (Oster, 1971, 1972, 1974a). Presumably, in the early stages of myocardial infarction, a plasmalogen-splitting enzyme, phospholipase A, is liberated which deacylates the plasmalogen to form lysoplasmalogen. Vinyl etherase catalyzes the hydrolysis of the lysoplasmalogen, freeing the plasmal aldehydes to diffuse and be oxidized by the ectopic bovine xanthine oxidase (Oster and Hope-Ross, 1966). The postulated alteration of the cell membranes may produce an initial lesion which could lead to inflammation or perfusion of the arterial endothelium or the myocardium, "cell proliferation," and possibly induce microthrombus formation (Ross et al., 1973). Because the catalytic oxidation of plasmal is one of the key events in the concept, the distribution of plasmalogens in normal and pathological tissues needs precise definition.

Plasmalogens are aldehydogenic lipids composed of an aldehyde linked to glycerol as an α, β-unsaturated ether. Most, but not all, are phosphatides. The deacylated derivatives are called lysoplasmalogen. Examples of native plasmalogen phosphatides are phosphatidal ethanolamine and phosphatidal choline.
The structure of phosphatidal choline is:

\[
\begin{align*}
\text{H} & \quad \text{H} \\
\text{CH}_2 - \text{O} - \text{C} & = \text{C} - \text{R}_1 \\
\text{R}_2 - \text{C} - \text{O} - \text{C} - \text{H} & \\
\text{H} & - \text{C} - \text{O} - \overset{\text{H}}{\overset{\text{H}}{\text{O}}} - \overset{\text{H}}{\overset{\text{H}}{\text{C}}} - \overset{\text{H}}{\overset{\text{H}}{\text{N(CH}_3)_2}}
\end{align*}
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The first isolation of a pure native plasmalogen (phosphatidal choline) was made by Gottfried and Rapport (1962). A comprehensive review of the chemistry, distribution in animal tissues, physical properties, histochemistry and biochemical reactions had been prepared by Rapport and Norton (1962). In this review, they noted that rabbit and rat brains contain relatively high quantities of plasmalogens, with decreasing amounts in tissues listed in the following order: heart muscle, lung, skeletal muscle and liver. In human brain white matter, spinal cord, and sciatic nerve, plasmalogens were reported to represent 30 - 35 percent of the total phosphatides. Twenty-eight percent of the phosphatide in horse myocardium was reported as plasmalogen, but only 4.4 percent in the mouse myocardium.

Hack and Ferrans (1960) observed a nitrogen-free plasmalogen in experimentally infarcted myocardium of dogs. Ferrans et al. (1962), using the plasmal reaction with fuchsin-sulfurous acid, reported the presence of plasmal aldehydes in the perimucular areas, sarcoplasm, sarcosomes and presumably, the intercalated discs of normal human myocardium and skeletal muscle. Identification of the precursor plasmalogens was by paper chromatography and multiple spot testing. Oster (1971) has stated that about 30 percent of the phospholipids in the human myocardium are plasmalogens, and that tissues with relatively high levels of xanthine oxidase are not likely to contain plasmalogens. For example, such metabolically active organs as the liver and small intestinal mucosa normally contain no plasmalogens (Oster, 1971). However, nearly pure phosphatidic acid preparations from rat liver contained 15 percent plasmalogen (Rapport and Norton, 1962). Using the fuchsin-sulfurous acid reaction for plasmal, Oster and Hope-Ross (1966) observed that plasmalogen had disappeared from the infarcted myocardium of a human patient whose tissues were obtained for analysis less than 2 hours after onset of the fatal heart attack. No histopathological changes were observed in this freshly infarcted tissue by standard light microscopy. Similarly, Oster (1971) reported the absence of plasmalogen in the atherosclerotic aorta of a 22-year-old drowning victim.

Plasmalogen content reportedly decreases in human atherosclerotic aortas, and with increasing age so that between 60 and 80 years, the plasmalogen content in the aortic wall decreases by as much as 40 percent (Buddecke and Andresen, 1959).
Analysis of 120 human aortas from persons ranging in age from newborn to 85 years showed significant decreases in plasmalogens (measured as aldehydegenic lipid) in atherosclerotic samples; however, plasmalogens were elevated in both normal and atheromatous aortic tissues in specimens from the 7th decade but were lower in atherosclerotic tissues up to the 5th decade (Miller et al., 1964). The authors did not comment on the lack of agreement between their findings and those of Buddecke and Andresen (1959) in tissues of subjects in the 7th decade.

In a brief report, Povoa et al. (1973) described an increase in blood plasma plasmalogen levels in 25 patients with ischemic heart disease as compared with the levels of 31 normal subjects. The authors considered that these findings confirmed the notion that plasmalogens are related to myocardial protection and that they disappear from cardiac muscle in ischemic states.

Phospholipase A, formed by the action of trypsin on pancreatic pro phospholipase A, acts only in the presence of bile salts and Ca$^{++}$ to liberate lysophosphatidylcholine from phosphatidylcholine (White et al., 1973). Phospholipase A occurs also in the small bowel. Phospholipases of the A class are also found in cobra and rattlesnake venoms (Baldwin, 1967; Lehninger, 1975). Oster (1971, 1972) holds that phospholipase A and vinyl esterase are present in many tissues including human heart muscle. Presumably, vinyl esterase has the capacity in vivo to cleave the aldehyde moiety from the lysoplasmalogen formed by the catalytic deacylation of plasmalogen by phospholipase A.

The analysis of concentrations of plasmalogens in tissues and histochemical methods for their detection have been reviewed by Rapport and Norton (1962). Their critique of the methodologies suggests serious discrepancies in the values reported in the literature. For example, the plasmal reaction methods introduce uncertainty because of the sensitivity of the reaction to artifacts produced during preparation of tissue samples. Wittenberg et al. (1956) noted the Schiff reagent used in the plasmal reaction tends to react with a variety of compounds other than aldehydes and that the presence of excess lipids in the sample interferes with the color development of the plasmal reaction.

In another criticism of the fuchsinsulfurous acid method for the identification of plasmalogens, Rapport and Norton (1962) observed that the introduction of an analytical method based on p-nitrophenylhydrazone formation by Wittenberg et al. (1956) was a notable advance because it increased specificity and gave reliable stoichiometry without sacrificing sensitivity. It appears that crucial studies directed to the assessment of the differences in the concentrations of the plasmalogens including proper evaluation of the validity, specificity and accuracy of the methods remain to be conducted.
Finally, Gotto and Jackson (1974) stated that they excluded plasmalogens from their review of biomedical phospholipids because they could find no reports of studies to implicate them in the two major topics reviewed, membrane enzyme function and lipoprotein structure.

2. **Xanthine Oxidase**

Although present in the human liver, kidney and jejunum, xanthine oxidase has not been reported in normal myocardium (Ramboer, 1969). In a comprehensive review of enzymes in the arterial wall, Kirk (1969) did not report xanthine oxidase in normal or arteriosclerotic arteries of human beings or experimental animals. He cited studies which showed the presence of an aldehyde oxidase in rabbits' aortas, and in this connection, noted that part of the aldehyde oxidation in aortic tissue may result from action of the hydrogen peroxide formed from monoamine oxidase reactions.

Ross *et al.* (1973) studied xanthine oxidase activities in the heart muscle of two patients that were autopsied following cardiovascular death. They found elevated levels of xanthine oxidase activity in the pericoronary myocardium specimen of one patient and in tissue from the apex of the heart (exhibiting no visible epicardial scarring) compared with normal-appearing myocardium from each heart. Ross *et al.* (1973) also compared xanthine oxidase activity of "grossly normal, less involved, pinkish" aortic tissues of five males with "tissues visibly containing yellowish atherosclerotic plaques" from the same aortas of the same patients. They found a significant elevation of enzyme activity in the abnormal tissues but not in the normal-appearing tissue of some of the subjects.

In these studies, Ross *et al.* (1973) measured xanthine oxidase activity by the Haining and Legan (1967) method, using a Beckman Ratio Fluorometer (Beckman Industries, Palo Alto, California). They provided data on enzyme activities in "normal" and "pathological" cardiovascular tissues; however, the enzyme activities were not reported in terms of actual protein concentrations in the samples. It is not known if protein concentrations, and hence enzyme activity, were similar in these samples taken from normal tissues and those taken from areas with atheromatous plaques. Reviewers of these studies have been critical of the very small number of cases and the small number of analyses on which conclusions were based. A paucity of detailed descriptive procedural information in this report has been alleged by some critics but denied by supporters of the hypothesis.

It seems reasonable that if xanthine oxidase is absorbed, transported in the plasma and deposited in the arterial tissue, then one should be able to detect xanthine oxidase activity in sera of patients with well-characterized atherosclerotic lesions. Despite the observations of Ross *et al.* (1973) on xanthine oxidase in tissues of persons with manifest atherosclerosis, a
study of the serum xanthine oxidase levels in patients with well-characterized atherosclerotic lesions revealed no elevation as compared with normal subjects (Clifford*). However, it is possible that no elevation of serum xanthine oxidase level is associated with the postulated deposition of the enzyme in the intimal walls and myocardium. Additional studies are needed to clarify this aspect of xanthine oxidase distribution as hypothesized by Oster.

The hypothesis holds that some of the xanthine oxidase, attached to micronized milk-fat globules resulting from homogenization, passes through the intestinal barrier and, via the lymph stream, into the arterial system. However, liberation of the enzyme from the bound to the free form by action of pancreatin casts doubt on this aspect of the Oster concept despite the fact that raw untreated milk was used in the pancreatin experiments. This and other questions about the fate of the enzyme in the gastrointestinal tract, as noted in Section IV, A, must be resolved before the question of possible absorption and mode of absorption can be answered.

Oster believes that the results of treating his patients with folic acid add support to his theory. The rationale underlying the treatment is based upon the concept that folic acid, or some derivative, is a xanthine oxidase inhibitor. He has stated that he is treating approximately 80 patients whose illnesses include symptomatic forms of atherosclerosis with combined folic acid-ascorbic acid. The ascorbic acid serves as a protective antioxidant. The results are described as gratifying in relieving symptoms and diminishing the progress of atherosclerosis (Oster, 1974a, 1974b).

The human nutritional requirement for folic acid is approximately 50 μg per day and the Recommended Dietary Allowance (RDA) is 400 μg per day for the adolescent and nonpregnant, nonlactating adult (National Research Council, Food and Nutrition Board, 1974). Tamura and Stokstad (1973) have shown that tissue saturation with folates is maintained with ingestion of 2 mg every other day after an initial loading dose. Excess dietary folate is presumably excreted in the urine (Herbert, 1975; Tamura and Stokstad, 1973). In view of these data, the rationale for use of doses up to 80 mg per day, that is, up to 1600 times the human nutritional requirement and 200 times the RDA, is not clear.

The inhibition of xanthine oxidase by derivatives of folic acid in vitro is well known (De Renzo, 1956); however, there is no direct evidence

*Unpublished data; presented by Dr. A.J. Clifford, Davis Campus, University of California, at a conference held on July 11, 1975 at the Federation of American Societies for Experimental Biology, Bethesda, Md.
that when ingested, these substances actually inhibit xanthine oxidase \textit{in vivo}. In addition, allopurinol is regarded as a more potent inhibitor of xanthine oxidase (Woodbury and Fingl, 1975). There is considerable information available on \textit{in vivo} inhibition of xanthine oxidase by allopurinol used in therapy of gout. No reports of an associated reduction in cardiovascular disease incidence or severity in subjects treated for gout with allopurinol have been found.

Even though the folic acid-ascorbic acid treatment is reported to be efficacious (Oster 1974a, 1974b), it is widely held that approximately one-third of patients with angina pectoris may have spontaneous remissions with any type of intervention including placebos. The placebo effect might be a factor in these cases of symptom alleviation. The beneficial experiences claimed by Oster for the folic acid therapy do not lend themselves to precise scientific appraisal, for only through carefully planned and executed laboratory and clinical investigations may the merits of a proposed therapeutic agent be evaluated. It is hoped that publication of these clinical studies will answer a number of questions concerning this treatment.

3. Epidemiological Data and Prevalence of Atherosclerosis

Several consultants questioned the selected epidemiological and food consumption data used by Oster (1971) to correlate arteriosclerotic and degenerative heart disease death rates with per capita consumption of milk and milk products. They indicated that a correlation would be at best only suggestive of possible relationships in 1967. Inasmuch as the Oster hypothesis does not identify consumption of homogenized milk and milk products as the single cause of arteriosclerosis, correlations between gross mortality data and milk consumption figures for populations should be viewed as neither supporting nor refuting evidence.

Available data on morbidity and death rates associated with cardiovascular disease, particularly arteriosclerosis, do not provide any definitive conclusions as to the possible association of the increased production and consumption of homogenized milk with concomitant rising occurrence of arteriosclerosis. There is no question that cardiovascular diseases account for more deaths each year than all other causes of death combined (Levy and Ernst, 1973; National Heart and Lung Institute (NHLI), Task Force on Arteriosclerosis, 1971). Mortality rates provide minimal direct information on morbidity and a national study on occurrence of arteriosclerotic events has not been conducted (NHLI, Task Force on Arteriosclerosis, 1971). However, most authorities agree that illness and disability from atherosclerosis are very prevalent in adults in the United States population.

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Keys et al. (1972) using populations of adult males from Europe and North America matched in regard to risk factors, found men in the United States had approximately twice as much coronary heart disease as their European counterparts. Levy and Ernst (1973) have estimated that over 4 million individuals have definite evidence of coronary artery disease and that 2.1 million of these are under 65 years old. It should be noted that this estimate does not include peripheral arteriosclerosis. There is no doubt that premature debilitation from cardiovascular diseases in general is a serious socioeconomic problem in the United States.

In 1967, approximately 84 percent of the cardiovascular mortality was related to arteriosclerosis and its complications (NHLI, Task Force on Arteriosclerosis, 1971). However, analyses of more current data collected over the past two decades indicate that the trend of age-adjusted death rate for arteriosclerotic heart disease including coronary heart disease is declining (Klebba et al., 1974). These data also from the National Center for Health Statistics, show an increasing mortality rate from 1950 to 1963 (185.2 versus 221.2 deaths per 100,000 population respectively). Since 1963, the trend has been reversed, and despite a revision in data coding and classification, most authorities agree that mortality rates for deaths resulting from arteriosclerotic disease have begun to decline (Klebba et al., 1974).

Based on their interpretation of several publications (Oster, 1971; 1972; 1973) suggesting homogenized milk as an etiologic factor, Bierman and Shank (1975) have pointed out that heart disease mortality rates began to rise prior to the widespread availability and use of homogenized milk in the United States. Based in part on these and other epidemiological data, they concluded currently available evidence does not support the contention that consumption of homogenized milk contributes to mortality from coronary artery disease via the absorption of xanthine oxidase.

In this connection, it is of interest to note the studies of Enos et al. (1953) and McNamara et al. (1971) on coronary artery disease in United States combat casualties in Korea and Vietnam, respectively. While the two groups of investigators used slightly different diagnostic criteria, they evaluated the degree of atherosclerosis in the right and left coronary arteries in young males (mean age 22.1 years) killed in action. Enos et al. (1953) found 77.3 percent had some degree of atherosclerosis and 15.3 percent had over 50 percent luminal narrowing related to plaque deposition. In the second study 18 years later, McNamara et al. (1971) observed coronary atherosclerosis in only 45 percent of the coronary arteries from 105 combat casualties and observed little or no luminal narrowing in all but one of the casualties. This decrease in incidence of atherosclerosis is highly significant and is important with respect to the validity of the hypothesis if one assumes that both groups were typical consumers of homogenized milk.
It has been suggested that the presence of atherosclerotic heart disease in a significant number of young ostensibly healthy adults is related to unidentified factors encountered during childhood and adolescence. Supporters of the Oster hypothesis point to homogenized milk consumption as one of these factors, but there is no definitive evidence to support this suggestion.

The concept of high incidence of atherosclerotic morbidity and possible decreasing mortality from such conditions is tenable because advances in preventive and supportive medical care have prolonged the lives of individuals affected with atherosclerosis and these individuals may subsequently die from other causes. There are few epidemiological data available that support this assumption even though the idea is generally accepted.

Oster (1971) has postulated that homogenization is important in regard to the bioavailability of the enzyme. The United States population has the second highest mortality rate but consumes less than half the amount of milk as the Finnish population on a per capita basis. These observations would seem to incriminate homogenized milk and lend support to the Oster hypothesis. According to Oster (1971), the higher mortality rate in Finland may be explained by the fact that although they consume only one-third of their milk in the homogenized form, their per capita intake is much higher than that in the United States. On the other hand, these epidemiological and consumption data can be interpreted to detract from the concept that homogenization is a causal or exacerbating factor because the Finnish people, even with the highest mortality rate, consume only one-third of the milk as homogenized milk. Regardless of the interpretation of such gross statistics, these data do not differentiate the milk drinkers from the abstainers nor the heavy and moderate consumers from those who use it occasionally.

Thus, epidemiological data on mortality and morbidity provide little direct evidence that either supports or refutes the Oster hypothesis. An indication of the validity of associating the incidence of atherosclerosis with the consumption of homogenized milk could only be demonstrated by an epidemiologic study of the disease morbidity and mortality in persons who drink pasteurized homogenized milk, those who drink only pasteurized milk, and those who do not drink milk.

4. Other Considerations

A recurrent question concerns the notion that the cleavage of plasmalogens, which occurs in vitro with dilute hydrochloric acid or mercuric chloride, may be expected to occur in vivo. However, the Oster hypothesis suggests that phospholipase A and vinyl etherase, respectively, catalyze the cleavage of plasmalogen in the arterial intima and myocardium to lysoplasmalogen and plasmals (such as palmitaldehyde). The available evidence for the presence of vinyl etherase and phospholipase A in these
tissues is limited. Warner and Lands (1961) reported the presence of an agent in rat liver capable of catalyzing the hydrolysis of the alkenyl ether linkage in various plasmalogens. Zieve and Vogel (1961) demonstrated the presence of lecithinase A (phospholipase A₂; E.C.3.1.1.4) in the sera of normal subjects and found that patients with pancreatitis had markedly elevated serum levels of the enzyme. Weglicki et al. (1973), from studies of rats and dogs, concluded that myocardial phospholipases are activated at acid pH in vitro and also activated in the isolated, perfused, ischemic dog myocardium. Moreover there is a breakdown of structural lipids of myocardial membranes under conditions of acidosis and ischemia that approach those seen in myocardial infarction. In their study of the influence of growth and age on phospholipase activity in aortic tissue, Eisenberg et al. (1971) refer to the presence of phosphatide acyl-hydrolase (phospholipase A₂) in the aortas of rats, rabbits and humans. From the results of these studies, the assumption could be justified that phospholipase A occurs normally in human myocardium and arterial walls; however, confirmatory studies should be done. For vinyl etherase, the meager available information leaves in doubt the question of its presence in normal human myocardium and arterial tissues.

If the hydrolysis of plasmalogens by phospholipase A and vinyl etherase occurs in vivo as postulated, it is difficult to imagine what extra effect oxidation of the aldehydes by xanthine oxidase would have on the tissue plasmalogen content inasmuch as both phospholipase A and vinyl etherase would catalyze essentially irreversible reactions, resulting in the loss of plasmalogens. However, Oster and his associates have pointed out that plasmal is the common intermediate in the in vivo synthesis and degradation of membrane plasmalogen; consequently, according to their concept, the hydrolysis of plasmal by xanthine oxidase would result in a net loss of plasmalogen.

The demonstration that the catalytic actions of several enzymes involve the production and reactions of the superoxide free radicals may be of significance with respect to reactions of xanthine oxidase (McCord and Fridovich, 1968, 1969; McCord et al., 1973). Xanthine oxidase can produce the superoxide free radical by action on its substrates, xanthine or acetaldehyde (Fridovich, 1975). Aldehyde oxidase also produces the superoxide free radical by action on its substrate N-methylnicotinamide as detected by chemiluminescence measurements (Arneson, 1970).

Superoxide dismutases in living organisms catalytically scavenge the superoxide radicals and prevent the intracellular toxicity of this form of oxygen (Fridovich, 1974; 1975). It is also speculated that antioxidants such as tocopherols and ascorbates could scavenge the remaining superoxide radicals and their products not acted upon by superoxide dismutases. Fridovich (1974) has suggested the possibility of irreversible cellular injury sustained as the result of the formation of these free radicals although he notes that this is speculation at this time.
In considering the possible actions of xanthine oxidase or aldehyde oxidase on cell function, it may be suggested that while oxidation of plasmal might cause the primary lesion, the simultaneous generation of the superoxide radicals could also produce cell membrane damage. In this connection, it is noteworthy that aldehyde oxidase may normally be present in the intimal wall (Kirk, 1969). Further study of these ideas should include appropriate consideration of the effects of the cellular superoxide dismutases.
V. CONCLUSIONS

Based on the existing biochemical, physiological and chemical data, most of the consultants expressed the opinion that there is considerable doubt that xanthine oxidase in cow's milk is significant as a causal or as a risk factor in atherogenesis. They indicated that whether the ingested enzyme or some substance arising from its presence in vivo plays a role is unknown because certain critical experiments have yet to be performed and others require confirmation. These consultants endorsed the need for additional research only because questions raised by the hypothesis include several unanswered questions which are critical to the understanding of the etiology of atherosclerosis.

Oster and his associates have concluded that the role of xanthine oxidase as a causal factor in atherogenesis is relatively well established and they endorse additional supportive research.

The Oster hypothesis advances a role for xanthine oxidase in atherogenesis and myocardial damage; however, the evidence is inconclusive. Analysis of the current state of knowledge about xanthine oxidase, as presented in this review, reveals that there is a paucity of evidence that small amounts of this enzyme may pass through the stomach, be available for absorption in the intestine, be actually absorbed intact, retain their biological activity in the blood, selectively oxidize plasmal in the arterial and myocardial membranes, and predispose to atherogenesis. Nevertheless, the hypothesis represents a different approach to the etiology of atherosclerosis and while scientific proof of its validity is lacking, the possibility that it might have some relationship to atherogenesis cannot be entirely ruled out with the evidence available. There is need for systematic, scientific elucidation of the fate of ingested bovine milk xanthine oxidase in the human body.

Although the concept may be speculative, the urgency to discover new approaches to elucidate the etiology of cardiovascular diseases warrants additional research on the topics that have been identified in this report. It would seem advisable that certain studies be done and for this reason a number of suggestions for research are listed in Section VI. These studies could answer the major questions and determine whether xanthine oxidase from bovine milk does play a role in atherogenesis.
VI. SUGGESTIONS FOR FUTURE RESEARCH

A number of research opportunities have been identified in this study; however, priorities have not been indicated. Because of the public health urgency of discovering means of preventing atherosclerosis, serious consideration should be given to investigating new concepts if they offer some opportunity for progress in understanding the basic mechanisms of atherogenesis. Results from studies of the type reviewed in this report must be available before a definitive conclusion can be made about the possible untoward effects of ingesting bovine xanthine oxidase in milk. The methodology exists to explore these issues and to develop a firm scientific basis for a definitive conclusion. The question of the validity of the proposition that orally ingested xanthine oxidase plays a role in the development of atherosclerosis in man can be answered.

NATURE OF XANTHINE OXIDASES

Information needed for an understanding of the possible biological role of exogenous xanthine oxidase in the human being includes knowledge of the subunits of the enzyme and techniques for identifying source specificity. Future research should include emphasis on:

- Confirmation of the existence of enzymatically active subunits of the 270,000-300,000 dalton xanthine oxidase molecule;

- Study of the biochemical behavior of xanthine oxidase subunits;

- A search for possible biochemical differences between bovine milk xanthine oxidase and xanthine oxidase from other bovine or human sources;

- Isolation, purification and characterization of human xanthine oxidases; and,

- Development of a reliable method for specific identification of bovine milk xanthine oxidase in biologic environments, such as in human blood serum \textit{in vivo}. 

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The fate of ingested bovine milk xanthine oxidase in the human body must be understood if the significance of this enzyme in the diet is to be identified. Homogenized pasteurized milk as well as other forms of bovine milk and cream consumed in the United States should be the test substances. Animal studies will likely yield clues to the types of investigations to be conducted in human subjects. Areas for future research should include:

- Quantitative determination of active xanthine oxidase remaining in chyme after gastric and small intestinal digestive processes;

- Determination of the ratio of milk fat-globule membrane-bound versus free xanthine oxidase after exposure to pancreatin and other enteric enzymes in vitro and in vivo;

- Determination of whether or not xanthine oxidase, freed from the fat-globule membrane by pancreatin, will recombine with the fat-globules in the small intestine;

- Establishing the proportion of ingested milk xanthine oxidase destroyed, enzymatically inactivated or otherwise catabolized during human gastrointestinal digestion;

- Investigation of the influence of acid curdling of milk on the bioavailability of xanthine oxidase in ingested bovine milk including the effects on milk fat globule membrane composition and globule size; and,

- Measurement of the biological half-life of intravenously administered bovine milk xanthine oxidase.

The absorption of ingested milk xanthine oxidase remains controversial, and is clearly a key question in the fate of the enzyme. In general, the gastrointestinal tract is considered impermeable to macromolecules of this size. Future research should include:

- Investigation of the possible absorption of large molecular weight proteins such as xanthine oxidase from the gastrointestinal tract during the life of the animal and whether or not this process resembles that of the neonate;

- Assay of human serum levels of xanthine oxidase in relation to milk ingestion. If a positive correlation is found, confirmation of source specificity will be necessary as well as differentiation between exogenous and endogenous sources; and,
In human subjects, confirmation of the existence of immuno-specific circulating antibodies to ingested bovine milk xanthine oxidase. If confirmation proves successful, investigation should be extended to find possible epidemiologic correlates for atherosclerosis.

SIGNIFICANCE OF XANTHINE OXIDASE IN ARTERIAL PATHOPHYSIOLOGY

Because relatively little is known about the presence of xanthine oxidase in normal and pathological cardiovascular tissues, and in view of the scarcity of information about the biological role of the cell membrane plasmalogens, future research should include:

- Measurement of xanthine oxidase in normal and atherosclerotic human arterial intimal and subintimal layers and in normal and ischemic myocardium, with differentiation between exogenous and endogenous enzyme;

- Using a source-specific method of identification, establishment of or ruling out the presence of ectopically deposited bovine milk xanthine oxidase in arterial and myocardial tissues of human milk consumers;

- Demonstration of whether or not exogenous xanthine oxidase is deposited in the cardiovascular tissues of experimental animals given amounts of milk that do not cause measurable changes in blood serum xanthine oxidase levels (because the concept holds that even very small amounts of the enzyme absorbed daily can produce atherosclerosis);

- Confirmation of reports of changes in plasmalogen content of atherosclerotic arteries and ischemic myocardium;

- Establishment of the occurrence and biologic significance of phospholipase A and vinyl etherase in normal and diseased human cardiovascular tissues;

- Confirmation that plasmals are found in vivo in normal human cardiovascular tissues, using a reliable method of detection;

- Better understanding of the biosynthesis and degradation of plasmalogens and proof of xanthine oxidase interaction with plasmalogens, particularly a demonstration that xanthine oxidase catalyzes the oxidation of plasmals in vivo;
Extension of the investigations of the biologic roles of plasmanogens, particularly in cardiovascular tissues including studies of intimal and myocardial cell membranes employing modern biophysical techniques such as charge transport measurement, fluorescence scanning microscopy, reporter groups at specific positions in protein molecules, and nuclear magnetic resonance studies of macromolecules;

Investigation of the possible adverse effects of orally and parenterally administered xanthine oxidase on the cardiovascular system of experimental animals in acute and lifetime studies; and,

Development of a histochemical method possibly using immunofluorescence principles to demonstrate the presence and locations of xanthine oxidase in atherosclerotic lesions at various stages of progression, especially during the early phases when it would involve the postulated initial insult to the endothelial and the subintimal cells.


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