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STUDIES ON XANTHINE OXIDASE IN COW'S MILK

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ABSTRACT

STUDIES ON XANTHINE OXIDASE IN COW'S MILK

By

Greg. S. G. Cheng

Xanthine oxidase (E. C. 1. 2. 3. 2.) was purified from fresh cow's milk by differential centrifugation and hydroxylapatite chromatography. The final product possessed A(280nm)/A(450nm)=4.84; A(1cm, 280nm, 1%)=11.9; activity/A(450nm)=141; specfic activity=3.59 IU/mg; and a detectable dehydrogenase activity. Purified enzyme was a reversible oxidase form and could be converted to dehydrogenase with 10mM dithiothreitol (DTT) or 1% mercaptoethanol (ME). Chemical analyses of the enzyme preparation indicated the presence of 14.8% protein nitrogen and the absence of lipid. The enzyme contained 82 sulfhydryl groups per mole (302,000 daltons) with 44 of these occurring in disulfide bonds. Amino acid composition revealed that the enzyme was hydrophobic in nature and contained lysine as its N-terminal residue.

Triton X-100 did not affect the enzyme activities while 1% mercaptoethanol enhanced its dehydrogenase activity. Six molar urea reduced the ability of oxidase to convert to dehydrogenase. An active monomer of XO with an estimated molecular weight of 155,000 was obtained from a Sephacryl S-200 gel filtration column in a 6M urea environment. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified enzyme revealed a single, sharp zone with a molecular weight of 151,000 $\pm$ 4,000. The enzyme retained its oxidase activity after limited proteolysis by trypsin, chymotrypsin, plasmin, pancreatin, pepsin and papain. The proteolyzed XO remained unresolved

in polyacrylamide gel electrophoresis with and without dissociating agents such as 1% mercaptoethanol or 6M urea. Three major zones with molecular weights of 85,000-100,000, 30,000-35,000 and 18,000-20,000 were commonly observed in sodium dodecyl sulfate gels. Amino acid content of the four principal subunits of trypsinized XO, e.g., 136,000, 85,000, 35,000 and 18,000 dalton species, indicated a hydrophobic nature and lysine as the N-amino terminal for all subunits.

Measurements of riboflavin and its derivatives in aging milk as well as the nature of its enzymic activity indicate that XO could not be the source of milk riboflavin. Surface and interfacial activities as well as its hydrophobicity indicate that the enzyme is lipophilic. Fat globules in an enzyme-butter oil emulsion were more stable than those in emulsions of casein- or whey-butter oil. The observed experimental evidence supports the hypothesis that XO serves to stabilize milk fat globules in its intracellular transport as well as in secreted milk.

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INTRODUCTION

Xanthine oxidase (XO) is widely distributed in most mammalian groups where it is involved in purine catabolism. Cow's milk is one of the richest sources of XO from which it can be readily purified in reasonable quantities.

XO is a versatile enzyme and performs a ping-pong binary kinetic mechanism which requires both reducing and oxidizing substrates. It is capable of catalyzing the oxidation of various purine and pteridine derivatives and many groups of aldehyde compounds such as aliphatic, aromatic and heteroaromatic aldehydes. A variety of electron acceptors such as oxygen, dyes and nitro-compounds can serve as the oxidizing substrate to accomplish the enzyme reaction. The reaction involving the oxidation of hypoxanthine and xanthine to uric acid, with oxygen serving as an electron acceptor, provides the basis for assaying the enzyme.

Molecular weight of milk XO has been reported, ranging from 275,000 to 370,000. This wide range results from the use of proteolytic enzymes in the isolation scheme. Generally, it is agreed that the molecular weight of XO is 300,000 and consists of two identical subunits of molecular weight 150,000-155,000. It is not known if the subunit is independently active. Active XO (300,000) contains FAD, Mo, Fe and labile sulfur in the ratio 1:1:4:4. Mo and FAD participate in the reducing and oxidizing substrate binding sites, respectively, whereas Fe and labile sulfur constitute the Fe/S center

serving as an electron reservoir.

The enzyme is enriched in the cream phase of milk. Approximately 8-10% of total protein of milk fat globule membrane preparations is XO which exists in two states: free and membrane-bound. Oxidase activity of the membrane-bound XO towards NADH is enhanced relative to that toward xanthine. The ratio of XO activities on xanthine-oxygen/NADH-oxygen constitutes, therefore, the assay for their distinction. Also, XO has been isolated as a reversible oxidase form from cow's milk treated with mercaptoethanol and is capable of converting to the dehydrogenase form. The interconvertibility between oxidase and dehydrogenase forms is a common property of XO from all mammalian sources investigated.

Fat globules in cow's milk are surrounded by two morphologically distinct biolayers. The outer layer consists of typical unit membrane materials and is considered to be the milk fat globule membrane. The inner coat consists of a proteinaceous materials enriched in XO and butyrophilin. The abundance of XO in the inner coat suggests that the enzyme may serve as the interfacial stabilizing agent for milk fat droplets. The fluorescent material found in the aqueous phase during the preparation of milk fat globule membrane has been attributed to the FAD moiety of XO. Thus, it has been suggested that XO may be a principle source of milk riboflavin. That the enzyme serves as a natural source of hydrogen peroxide for the lactoperoxidase system in milk has been suggested. The correlation between the activity of XO with the onset of spontaneously oxidized flavor in milk, as well as its involvement in atheroscloersis have been postulated by several researchers.

Although XO has been extensively studied, especially the mechanism of its enzymic reaction, much work remains to further elucide its biological and physical role in milk and its proteinaceous properties. The objectives of this study were: first, to determine if milk endogenous XO is a dehydrogenase or a reversible oxidase form; second, to elucide the nature of the protein and its functional parameters; third, to assess the relationship between XO and "free" milk riboflavin; and, lastly, to evaluate the hypothesis that fat globules are stabilized by XO.

LITERATURE REVIEW

Occurrence in Milk

Xanthine oxidase (xanthine: oxygen oxidoreductase; E.C. 1.2.3.2.;) is normally considered to be one of a group of enzymes required for the degradation of purines. It is capable of catalyzing the oxidation of many purine and pteridine derivatives and aldehydes by various electron acceptors such as oxygen, certain dyes, and nitro-compounds. The enzyme is widely distributed in animal tissues and blood, particularly in liver (Al-Khalidi, 1965). XO is also present in the milk of cow, goat, sheep, and rabbit but is absent from human, mare and sow milk (Modi et al., 1959). However, Zikakis et al. (1976a) recently reported that human milk contains XO but with a lower activity compared to that in other milk.

Bovine milk is one of the richest sources of XO, containing about 160 mg/ml (Ball, 1939). Kitchen et al. (1970) found that approximately 54% of whole milk XO activity was in the skim milk and 21% in the cream. Churning the cream released 65% of the cream XO into buttermilk. In skim milk the enzyme was not associated with casein and was precipitated at 45% saturation with ammonium sulfate.

Ball (1939) noted that the activity of XO in skim milk increased as the milk aged, especially at low temperatures. He proposed that the enzyme was adsorbed on the fat globule and could be forced into solution if the adsorption surface was decreased by causing fat globules to coalesce. Worden (1943) confirmed this spontaneous increase in the activity of XO when whole milk was cooled or agitated.

He suggested that the release of XO activity accompanied aggregation of the milk fat globules and coincided with disruption of their surface membrane.

Morton (1953) was the first to demonstrate that XO activity was associated with the lipoprotein complex constituting microsomal particles isolated from milk. He reported that the particles were adsorbed on the protein membrane surrounding the milk fat globule (Morton, 1954).

Zittle et al.(1956) indicated that XO activity was concentrated in the cream phase. The pelleted fraction resulting from the centrifugation of cream possessed the highest specific activity among other fractions. They found that XO in the skim milk was not associated with casein or whey protein. Herald and Brunner (1957) reported that the centrifugation of purified membrane material resulted in the concentration of XO activity in the insoluble, pelleted fraction. Mangino and Brunner (1977a) and Briley and Eisenthal (1975) postulated that XO serves as an integral part of the intracellular milk fat globule membrane (MFGM). Although XO has not been detected in the plasma membrane of any tissue, preparations of MFGM contain approximately 8 to 10% of the enzyme.

Because all the major milk proteins function as activator or inhibitor of XO--depending upon their concentration--and the presence of a non-dialyzable inhibitor in the protein-free milk fraction, the true distribution of the activity of XO as it exists naturally in milk may be different from the activities found in separated fractions (Hwang et al., 1967).

Morell (1952) was the first to suggest the existence of an

inactive form of XO enzymic preparation. Avis et al. (1956b) studied the molybdenum content of crystallized XO and other XO preparations. The occurrence of non-stoichiometric FAD/Mo ratios and variable specific activities in crystallized enzyme preparations led them to postulate the presence of two inactive forms of the enzyme. They suggested that the two inactive forms are one containing, and the other deficient in, molybdenum.

Hart et al. (1970) reinvestigated the enzyme prepared by different isolation conditions including a selective denaturation step in the presence of sodium salicylate. They referred to the two unfunctional forms of XO as "inactivated" and "demolybdo" XO. Their data provided clear evidence for the existence of the demolybdo form, although they were unable to isolate it free from other forms. It was found that the amount of demolybdo XO, relative to other forms, never changed in the purification procedure or handling of the enzyme. From extended studies of the enzyme from individual cow's milk, they indicated that the demolybdo XO is a natural product secreted together with the active enzyme and that the relative amount is nutritionally rather than genetically determined. They also reported that even the best purified XO samples are contaminated by a significant amount of the inactivated enzyme. This characteristic may be used to explain the changes in the enzyme which take place during the slow phase of reduction by substrate. McGartoll et al. (1970) confirmed that active XO is rapidly reduced by substrate while inactive enzyme is only slowly reduced.

The inactivated form of XO is being referred to as "desulfo" xanthine oxidase and is normally a preparation or storage artifact.

Conversion of active enzyme to the inactivated form may be minimized by handling the enzyme throughout in the presence of salicylate or ETDA (Bray, 1975).

Edmondson et al. (1972) successfully separated the fully functional XO from unfunctional enzyme with affinity chromatography using allopurinol, an analog of XO inhibitor, as a ligand which bound active enzyme only. They were the first to prove the correctness of Morell's hypothesis. Recently, Nishino et al. (1981) confirmed the observations of Edmondson et al. (1972).

Zikakis and Treece (1971) reported the presence of two XO polymorphs in cow's milk: a high and a low activity species. They claimed that the heterogenous activity of XO in cow's milk is under direct genetic control. The occurrence of genetic polymorphism of XO was supported by Dvorak et al.(1980).

Briley and Eisenthal (1974) studied the catalytic properties of XO in bovine MFGM. They indicated that XO exists in two states in the MFGM. They designated them as free and membrane-bound enzymes which were differentiated by the ratio of activities in xanthine-oxygen and NADH-oxygen reactions. This ratio is the most sensitive assay for distinguishing between free and membrane-bound XO in milk fractions.

Studies have shown a direct relationship between XO activity and the stage of lactation. The change of feeds did not alter the relationship (Rajan et al., 1962). Stannard (1975) reported that the variation of XO content in milk was a function of the stage of lactation and not season. The enzyme activity was found to be minimal at mid-lactation.

Xanthine Oxidase and Milk Membranes

Milk Fat Globule Membrane (MFGM)

The membrane materials surrounding fat droplets in milk is derived from both apical plasma membrane of mammary secretory cells and membrane materials of the Golgi apparatus. Patton and Fowkes (1967) showed the similiarity between the phospholipids of the surface coat of milk fat globules and those of the plasma membrane of mammary tissues. They found that fat droplets within the cell were relatively devoid of certain membrane constituents, i.e., phosphatidyl ethanolamine, and carotenoids, which are characteristically present in the MFGM. It was indicated that membrane materials surrounding the fat droplet were acquired at secretion. The removal of plasma membrane caused by fat secretion is replenished by addition of membrane derived from the Golgi apparatus (Moore et al., 1971)

Dowben et al. (1967) found that many enzymes present in MFGM were also present in the fraction of tissue containing plasma membrane. They reported that enzymes which characterize other tissue fractions were generally absent from MFGM. Agglutination and hemolyzation of bovine erythrocytes by antibody of MFGM provided further evidence that plasma membrane constitutes the source of the MFGM.

Keenan and Huang (1972) compared protein and lipid compositions of MFGM, Golgi apparatus, rough endoplasmic reticulum, and plasma membrane. They found that the phospholipids of MFGM were almost identical to those of plasma membrane. Their distribution in the Golgi apparatus was intermediate between plasma membrane and endoplasmic reticulum. Comparisons of the molecular weight profiles of membrane

proteins from SDS-polyacrylamide gels lead to a similar conclusion. Eight identical electrophoretic protein bands were found. Also, the membrane derived proteins had similar amino acid compositions.

Mangino and Brunner (1977b) employed the statistical difference method (S▲Q) of Marchalonis and Weltman (1971) to analyze the relatedness between MFGM and three different types of plasma membrane, i.e., cardiac plasma membrane, human erythrocytes membrane, and Ehrlich ascite plasma membrane. The data showed a high degree of compositional similarity among these membranes. The characteristics and composition of MFGM has been reviewed in detail by Patton and Keenan (1975), Brunner (1974), and Anderson and Cawston (1975).

XO is one of the most interesting enzymes present in MFGM preparations. The abundance of the enzyme in MFGM, about 8 to 10% of total membrane protein (Swope and Brunner, 1968), indicates that XO may be of structural and/or functional significance.

Briley and Eisenthal (1974) found that approximately half of XO present in buttermilk was associated with MFGM, whereas the remaining portion was free enzyme. Studying the nature of the XO-membrane association, they concluded that most of the enzyme in milk appears to be an integral part of the fat globule membrane. They suggested that XO might be used as a tool to investigate the origin of the fat globule membrane (Briley and Eisenthal, 1975).

Freudenstein et al. (1979) studied the ultrastructure of MFGM from bovine and human milk. They found that the surface layer on fat globules consisted mainly of an outer layer with a typical "unit membrane" appearance, considered as the real MFGM, and an internal proteinaceous coat. This observation was further supported by Buchheim

(1982) who used a freeze-fracture technique instead of thin-sectioning for preparing the sample. The coat material was tightly attached to the membrane and survives isolation and extensive washing of the isolated MFGM. This inner coat material was devoid of identifiable membrane structure and was enriched in XO activity and butyrophilin. It was suggested that MFGM coat complex might represent an unusual altered component of membrane-type proteins and that such a special structure might serve a special function in the process of milk fat globule budding.

Franke et al. (1981) and Jarasch et al. (1981) employed antibodies of butyrophilin and XO purified from MFGM coat material to investigate the location of these two proteins in the mammary gland. Sigificant antibody-antigen reaction of butyrophilin was found in milk secreting epithelial cells and not in other cell types of the mammary gland and various epithelial tissue. In milk secreting cells, the staining of antibody-antigen complex was only revealed at the apical cell surface, including budding milk fat globule, and the periphery of the milk fat globule in alveolar lumina. They found no evidence of stained complexes at the basolateral surface of endoplasmic reticulum and/or the Golgi apparatus in secretory cells. They suggested that butyrophilin was involved in the vertical discharge of milk fat globules. For the XO, they found the antigen in milk secreting epithelial cells but not in epithelial cells of several other tissues. In other tissue, antibody/XO complexes were observed only in capillary endothelial cells. In both milk secreting epithelial and capillary endothelial cells, XO was distributed throughout the cytoplasm. They concluded that XO in the lactating cells was similar to the enzyme

presented in other tissue and may serve similar redox functions.

Above evidences supported the proposal made by Mangino and Brunner (1977a) that the intracellular fat droplets are stabilized with a layer enriched in XO and an additional layer of plasma membrane is added during the secretion. This model would explain why the membrane coat material is rich in XO and butyrophilin and devoid of membrane matrix. Thus, XO may serve as a principal stabilizing agent to the lipid phase in unprocessed milk.

Skim Milk Membrane

Stewart et al.(1972) examined the fluff layer obtained by ultracentrifugation from skim milk. Through electron microscopy, they concluded that the fluff material was composed of membrane-like structure. They believed that most of this material was derived from the plasma membrane, or possible the Golgi vesicle of the secretory cell. A comparison of the lipid composition of a number of membrane systems from milk and mammary gland showed that skim milk membrane was similar to the plasma membrane of the lactating cell but was significantly different from MFGM. From membrane marker enzyme assays, it was concluded that skim milk membrane material and the plasma membrane of the secretory cell share a co-identity (Plantz and Patton, 1973a; Plantz et al., 1973b).

Kitchen (1974) isolated skim milk membrane from skim milk treated with rennet. Compared to the cream membrane, he found that the overall protein composition of both membrane systems was quite similar, but that the major protein components were different. A molecular weight of 70,000 was estimated for the major protein present in the cream membrane, whereas a 85,000 dalton species represents the major

protein in the skim milk membrane which is almost absent in the cream membrane. Compositional data on lipid, protein, and carbohydrate components showed that skim milk membrane differs from cream membrane and indicated that the former was more closely related to secretory plasmalemma than to the cream membrane. Also, he reported that activities of most enzymes were higher in skim milk membrane, except for diaphorase, acid phosphatase, and xanthine oxidase which were 2 to 4 times less than observed in the cream membrane.

Janolino and Swaisgood (1982) found no detectable XO activity but significant high activity of sulfhydryl oxidase in skim milk membrane isolated from either whey or whey retentate.

XO activity is barely detectable in the apical plasmalemma of the secretory cell. If skim milk membrane is derived from plasma membrane only, XO activity would not be found in the membrane. However, since XO and/or membrane fragments containing membrane-bound XO could be release into skim milk, the skim milk membrane might be a mixture type of membrane materials and hence, contains XO activity. Therefore, the presence of XO in skim milk membrane requires further investigation. The decisive factor for the presence or absence of XO activity in skim milk membrane is its definition and/or its isolation procedure.

Isolation of Xanthine Oxidase

Because XO is highly concentrated in the MFGM, fresh cream constituted the usually starting source for isolation procedures. The advantage of using this source is the removal of a large fraction of milk proteins during its preparation. Methods of isolation may be grouped into those that involve a proteolytic digestion step and those

which avoid enzymic proteolysis.

Ball (1939) originally introduced the proteolytic digestion step in the preparation of XO. He incubated buttermilk obtained from washed cream with pancreatic lipase to remove residual casein and to increase yield of XO. The proteolytic species of interest in pancreatic lipase is primarily trypsin. Based on this principle, Ball (1939) obtained a 540 fold purification and 25% yield for a XO preparation from whole milk. Corran et al. (1939) obtained a 1000-fold purification of XO isolated from fresh whole milk without treatment with proteolytic enzymes. Milk was saturated with NaCl at 30 C and the precipitate was removed. XO in the supernatant was precipitated with ammonium sulfate and redissolved prior to adsorption by an alumina gel. After elution from the alumina gel, the enzyme was precipitated again with ammonium sulfate. A yield of approximate 1.8% was obtained.

Avis et al. (1955) starting with pancreatin-treated buttermilk, employed calcium phosphate column chromatography to remove contaminating proteins prior to a final crystallization of XO. They obtained approximate 8% yield and the enzyme approached 100% apparent homogeneity by various criteria. Electrophoretic and sedimentation analyses, and the solubility behavior of the crystallized XO indicated that at least 90% of the final product showed physico-chemical characteristics of a single component (Avis et al., 1956a). Enzymic measurements indicated that both active and closely related inactive forms of XO are present in the final product (Avis et al., 1956b). A high-yield preparation, approximating 70-90% XO, was reported by Gilbert and Bergel (1964) who "improved" the method by adding salicylate, EDTA and cysteine into buttermilk prior to pancreatin

digestion. Salicylate acted here as a stabilizing agent to the enzyme (Bergel and Bray, 1959). The function of cysteine was to release the XO from its association with particulate matter. The purity of the final XO product was in the range of 70-85%.

Except the procedure of Corran et al. (1939), the above purification schemes included a proteolysis step. More recent studies have shown that such treatment produces several forms of XO. Carey et al. (1964) demonstrated that XO prepared by Ball's (1939) procedure could be resolved into several components of similar enzyme activity by gradient elution from hydroxylapatite. XO prepared without proteolytic digestion was eluted as one sharp peak followed by a shoulder containing one third of total enzyme activity. Protease digestion converted an apparently homogeneous preparation of XO into several separable but active forms. Non-digested XO was much more active in catalyzing the reduction of cytochrome c by DPNH and migrated faster in electrophoretic gels. They suggested that the shoulder peak observed with the non-digested XO could have been generated by a native proteolytic enzyme within the milk system. This postulate was supported by the later work of Mangino and Brunner (1977a) who showed that an endogenous membrane-associated protease in milk was co-isolated with XO and degraded the XO stored at 37 C for 24 hr or at 4 C for 30 days.

Nathans and Hade (1975) proved that the proteases from pancreatin were co-isolated with XO in the final ammonium sulfate step. Further purification to remove residual proteases is often carried out with hydroxylapatite or tricalcium phosphate chromatography. However, the effect of these proteases on the XO may have already occurred by this

point in the isolation procedure. Electrophoretic results showed that XO isolated with pancreatin digestion resulted in a loss of the larger components, i.e., molecular weight of 155,000, 125,000, etc. Addition of a serine protease inhibitor during the isolation XO did not assure the survival of the larger size components. They also demonstrated that a commercial XO exhibited significant effects of proteolytic activity.

Massey et al. (1969) and Hart et al. (1970) did not find spectral or catalytic differences between XO purified with and without pancreatin treatment. However, the importance of the effect of proteases during preparation of XO has been shown by the work of Battelli et al. (1973) who were the first to indicate significant functional differences between enzyme preparations obtained by these two methods. They found that milk XO prepared in the absence of protease is a reversible oxidase form which can be converted into a dehydrogenase by treatment with dithioerythritol or dihydrolipoic acid. The dehydrogenase or type D form is NAD^+-dependent while the oxidase form (type O) is not stimulated by the addition of NAD^+. The enzyme can be converted into an irreversible oxidase form by proteolysis with chymotrypsin, pepsin, or subtilisin, but only partially with trypsin. A major fast band and a minor slow band were revealed by gel electrophoresis of purified XO. They observed that the slower band was greatly reinforced after the enzyme was converted to the irreversible type O form by chymotrypsin. The kinetic constants of the two forms of XO are similar to those of the corresponding forms of rat liver XO. The interconvertibility between D and O forms is a common property of XO from all mammalian sources investigated so far

(Waud and Rajagopalan, 1976c; Coughlan, 1980). A commercially prepared XO which included treatment with a proteolytic enzyme could not be converted into a dehydrogenase.

Silver and Zikakis (1979) reported that proteolysis modified substrate binding to the enzyme, resulting in a reduction of its maximum velocity. Temperature, pH optima, Michaelis constants and maximum velocities varied for non-proteolytically- and proteolytically-purified preparations.

For the above reasons, XO to be used in studies of its physical, chemical, and biological characteristics should be isolated by a non-proteolytic procedure. Waud et al. (1975) reported that an electrophoretically pure XO on disc-PAGE and SDS-PAGE was obtained by using a non-proteolytic isolation procedure. They fractionated cream with cold butanol and ammonium sulfate. After chromatography on the removel of residual butanol with Sephadex G-25, DEAE-Sephadex A-50 and Sephadex G-200 chromatography was employed. Cysteine, EDTA, and sodium salicylate were used in all steps except the final G-200 elution. A 10% yield with respect to the first ammonium sulfate precipitation was obtained. Mangino and Brunner (1977a) obtained a 12% yield of the endogenous enzyme, utilizing a non-proteolytic isolation. A 100S pellet obtained from buttermilk was made to 0.1 mg sodium deoxycholate per mg protein. After two steps of centrifugation, the supernatant was submitted to hydroxylapatite chromatography. The purified enzyme was shown to be electrophoretically pure in SDS-PAGE and was purified 340 fold from the original buttermilk.

Nathans and Hade (1978) introduced an ultrafiltration method to purify milk XO. Milk fat globule membrane obtained from raw milk was

concentrated through an Amicon XM-100A membrane. The ultrafiltrate was concentrated again through an Amicon PM-10 membrane. The final enzyme product was revealed as a single band in SDS-PAGE. Compared with the enzyme purified by the conventional non-proteolytic procedure, both preparations were similar according to empirical criteria of homogeneity based on size and absorption spectra. Also, XO isolated by both methods displayed a single, symetric schlieren pattern when assayed by sedimentation-velocity.

A combination procedure of ultrafiltration and column chromatography, including gel filtration and ion-exchange has been reported by Zikakis (1979a). The purified enzyme was undenatured and the yield, 21%, was claimed to be significantly higher than that obtained by other processes.

Affinity chromatography has been introduced to isolate highly active XO from milk. Edmondson et al. (1972) employed a cyanogen bromide activated Sepharose 6B with a ligand of 3-(1-H-pyrazolo(3,4-d)pyrimidin-4-ylamino)-1-propyl-6-amino-hexanoate, an analog of allopurinol, as the affinity column. The non-functional (desulfo) XO does not contain the cyanolyzable persulfide in its active center and hence does not bind to the ligand. A fraction with greater than 95% active species was eluted from the column. Nishino et al. (1981) employed a folate, which is known to be a competitive inhibitor of XO, as a ligand on an AH-Sepharose 4B gel. They demonstrated a successful resolution of active and inactive XO. The active fraction had an extremely high specific activity and was very close to 100% active based on the activity to flavin ratio (AFR). Other ligands, i.e., 9-(p-aminoethoxy-phenyl)guanine (Baker and Siebeneick, 1971) and

N-(6-bromomethyl)-benzylnicotinamide (Chu and Chaykin, 1974) have been used for the isolation of liver XO. Also, immobolized monoclonal antibody to XO on Sepharose has been employed for the preparation of XO (Mather et al., 1980).

Chemical and Physical Properties

Molecular weight of milk XO is 275,000-370,000 which contains two identical subunits with molecular weight of 150,000-155,000 (Mangino and Brunner, 1977a; Andrew et al., 1964; Waud et al.,1975; Nathans and Hade, 1978). The enzyme possesses a sedimentation coefficient of 11.3S and a specific volume of 0.737 cm /g. Extinction coefficient and absorption ratio of pure XO are A(1%,1cm,280nm)=11.7 and A(280nm)/A(450nm)=5.0, respectively. On complete reduction of the enzyme, absorption at 450nm decreases by 70%. Isoelectric point in acetate buffer, ionic strength 0.2, is 5.3-5.4 (Bray, 1975) while Jarasch et al. (1981) found XO migrated to pH 7.2-7.7 when MFGM was subjected to isoelectric focusing.

In general, it is agreed that milk XO free from the demolybdo form contains FAD, Mo, Fe, and labile sulfur in the ratio 1:1:4:4. The enzyme has two identical independently active sites each comprising 1 Mo, 1 FAD, and 4 Fe atoms (or 2 Fe/S centers- each with 2 Fe and 2 S), and a persulfide group. It is not clear if each subunit (150,000) of XO has one active site or is a functional monomer. The removal of the persulfide group resulted in an inactive form (desulfo) of the enzyme. Amino acid analysis shows no striking features other than a rather low tryptophan content (Bray, 1975). Only total half-cystine has been reported. The SH groups of the enzyme can be divided into two groups

according to reactivity with PCMB: those which react rapidly with PCMB at pH 7-12.9 and those reacting slowly with PCMB only at pH>11.7 (Nagler and Vartangan, 1973).

The activity of the enzyme investigated over a pH ranging from 5 to 12, using 0.1M Tris-HCl, Tris-NaOH, Tris-acetate buffer to cover the pH spectrum, showed an optimun pH of 8.3 (Mangino and Brunner, 1977a). Greenlee and Handler (1964) reported that V_{max} of xanthine oxidation is constant from pH 11.0 down to 8.5 and was decreased by a factor of 2 on lowering the pH to 7.0, and by an additional factor of 2 at pH 5.0. The activation energy of the enzyme reaction derived from an Arrhenius plot of initial velocity across a temperature range of 15 to 40 C as 14.1 kcal/mole (Mangino and Brunner, 1977a), or 14.5 kcal/mole for the range of 10 to 38 C (Massey et al., 1969).

Temperature stability of the pure XO has not been studied extensively. Most work on the heat inactivation and cooling effect of XO has been investigated in the milk system. XO activity can be detected in whole milk heated at 170 F (76.7 C) for 15 min. The enzyme was inactivated after heating to 195 F (90.1 C) for 15 sec and could be partially reactivated when the same milk was condensed to 50% total solid and homogenized at 4500 psi. Further increasing homogenization pressure did not enhance the amount of activation (Greenbank and Pallansch, 1962). They suggested that an inactive form of XO which associated closely with fat globules is displaced from the fat globule by homogenization and hence is activated. Excessive homogenization, therefore, not only would free the enzyme but rapidly denature it since free enzyme is apparently sensitive to heat denaturation.

Gudnason and Shipe (1962) found that XO activity in fresh milk

increased after storing at 4 C, heating to 70 C for 5 min, or homogenization. XO activity was also increased when fresh milk was incubated with commercial lipolytic and proteolytic enzymes such as steapsin, lactivase, and pancreatin. The activity of both milk and cream increased on storage, whereas there was no activity increase in skim milk. However, skim milk obtained from milk stored at 4 C for 24 hr showed an increase in XO activity while the corresponding cream showed a decrease. These investigators supported that some of XO is not active in fresh uncooled milk and the observed increase in activity was related to the redistribution of the enzyme. This redistribution was believed to be associated with the dispersal of MFGM in milk after cooling, heating, or mechanical stress.

Bhavadasan and Ganguli (1980) studied the temperature effect of XO activity in milk by investigating the changes of activities of free and membrane-bound XO. They found an increased activity of membrane-bound XO and a decreased activity of free XO in buttermilk concomitant with a significant increase in the activity of free enzyme in skim milk after whole milk stored at 5 C for 24 hr. These observations indicated that the increase of XO activity in whole milk could not be a consequence of desorption of microsomes from fat globules and their release to skim milk as suggested by Gudnason and Shipe (1962). They suggested that the increased activity of membrane-bound XO in buttermilk was due to structure changes occurring in MFGM as a result of cold storage of milk (Coleman, 1973). The increased activity of the free enzyme in skim milk and its decrease in buttermilk was attributed to a distribution of part of the free enzyme from the fat phase into the milk serum during cold storage of milk. They also found a

significant increase of XO activity in the cream phase and a slight increase of the activity in skim milk after heating of milk at 60 C for 5 min. The observation of the state of XO activity in skim milk from heated reconstituted milk led them to conclude that the increased XO activity in milk after heat treatment was not due to the release of the enzyme from the fat phase to the serum phase. They suggested that the increase of XO activity on heated milk was possible due to the presence of a heat resistant activator in milk which reactivated the enzyme or the inactive form of XO became activated during heating of milk.

Bhavadasan et al. (1982) reported that agitation of milk at low temperature, 10 to 25 C, resulted in high lipolysis of milk fat concided with a great release of membrane-bound XO from MFGM into skim milk. They suggested that release of membrane-bound XO from MFGM was due to the disruption of MFGM by agitation. This disruption of MFGM increases the susceptibility of milk fat to the lipase system and hence, enhanced the milk lipolysis.

Mechanical force used in homogenization of milk is much stronger than that used for agitation. During homogenization of milk, MFGM is subjected to an extensive rupture which causes the release of free and/or membrane-bound XO from the fat phase and subsequently increases the enzyme activity in milk. Homogenization studies on milk heated at 48 C for 5 min showed that increase of XO activity was a linear function of the pressure between 70.3 and 281.2 kg/cm^2. Each additional kg/cm^2 of pressure resulted in additional 0.16 milliunits of activity per ml of milk (Demott and Praepanitchai, 1978).

Enzymic Characteristics

Substrates and Inhibitors

Xanthine oxidase is capable of reacting with a wide variety of substrates, requiring two substrates, i.e., both reducing and oxidizing, to complete its reaction. Generally, the reducing substrates are purines, pteridines and their derivatives as well as aliphatic, aromatic, and heteroaromatic aldehydes which can also be the substrate of aldehyde oxidase. Bray (1975) listed a number of reducing substrates and demonstrated the difference of the substrate specificity between XO and aldehyde oxidase. Reducing substrates bind to the molybdenum active site except for NADH which interacts with the enzyme via the flavin molecule. Bunting et al. (1980a) found that nitrogen heteroaromatic cation substrates reacted with the enzyme only at pH values above 9.6. They attributed this change in specificity to an alteration in the substrate-binding site at high pH. This phenomenon provided them with a mechanism for investigating the reducing substrate binding site of XO. They proposed that an electronegative atom (oxygen or nitrogen) of the substrate interacts with a hydrogen-bond donor in the active site during the reaction. This interaction allows the sequence of oxidation at C6, C2, and C8 of purines and C4, C2, and C7 of pteridine. For example, uric acid is generated from xanthine, xanthine is formed from hypoxanthine, and hypoxanthine is produced from oxidation of purine (Bunting and Gunasekara, 1982; also see the Appendix, Figure A4 and A5).

Oxidizing substrates acting as an electron acceptors are oxygen, ferricyanide, ferritin, cytochrome c, and a large number of dyes such

as methylene blue, indophenols, etc. Interaction of oxidizing substrates and the enzyme is via the flavin active site.

The enzyme specificity varies depends on not only the differences in the substrates encountered but also on the concentration of both reducing and oxidizing substrates and the pH values employed in assay conditions. Most reducing substrates such as purines and aldehydes are most rapidly oxidized at neutral pH while the quaternary heterocyclic compounds only react with XO at pH>9.6 (Greenlee and Handler, 1964a; Bunting et al., 1980a,b). Also, the XO reaction is susceptible to inhibition by excess substrates (Bray, 1975).

Inhibitors of the enzyme consist of amino group reagents such as 2,4-dinitrofluorobenzene, 2,4,6-trinitrobenzenesulfonate, benzylaldehyde (Greenlee and Handler, 1964b), alloxantnine (1H-pyrazolo(3-4-d)pyrimidine-4,6-diol) (William and Bray, 1981), 9-(p-aminoethoxy-phenyl)guanine (Baker and Siebeneick, 1971), and allopurinol (4-hydroxylpyrazolo(3,4,d)pyrimidine) (Bray, 1975). Cyanide can inactivate the enzyme due to cyanolysis of a persulfide group in the molybdenum active site. Cyanide-inactivated enzyme can be largely reactivated by incubation with Na_2S (Massey and Edmondson, 1970). Hwang et al. (1967) found that all the major protein fractions in milk can act as an activator or inhibitor of the enzyme depending upon their concentration in the reaction mixture.

Assay

Since XO catalyzes oxidation of a large group of substrates, a number of enzyme assays have been reported. Assays of XO or dehydrogenase generally present no unusual problems. Assays quantitating the catalytic activity of XO through the rate of

substrate disappearance or product appearance are normally employed. The most widely used procedure utilizes xanthine as reducing substrate and oxygen as oxidizing substrate; liberating uric acid. An international unit (IU) of enzyme activity is defined as the amount of the enzyme needed to convert one micromole of xanthine to uric acid per minute at 23.5 C in a pH 8.3 solution of 0.1 mM xanthine and saturated with air (Bray, 1975).

Avis et al. (1955) introduced a spectrophotometric method to monitor the appearance of uric acid by absorption measurements at 295nm under conditions specified to yield results in IU. An initial rate measurement via A(295nm)/min allows a direct calculation of the enzyme activity, utilizing the molar absorption coefficient difference between xanthine and uric acid, e.g., 9.6×10^3 $cm^{-1}M^{-1}$ (Kackler, 1947). This method has been widely adopted by later researchers. Other spectrophotometric methods reported measure the color reactions of methylene blue (Dixon and Thurlow, 1924), triphenyl tetrazolium chloride (Zittle et al., 1959), cytochrome c (Horeker and Heppel, 1949), conversion of ferricyanide to ferrocyanide (Krenitsky et al., 1972), and conversion of vanillin to vanillic acid which reacts with 2,6-dibromoquinonechloroimide (Kuramoto et al., 1957). The dye reaction of tetrazolium chloride with the enzyme has been utilized qualitatively to visualize the active XO species in gel electrophoresis (Zikakis, 1979a).

Ball (1939) was the first to use a manometric technique to monitor the disappearance of oxygen in the reaction. Zikakis and Treece (1971) developed a polarographic technique to measure the oxygen consumption by using a Clark oxygen electrode. This method is claimed to be more

rapid than spectrophotometric methods. Three milliliters of sample and 0.2 ml of 0.1M phosphate buffer, pH 7.2, are equilbrated at 38 C with air purging. After inserting the electrode, 50 ul of 3.13×10^{-3} M hypoxanthine are added to start the reaction. Completion of the typical reaction requires two to ten minutes. The electrode has been incorporated into an integrated automatic system (Yellow Springs Instrument Biological Monitor, Model 53) which is capable of plotting the oxygen uptake. Results in microliter of oxygen per ml sample per minute (polarographic unit) are readily converted into the conventional I.U. by using the gas law of Boyle and Charles (Zikakis, 1979b).

A radiochemical assay employing (^{14}C)-labeled xanthine was reported to be approximate 4,000 times more sensitive than either one of the above assays but required more analysis time (Dougherty, 1976). However, the polarographic method, following oxygen consumption, and the spectrophotometric technique, following the appearance of uric acid, are the most frequently used assays. Both provide accuracy and convenience since the results are expressed directly in or readily converted to I.U. of XO activity.

Assay for xanthine dehydrogenase activity generally employs xanthine and NAD^+ as substrates. Measuring the formation of NADH at 340 nm is the most commonly way to obtain dehydrogenase activity.

Catalytic Kinetics

Analysis of steady state kinetics of XO showed a ping-pong binary mechanism which involves two half-reactions separated from each other. The half-reactions are reduction of the enzyme by xanthine followed by release of uric acid and reoxidation of the enzyme by oxygen with subsequent dissociation of hydrogen peroxide (Massey et al., 1969;

Edmondson et al., 1972)

Each half molecule of XO contains two sites for interaction of the various reducing and oxidizing substrates. These are the flavin site whose function depends on the presence or absence of a nearby thiol group and a molybdenum site whose functioning is dependent on having a nearby persulfide group intact. The reducing substrates, except NADH, interact at the molybdenum site, whereas NADH and oxidizing substrates interact at the flavin site. Evidences relating to the roles of these sites are: [1] the demolybdo enzyme is inactive toward xanthine, [2] reducing substrates influence Mo(V) electron paramagnetic resonance signals from the enzyme in a number of specific and direct ways but have no such effects on signals from other chromophores, [3] substrates, except NADH, no longer reduce the enzyme after removal of the persulfide group by cyanide, [4] substrate analogs, particularly allopurinol, inhibit reduction of the enzyme by normal substrates but not by NADH, [5] removal of the flavin abolishes reoxidation of reduced enzyme by oxygen, but does not affect its reducibility by xanthine (Bray, 1975; Komai et al., 1969; Massey and Edmondson, 1970).

Olson et al. (1974b) described the reaction of XO in terms of relative reduction potentials of the various electron acceptor groups. The mechanism of the XO reaction is best described by the order of the electron affinity constants which is roughly FAD$\geq$Fe/S$\geq$Mo. Differences between reductive substrates can be interpretated in terms of differential perturbation of these reduction potentials when substrate is bound to the enzyme. The results of these studies supported the conclusion of Edmondson et al. (1972) that the rate-limiting step in both reduction and oxidation is the decay of ES-complex which is

accompanied with rapid electron redistribution among Mo, the Fe/S center and FAD. The Fe/S centers act as electron reserviors functioning to maintain Mo(VI), (for efficient reduction) and flavin as $FADH_2$, (for efficient oxidation). Each half molecule of XO contains one 2-iron labile sulfur center, thus each half molecule is capable of accepting any number of electrons between 0 to 6 from reducing substrate (before reoxidation)-2 on 2 Fe/S, 2 on Mo, and 2 on FAD, a total of 6 electrons.

XO is capable of reducing oxidizing substrates in one-electron, two-electron, or both reactions. With oxygen, superoxide is produced in a one-electron reaction whereas hydrogen peroxide is produced via a two-electron transfer. High pH, high oxygen concentration, and low xanthine concentration all tend to favor the one-electron pathway relative to the two-electron pathway. Both one-electron and two-electron transfer occur at the flavin molecule (Olson et al., 1974a; Bray, 1975; Hille et al., 1981a). Porras et al. (1981) and Hille and Massey (1981b) have proven Olson's hypothesis of one- and two-electron transfer on reoxidation of the enzyme by molecular oxygen. They demonstrated that the electron transfer followed a sequential removal of electrons, e.g., 6--4--2--1--0. The first two steps of the two electron oxidation forms hydrogen peroxide and the last two steps represent a one-electron transfer to form superoxide.

The schemes of the reduction and oxidation mechanism of XO and electron transfer proposed by the above researchers are illustrated in the Appendix Figure A's. The pathway of internal electron transfer within XO molecule is beyond the scope of this discussion, but has been discussed by Barber's group (Barber and Siegel, 1982a,b; Spence

et al., 1982).

Oxidase Dehydrogenase Conversion

Stirpe and Della Corte (1969, 1970) found that many mammalian xanthine-oxidizing enzymes exist in vivo and freshly prepared tissue extracts as NAD^+-linked dehydrogenase. During isolation and storage, the activity with NAD^+ is gradually lost while that with oxygen increases. Conversion of the dehydrogenase (type D) to the oxidase (type O) can be brought about by heating, proteolysis, storage at -20 C, aerobiosis, organic solvents, incubation with subcellular fractions, and sulfhydryl modifying reagents. This conversion, except for proteolysis, is reversed by treatment with dithiothreitol or dithioerythritol. It was suggested that a specific thiol group(s) in the vicinty of the flavin active site is essential to utilization of NAD^+ as an acceptor. Modification or oxidation of this thiol group(s) results in the loss of reduction of NAD^+ and an increase in the affinity to use oxygen as an acceptor. Proteolysis is assumed to remove the thiol group or displace it from the vicinity of the flavin (Della Corte and Stirpe, 1972; Waud and Rajagopalan, 1976a). These finding were recently confirmed by Waud and Rajagopalan (1976b) and Coughlan and Cleere (1976). Both teams proposed that the conversion involves a formation of disulfide bonds from the vicinal sulfhydryl groups when the dehydrogenase converted into the oxidase. Proteolysis removes a peptide containing some free sulfhydryl groups which may function to stabilize the dehydrogenase formation, and thus results in an irreversible oxidase form (Coughlan, 1980).

Most evidence relating to the interconversion between XO and dehydrogenase are based on the investigation of liver XO. Battelli et

al. (1973) was the first to provide evidence that milk XO possessed NAD^+-reductive activity. They found both crude and purified milk XO could be converted almost completely into the type D form by treatment with dithioerythritol or dihydrolipoic acid, but only to a small extent by other thiols. Results from their kinetic studies and proteolytic treatment of the enzyme are similar to those reported previously on liver XO.

Clare et al. (1981) treated xanthine dehydrogenase purified from various mammalian tissues with an immobilized preparation of crude bovine sulfhydryl oxidase. A Comparison of the rates of conversion of the D form to the O form in the presence and absence of the immobilized enzyme indicated that sulfhydryl oxidase catalyzes the conversion. The D form (dehydrogenase) of milk xanthine oxidase, obtained from purified milk XO (type O) treated with dithiothreitol was also enzymically converted back to the O form with the concomitant disappearance of sulfhydryl groups. They suggested that sulfhydryl oxidase may serve an important role in the formation of the O form of the enzyme in a given tissue.

Biological Role and Applications

XO in mammals exists in vivo as a dehydrogenase which performs a reduction-oxidation reaction in purine metabolism in the cell. Some microorganisms can utilize purines as their major source of carbon and nitrogen through XO/dehydrogenase reactions which may also balance the nucleotide pool (Coughlan, 1980). The real biological significance of XO in mammals is still not clear since Johnson et al. (1974) found that rats grow and reproduce normally after feeding sufficient

tungsten to destroy all XO activity. Fired et al. (1973) suggested that the main function of XO is to provide a source of hydrogen peroxide and superoxide radical, both of which would then be available for coupling biological oxidation. Bray (1975) questioned the validity of this proposal since, as mentioned above, the enzyme exists as a dehydrogenase in vivo. However, Fired et al. (1973) pointed out that one advantage to the proposed mechanism is that a large amount of substrate can be oxidized by a proportionally small amount of oxygen. Part of the oxygen is regenerated from the hydrogen peroxide and/or superoxide by catalase and superoxide dismutase and can, therefore, be utilized for further reaction. These two enzymes are widely distributed and could be part of an overall reaction scheme whereby XO participates as a generator of an oxidizing agent which is deactivated by specific enzymes. Based on this theory, Bjorck and Claesson (1979) suggested that XO serves as a natural source of hydrogen peroxide for the lactoperoxidase system in milk. The lactoperoxidase system is proposed to function in the preventing of bacterial infections in the gastrointestine tract of the neonate (Reiter, 1978). The system catalyzes the oxidation of thiocyanate by hydrogen peroxide to hypothiocyanate ($OSCN^-$), which is bactericidal for enteric pathogens including multiple antibiotic resistant strains of E. coli. Lactoperoxidase and thiocyanate are natural constituents of milk whereas hydrogen peroxide is not. It was found experimentally that hydrogen peroxide or superoxide which was generated by XO reacting with low concentrations of free purines (hypoxanthine or xanthine) had little effect on bacteria. But indirectly, in combination with thiocyanate and lactoperoxidase, gave rise to a substantial

antibacterial effect. A decisive factor for the validity of this hypothesis is the availability of substrates for XO. The activity of XO necessary for this function is less than half of that normally found in milk. This may be due to other functions of the enzyme in bovine milk. As previous discussion, XO serves as an integral part of the intracellular milk fat globule membrane. Stabilization of fat globules by the enzyme may, presumably, be one of its biological roles in milk.

Presumably, XO contributes to spontaneous oxidized flavor in milk. Aurand and Woods (1959) indicated that the occurrence of a spontaneously oxidized flavor in milk was dependent upon a high level of XO activity. The use of an enzyme inhibitor prevented flavor development. They suggested that an intermediate product of hydrogen peroxide served as the oxidant for developing flavor. Smith and Dunkley (1960) did not find a high correlation between XO activity and spontaneous development of oxidized flavor. XO may be involved as a catalysis of oxidized flavor but it was not a limiting factor. Ascorbic acid and copper are the essential reactants for spontaneous flavor development (King and Dunkley, 1959; Smith and Dunkley, 1962). However, Aurand et al. (1967) found that milk with a level of XO activity adjusted to greater than 120 ul uptake of oxygen per ml per hour developed oxidized flavor. Flavin-free xanthine oxidase added to raw milk at the same level had no effect on off-flavor development. They suggested that endogenous acetaldehyde was the substrate used by XO to produce oxidized flavor.

Pederson and Aust (1973) found that superoxide produced by XO can form singlet oxygen which promotes the peroxidation of unsaturated

lipids. Kellogg and Fridovich (1975) also found that XO, acting aerobically upon acetaldehyde, caused the peroxidation of linolenate. Since superoxide and hydrogen peroxide can directly give rise to singlet oxygen, Aurand et al. (1977) proposed that superoxide anion produced by XO in milk may undergo non-enzymic dismutation to form singlet oxygen which could catalyzes lipid oxidation. They reported that the addition of XO with hypoxanthine in milk resulted in increased lipid peroxidation, whereas heat inactivated XO added to milk inhibited the oxidation. More recently Allen and Wrieden (1982) investigated the lipid oxidation influenced by milk proteins and concluded that under normal condition XO may not be a very significant factor in lipid autooxidation. However, a strongly pro-oxidation effect was found in the presence of 10 uM Cu^{+2}. More interestingly, the pro-oxidation effect of the enzyme was greatly enhanced by heat denaturation. They concluded that the role XO plays in lipid peroxidation could be very important when cupric ions are added to milk for nutritional reasons or by contamination. The proteins of the MFGM are particularly effective at binding copper.

Initiation of lipid peroxidation by singlet oxygen is commonly induced by light, metal and/or enzyme. XO may involve the enzymic induction of lipid oxidation. However, superoxide dismutase, a native constituent of milk and usually considered together with XO in the reaction system, would catalyzes the conversion of superoxide anion into triplet oxygen and hydrogen peroxide (Shipe, 1977; Hicks, 1980). Ascorbic acid in the milk would be oxidized by superoxide anion prior to oxidation of lipid (Nishikim, 1975). Therefore, more research is required to determine the role of XO in lipid peroxidation and

oxidized flavor development. It appears that non-enzymic as well as enzymic roles are played by the enzyme.

Oster (1971) postulated that XO in homogenized bovine milk is involved in the development of atherosclerosis in humans. According to his hypothesis, XO survives through the gastrointestine tract and passes through the intestine barrier, especially the micronized droplets found in homogenized milk. After entering the circulatory system, XO is deposited in the arterial wall and heart muscle. At these sites, the enzyme causes a depletion of the phospholipid plasmalogen, an important constituent of arterial wall and the myocardial cell, by oxidizing the aldehyde moiety-plasmal. Irreverisible removal of plasmal by XO produces alterations in phospholipid balance and changes in the structure integrity of the cell membrane. Defective cell membrane causes failure in the active transport system and eventual cell death (myocardial infarction). In the vessel wall, cholesterol ester and other lipids accumulate at the site of injury as a compensatory repair mechanism and, hence, initiate the formation of atherosclerotic plaque.

After Oster's presentation, several articles have been presented which refute this theory; others support it without actual evidences. Oster (1971, 1976a) assumed that human milk does not contain XO and found a correlation existed between the titer of antibody to XO in human sera and milk consumption. In fact, in 1977, Zikakis et al. reported the XO activity in human milk. Both active and inactive forms of milk XO elicit a similar antigenic response (Ultmann et al., 1962; Zikakis and Rzucidlo, 1976b). Hence, a high antibody titer does not necessarily indicate a high level of biologically active XO. The main

issue between these two opposite groups is whether XO can be absorbed through the intestinal wall in an active state.

Volp and Lage (1977) presented evidence that XO does not retain its activity in the stomach and is not detectably absorbed by the small intestine. McCarthy and Long (1976) also reported that there was neither causal nor statistical significance between XO activity in blood and average daily milk consumption, age, or sex. Mangino and Brunner (1976) found that XO was completely inactivated below pH 3.2. Gastric juice has a pH of less than 2 and with a high volume to volume ratio to milk XO activity is destroyed completely. However, Zikakis et al. (1977) found that the pH was reduced to 5.16 when simulated gastric juice mixed with milk with a ratio of 1:2 and 14.2% of the enzyme activity remained viable. Ho and Clifford (1976) reported that a very small amount of the active enzyme could reach the intestinal wall and was absorbed as intact enzyme.

Evidences from animal studies of the intestinal absorption show that the size of proteins no greater that 80,000 molecular weight could be absorbed. Ho and Clifford (1976) found that only a very small amount of XO with a molecular weight of 300,000 could be absorbed. Zikakis et al. (1977) indicated that the probability for XO absorption may be greater if it can exist in the low molecular weight (<75,000) form which was isolated by Biasotto and Zikakis (1975). Oster (1971) believed that the small XO-containing fat globules (<1 u) in homogenized milk could pass through the intestinal wall. Clark and Pratt (1976) suggested that administration of half cream and half milk (H/H) supplemented with XO may cause the absorption of intact XO across the gastrointestinal tract. But it is inconclusive, since they

also found that H/H stimulated endogenous XO activity. Gandhi and Ahuja (1979) indicated that XO is not destroyed completely in gastrointestinal digestion and is absorbed when associated with milk fat globules. Ross et al. (1980) demonstrated that absorption of XO-containing particles in homogenized milk was linked to liposomes as a vehicle for the persorption. Hence, they suggested that active XO could be readily absorbed in this form. It appears that intact, active XO may be absorbed, at least in small amounts, and enters the circulatory system. However, the initiation of atherosclerosis by this absorbed XO is still questionable. Ho and Clifford (1977) found that large intravenous doses of XO over a prolonged period did not deplete arterial or coronary tissue plasmalogens and did not induce the formation of arterial plaques.

Though the evidence is issufficient and unsatisfactory to support Oster's hypothesis, it caused some concern among physcians, dairy manufacturers and consumers. Most dairy products with the exception of powdered and evaporated milk products and all butter contain detectable XO activity (Zikakis and Wooters, 1980). Deeth (1981) published a critical review on homogenized milk and coronary heart disease suggesting that milk XO-induced atherosclerosis deserves further examination. He concluded that until experimental evidence is presented which proves that absorbed milk XO causes tissue damange directly related to atherogenesis the Oster hypothesis cannot be accepted.

The suggested food applications of XO are limited. Actual food application of the enzyme are unknown due to a wide range of substrates and their low specificity for the enzyme.

Alfa-Laval (1977) indicated that native XO in milk could generate hydrogen peroxide upon the addition of hypoxanthine (0.5% w/w). This enzyme reaction could improve the keeping quality of milk through cold pasteurization and has been suggested as a method for preserving milk on the farm. Xanthine oxidase can be used as a marker enzyme to assay for the churning of milk. The release of XO from the fat globule into skim milk indicates that churning has occurred (Stannard, 1975). Groman and Groman (1975) suggested that quantitation of XO in the skim milk due to agitation could be used for assessing the correctness of stirrer choice for dairy storage and fermentation tanks.

The level of hypoxanthine in fish muscle is an index of fish quality or freshness. Burt et al.(1968) introduced an automatic colorimetric method to measure the hypoxanthine concentration in fish tissue by the XO reaction. Uric acid produced by XO induced a color change in the redox dye 2,6-dichlorophenolindophenol. Jahns et al. (1976) developed a rapid and simple semi-quantitative test utilizing dry strips containing XO and dye, a sort of "litmus" test. The freshness of fish products was estimated by a color change of the stripe within a short time.

EXPERIMENTAL

Materials and Chemicals

The milk used in this study was obtained from Holstein cows of the Michigan State University dairy herd. No attempt was made to collect milk from a specific cow except for the riboflavin study.

The principal chemicals and their sources used in this study are listed in the Appendix, Table A1. All chemicals are reagent grade unless otherwise specified. Deionized water was used in the preparation of all buffers and solutions. Equipment regularly used in the course of this study is listed in the Appendix, Table A2. Instrumentation specific for a certain experiment will be referred to the appropriate section.

Preparative Procedure

Fractionation of Whole Milk

Milk was collected immediately after milking and separated as soon as possible at 40-45 C with a Westfalia separator. The cream was collected and washed by adding three volumes of deionized water at 40-45 C followed by gently mixing and reseparation. This washing step was repeated three more times to ensure adequate removal of casein and whey protein from the cream. Following storage overnight at 4 C, the washed cream was churned at room temperature. After the fat emulsion was broken, the aqueous phase was filtrated through four layers of cheesecloth. Unchurned butter granules were removed by centrifugation at 1,000xg for 20 min. The resulting aqueous suspension served as

buttermilk for subsequent fractionation and analysis.

Butter oil was prepared from churned, washed milk fat globules. The churned fat was melted at 55 C and washed with 55 C deionized water. The mixture was centrifuged at 1,000xg for 15 min to separate butter oil from the aqueous phase. Butter oil was stored at cold room for further use.

Whole casein was prepared by adjusting pH of skim milk to 4.6 with 1N HCl. The casein precipitate was collected and washed 2 times with a volume of deionized water equal to that of the original skim milk. Washed casein was resuspended in deionized water by adjusting pH to 7.0 with 1N NaOH and reprecipitated at pH 4.6 with acid. After washing 2 times with deionized water, the casein was solubilized, dialyzed against deionized water at 4 C for 48 hr and lyophilized. Whey protein was obtained by dialyzing the milk serum after the removal of casein at 4 C for 48 hr and dried by lyophilization.

Isolation of Xanthine Oxidase

The differential centrifugation method of Mangino and Brunner (1977a) with slight modifications was employed to isolate bovine milk XO. A Beckmen L2-65 preparative ultracentrifuge and a Type 30 rotor were used.

Buttermilk obtained as previously described was centrifuged at 27,500 rpm (80,000xg) for 150 min at 10 C. The pellet-material (100S) was purified by additional centrifugations and by hydroxylapatite adsorption chromatography. The amount of wet hydroxylapatite gel added to the extract was 50 mg/ml. The purified XO was stored at 4 C in 25% (w/v) ammonium sulfate solution pending further analysis. When required, the enzyme was lyophilized after dialysis against deionized

water at 4 C for 24 hr.

Isolation of Polypeptide Fragments of XO Cleaved by Trypsin

Twenty electrophoresed SDS-gels of trypsin digested-XO were sliced into segments corresponding to a reference gel from the same run which was visualized with Coomassie Blue R-250. Gel segments representing discrete polypeptide bands were combined and diced into small pieces. Polypeptide was extracted by overnight soaking diced gels with boiled deionized water containing 0.05% SDS at 37 C. The mixture was centrifuged and the gel pellet was washed three times with the same solution. The clear supernatant fractions were combined, dialyzed and dried with vacuum evaporation in preparation for N-amino terminal analysis.

For amino acid composition analyses, a homemade plexiglass electrophoretic elution apparatus was used to elute the polypeptide from SDS-gels (see Figure 1). About 100 SDS-gels were cut to correspond with stained zones in a reference gel pattern. Gels representing each band were combined and immersed in a small volume of boiled deionized water to avoid dehydration of the gels before subsequent elution.

A layer about 0.3 cm thick of 1.5% agarose in veronal buffer, pH 8.6, ionic strength 0.02, was formed at the bottom of the elution cell. Polyacrylamide gel pieces were dispersed on top of the agarose layer. The free water phase was mixed with agarose and veronal buffer to make a final solution containing 1.5% agarose solution which was poured into the elution cell, covering the PAG fragments to a depth of 1.5 cm. About 10 ml of veronal buffer was layered on top of the gel for collecting eluted polypeptide. The bottom piece of the elution

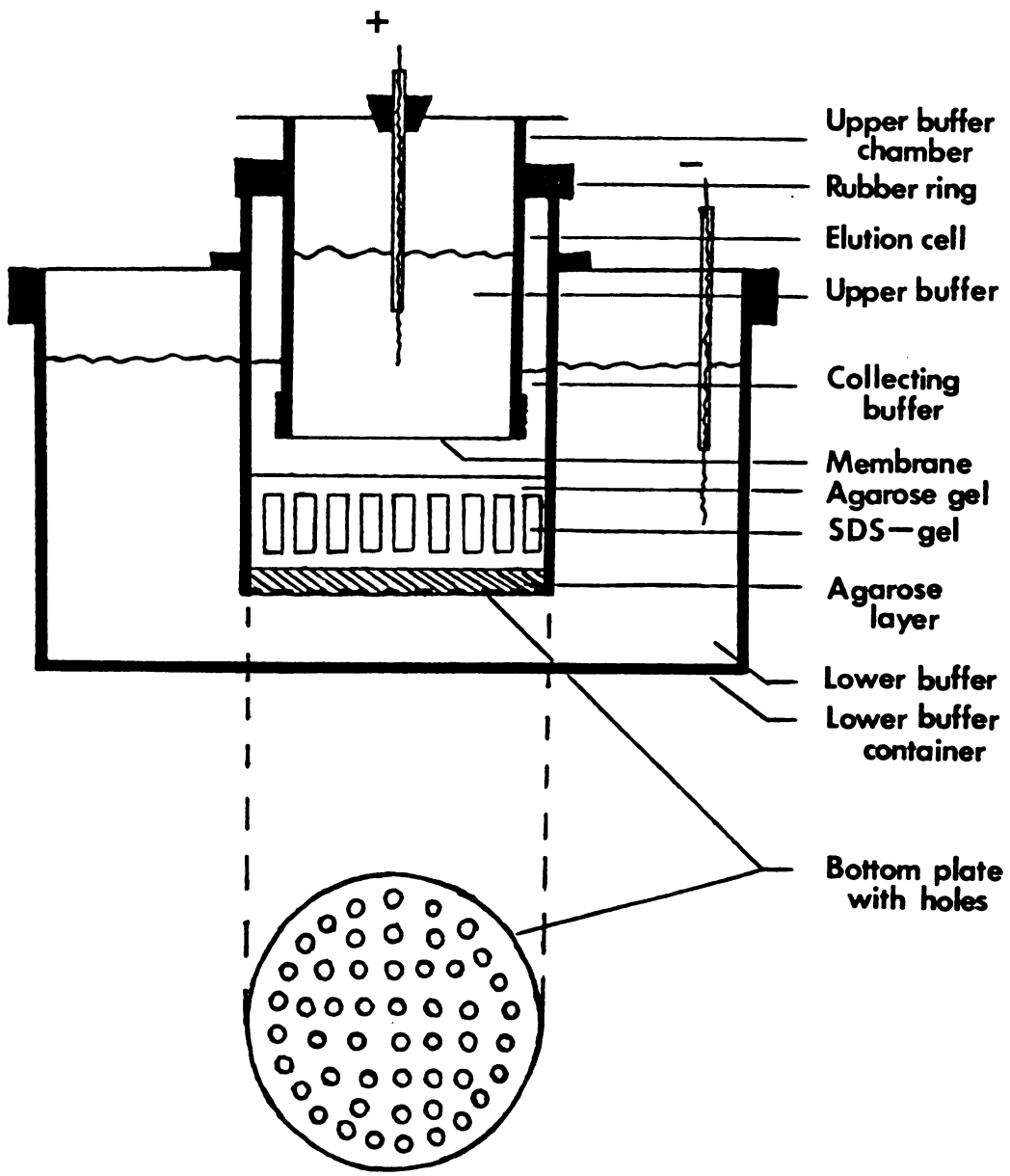

Figure 1. The electrophoretic apparatus used for the elution of polypeptide from SDS-gel fragments.

chamber was perforated with evenly distributed 3 mm holes. The upper buffer chamber (anode), constructed as an open-ended plastic cylinder with a dialysis membrane sealed at one end with rubber bands, was filled with about 40 ml of the veronal buffer and inserted into the elution cell until membrane of the cylinder was immersed in the collecting buffer to complete the electric current. The lower chamber consisted of a plastic container with a cover arranged to hold the elution cell away from the bottom of the container. Veronal buffer, similar to that used in the upper chamber, was added until contact was made with the bottom of the elution chamber. The final set-up of this system is shown in Figure 1.

Electrophoresis was conducted with a constant current of 100 mA for 3 hr. Polypeptides in the polyacrylamide gel pieces migrated upward into the collecting buffer. After electrophoresis, the upper chamber was removed and the collecting buffer was poured through a Whatman No. 1 filter paper. The filtrate was dialyzed exhaustively, dried with a rotatory vacuum evaporator and hydrolyzed in 6N HCl for amino acid analyses. The above described process was repeated for all zonal fractions.

One gel, run only with SDS-sample buffer, from each electrophoretic run served as a reagent and process blank in the above N-terminal and amino acid composition analyses.

Physical Methods

Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE)

All electrophoretic experiments were performed in 6 mm I.D., 2 mm walled and 75 mm length glass tubes. The tubes were washed with

detergent, immersed in chromic acid, rinsed with deionized water, treated with Kodak Photoflo (1:200) and dried before using.

Disc gel electrophoresis was conducted as described by Melachouris (1969) with two modifications: (1) the acrylamide:bisacrylamide ratio was kept at 37:1 to achieve a 2.6% crosslinked gel, and (2) no urea was incorporated into the gel buffer. Electrophoresis was initiated at 2 mA/tube and increased to 3 mA/tube when the tracking dye entered the separating gel.

Gels were stained for protein in a solution of Coomassie Blue R-250 (Weber and Osborn, 1969). Specific staining for XO activity was conducted by immersing gels in a solution of 25 mM NaOH, pH 8.3, containing 10 mM xanthine and 250 mg neotetrazolium chloride. Detection of active XO by neotetrazolium involves transfer of electrons to neotetrazolium after the reaction of XO and xanthine, resulting in the formation of a purple colored precipitate (formazan).

Gels stained with Coomassie Blue were destained by diffusion in a solution of 7% acetic acid and stored in the same solution. Gels visulized by enzyme activity were stored in the reaction mixture or deionized water at 4 C.

Urea Disc-PAGE

Melachouris' (1969) method of urea gel electrophoresis was modified by using a ratio of 19:1 - acrylamide: bisacrylamide - and 6M urea incorporated into the gel formula. Samples were equilibrated with gel buffer containing 6 M urea for 24 hr at room temperature prior to electrophoresis.

SDS-PAGE

Disc gel electrophoresis in the presence of sodium dodecyl sulfate

(SDS) was performed according to the method of Laemmli (1970). A 9% total gel concentration (T) with 2.6% crosslinker (C) was used in the separation gel whereas the stacking gel contained 5% T and 20% C.

The enzyme samples were equilibrated against sample buffer for 24 hr. Two drops of tracking dye, 1% bromophenol blue, were added to 2 ml sample and the mixture was heated for 5 min in boiling water prior to being applied to the top of gels. Electrphoresis was carried out with a current of 1.5 mA/tube for stacking and 3 mA/tube during separation. Gels were stained with Coomassie Blue R-250 (Weber and Osborn, 1969), destained by diffusion and stored in 7% acetic acid.

Molecular weight of protein bands was estimated from a plot of relative mobility (R_f) vs the log of molecular weights of standard proteins. The standards used in this study were thyroglobulin (330,000), ferritin (half unit, 220,000), phosphorylase (94,000), bovine serum albumin (67,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and alpha-lactalbumin (14,400).

Surface and Interfacial Tensions

All surface and interfacial tension measurements were conducted with a Cenco-duNouy Tensiometer (Precision Direct Reading Model) with a 4 cm circumference platinum ring. Isoelectric casein, whey protein, purified XO and butter oil were prepared as previously described. The instrument was calibrated with known weights according to directions provided by manufacturer.

Different concentrations of protein solution were poured into the watch glass carried by the platform. The platform was raised until the surface of the sample solution touched the platinum ring hanging from

the torsion hook. The ring was pulled from the surface by applying tension to the torsion wire. The surface-breaking force in dynes/cm was recorded to the nearest 0.10 as the surface tension of the protein samples.

Interfacial tension between proteins and butter oil was determined at 45 C to avoid solidification of butter oil. A lab-jack was used to support a 45 C water bath instead of using the instrument platform. Different concentrations of protein samples were poured into a 50 ml beaker up to 35 ml marker. After temperature of the solution reached 45 C, a platinum ring was dipped into the solution to about 7 mm depth by raising the lab-jack. The 45 C butter oil was poured carefully along the wall of beaker to provide an oil layer 10 mm in depth. The ring was positioned at the interface between protein solution and butter oil by adjusting the lab-jack. The calibrated indicator dial was turned slowly until the ring separated from the interfacial surface. The dial reading in dynes/cm was recorded as interfacial tension.

Enzyme Assay

Xanthine Oxidase

XO activity was assayed according to the procedure of Avis et al. (1955) which monitors the conversion of xanthine to uric acid at 295 nm. The concentration of uric acid formed was determined by using a molar absorption coefficients difference between xanthine and uric acid of 9.6×10^3 cm^{-1} M^{-1}(Kackler, 1947). Enzyme activity was reported as micromoles substrate converted/min/ml sample (unit or IU/ml) at 23.5 C in 0.1 M pyrophosphate buffer, pH 8.3.

Xanthine Dehydrogenase

Xanthine dehydrogenase (type D XO) activity was measured by monitoring the initial rate of formation of NADH at 340 nm (Stripe and Della Corte, 1969). A molar absorption coefficient of 6.22 X 10^3 cm^{-1} M^{-1} of NADH at 340 nm was used to calculate the amount of NADH produced. Enzyme activity was expressed either as unit/ml sample or unit/mg protein. One unit of the enzyme activity is defined as one micromole NADH formed/min.

NADH-Ferricyanide Reductase

The activity of XO reacting NADH and ferricyanide was assayed as described by Komai et al. (1969). The reaction mixture, about 2.8 ml, containing 0.1 M acetate buffer, pH 4.6, 600 nmole of NADH and 2 micromoles potassium ferricyanide. The enzyme reaction was started by addition of 0.2 ml of enzyme sample and was monitored by measuring absorbance at 420 nm for 10 min.

Chemical Methods

Nitrogen Content

Nitrogen content in the sample preparations was determined by semimicro-Kjeldahl procedure utlizing $CuSO_4$ and SeO_2 in concentrated sulfuric acid as the digestion mixture. Protein nitrogen was calculated by substracting the nitrogen content of the sample from the nitrogen content of the supernatant of 12% TCA-treated sample. The range of recovery on a tryptophan standard was 95 to 99%.

Lowry Determination of Protein

The Folin-phenol protein determination method of Lowry et al. (1951) was used in this study. Quantitation was achieved with a

standard curve prepared by employing bovine serum albumin over a concentration range from 0 to 300 mg.

Available and Total Sulfhydryl Groups

Determinations of the available and total sulfhydryl groups present in the protein preparations were estimated by methods described by Habeeb (1972). A molar extinction coefficient of 13,600 at 412 nm as reported by Ellman (1959) and a molecular weight for the purified XO of 302,000 were used for calaulating numbers of sulfhydryl groups. Determinations were made in triplicate and a reagent blank was submitted concurrently with the samples.

Disulfide Groups

The principle of the disulfide group determination is based on the reduction of disulfide bonds to sulfhydryl groups by a strong reducing agent. Then, total sulfhydryl groups are determined as described above. The method employed here was adopted from a procedure developed by Cavallini et al. (1966).

Determinations were conducted at least in triplicate and absorbance was measured at 412 nm against an appropriate blank. A molar absorptivity of 12,000 was used for calculating the number of sulfhydryl groups formed after reduction. The number of disulfide bonds is equal to half of the difference between numbers of total sulfhydryl groups before and after reduction.

Amino Acid

Amino acid analyses were performed on 24 hr protein hydrolysates employing a Beckman Automatic Amino Acid Analyzer, Model 120 C (Moore et al., 1958).

Typically, a protein sample was mixed with 5 ml 6N HCl in

Pierce's screw cap septum vials. After 20 min of nitrogen flush, vials were capped with a Teflon-silicone disc inside and placed in an oil bath at 110 C for 24 hr. After hydrolysis, 1 ml (=2.5 umole) of N-leucine solution was added to the hydrolysate as an internal standard. The sample was transferred into a pear-shaped flask and dried with a vacuum rotary evaporator. The residue was washed three times with a small volume of deionized water and evaporated again to ensure the removal of all residual HCl. The final dried material was made up to 1 or 5 ml with a 0.067 M citrate-HCl buffer, pH 2.2. An aliquots of 0.3 ml were applied to the ion-exchange column for analysis.

Half cystine and methionine contents were determined from oxidized and hydrolyzed specimens according to procedure described by Lewis (1966). Five milliliters of performic acid (90 ml formic and 10 ml hydrogen peroxide) was added to a weighed specimen in a pear-shaped flask. Oxidation was accomplished at 4 C for 15 hr. The oxidized mixture was mixed with 0.6 ml HBr while in the cold room. After 10 min, 1 ml of N-leucine internal standard was added prior to evaporation to dryness. The dried sample was transferred with 5 ml 6N HCl into a hydrolysis vial (Pierce), flushed with nitrogen for 20 min, capped and placed in a 110 C oil bath for 24 hr hydrolysis. Subsequent procedures were similar to those employed for the acid hydrolysate. Half cystine was calaulated as cysteic acid and methionine as methionine sulfone, both eluted with the pH 3.28 buffer.

The amino acid composition was expressed as moles of residue/100 moles of total residues.

Tryptophan

The alkaline hydrolysis method of Basha and Roberts (1977) was

employed for the determination of tryptophan.

Samples were hydrolyzed with 5M KOH in Pierce's hydrolysis vials at 110 C for 10 hr. A slight molar excess of HCl was added to the hydrolysate. Centrifugation was employed to removed the colloidial material and the pellet was washed three times with deionized water. Clear supernatant fractions were combined and made to a known volume (normally 3 ml). An aliquot of 0.5 ml was mixed thoroughly with 0.5 ml of 4 N HCl, then, 0.5 ml of sodium nitrite (0.4%, w/v) was added. The solution was mixed well and allowed to stand at room temperature for 40 min. One milliliter of ammonium sulfamate (0.5%, w/v) was added to destroyed excess nitrite. After 10 min, 2 ml of N-1-(naphthyl) ethylenediamine dihydrochloride (0.05% in ethanol, w/v) was added and mixed. Absorbance was measured after 40 min at 550 nm with a Baush and Lomb Spectronic 21 spectrophotometer.

A standard curve was developed from analyses of authentic tryptophan, 0 to 100 ug, as described above but without alkaline hydrolysis. The tryptophan content of the sample, thus determined, was combined with the data of amino acid analysis and recalculated to express the complete amino acid composition in residue mole percentage.

N-Amino Terminal

A modification of the polyamide chromatography method of Woods and Wang (1967) was used to characterize the N-terminal amino acid of XO and its trypsin digest fragments.

Samples were dissolved in 20 ul of 0.1 M sodium bicarbonate and 20 ul of dansyl chloride (DNS-Cl, 100 mg/ml acetone) was added. Dansylation was conducted at 37 C for 2 hr. Typically, a dansylated sample was dried with vacuum evaporation prior to acid hydrolysis with

6N HCl. Hydrolysis was performed at 110 C for 16 hr, then, cooled and evaporated to dryness. The sample was transferred with acetone to a 5X5 cm micropolyamide sheet.

Ascending chromatography in four successive solvents as described by Weiner et al. (1972) was employed to separate the dansylated derivatives. Solvent 2, 3 and 4 were run in the same direction but prependicular to and subsequent to the use of solvent 1.

Solvent 1 redistilled 88% formic acid : distilled water; 3:200

Solvent 2 redistilled benzene : glacial acetic acid; 9:1

Solvent 3 ethyl acetate : glacial acetic acid : methanol; 20:1:1

Solvent 4 0.067 M sodium phosphate (tribase) : 100% ethanol; 3:1

The results were compared with blanks and the N-amino terminal of samples was identified by running a dansylated amino acid standard on the reverse side of the polyamide sheet.

Free Riboflavin, Flavin Mononucleotide (FMN) and Flavin Adenine Dinucleotide (FAD)

A fluorometric assay according to the method described by Baker and Frank (1975) was employed. Alummium foil was wrapped around the sample container to protect contents from light throughout the course of this study. Milk was diluted 2.5 folds at cold room temperature and a concentrated solution of TCA was added to a final concentration of 11%. The mixture was allowed to stand at 4 C for 10 min then centrifuged with a clinical centrifuge operated at maximum speed for

10 min. The supernatant was filtrated through Whatman No. 1 paper and subjected to the following procedures prior to a fluorometric measurements of riboflavin: (A), A portion of the filtrate was sealed with Parafilm and incubated at 37 C overnight to permit hydrolysis of FAD. The hydrolysate was neutralized with 4M dibasic potassium phosphate (about 1/4 volume of the hydrolysate volume) to pH 6.8 prior to measurement; (B), the remaining filtrate was neutralized with 4M dibasic potassium phosphate immediately after filtration to prevent hydrolysis of FAD. The solution was kept cold before the measurement and following extraction. (C), Two milliliters of sample from (B) was mixed vigorously with 2.5 ml water saturated benzyl alcohol to extract free riboflavin. The mixture was centrifuged at 3,000 rpm for 3 min and its aqueous phase mixed thoroughly with water-saturated chloroform and centrifuged to remove residual benzyl alcohol. The aqueous phase of the chloroform extract was transferred for fluorometric analysis.

Fluorescence measurements were conducted with a Beckman Ratio Fluorometer, Model 772 equipped with a mercury vapor source lamp wrapped with a phosphor sleeve (set up to 360 nm), a primary filter of 250-400 nm and a secondary filter of 520 nm. The original instrumental reading substracted from a reading obtained after the addition of 20 mg sodium hydrosulfite represented the fluorescence reading of riboflavin in a sample. A solution contained 10% TCA and 0.08 M dibasic potassium phosphate was also measured as a blank. A standard curve was established from measurements of authentic riboflavin, 0 to 3 ug, dissolved in the blank solution carried through the entire procedures.

Data obtained from procedure A represented the total amount of

riboflavin including free riboflavin, FMN and hydrolyzed FAD. Data from procedure B showed the amount of free riboflavin plus FMN and 14% FAD since FAD has a fluorescence equal to 14% of that of riboflavin. The FAD content of the sample is equal to (A-B)/0.86. The partition coefficients between benzyl alcohol and 10% TCA for riboflavin, FMN and FAD neutralized as previously described are 4.1, 0.032 and 0.02, respectively. The free riboflavin extracted by benzyl alcohol can be calaulated and is equal to (4.1X2.5)/(2+4.1X2.5) X100%=84%, leaving 16% in the aqueous phase. Based on these calculation, 3% FAD was extracxted and 97% FAD remained in aqueous extract while 2.4% FMN was extracted with 97.6% in the aqueous phase. Thus, data from procedure C represented 0.16 riboflavin plus 0.976 FMN and 0.136 FAD (0.136=97%X14%). It is more convenient to express the data from A, B and C as follows:

A = free riboflavin + FMN + FAD

B = free riboflavin + FMN + 0.14 FAD

C = 0.16 free riboflavin + 0.976 FMN + 0.136 FAD

Combining these three equations, the solutions are:

FAD = (A - B)/0.86

Free riboflavin = 1.197 B - 1.225 C - 0.01 A

and FMN = A - FAD - Free riboflavin

or FMN = 1.225 C - 0.162 A - 0.034 B

Limited Proteolysis of XO

Effect on the enzymic and molecular properties of XO by proteases was conducted at pH 6.8 in 0.1 M sodium phosphate buffer. Trypsin, chymotrypsin, plasmin, pancreatin, pepsin and papain were the

proteolytic enzymes selected for evaluation in this study.

Purified XO was equilibrated at 4 C against the phosphate buffer, containing 5 mM sodium salicylate. A selected protease was added to the sample solution to yield a protein/protease ratio of 5 to 1. Proteolysis was carried out at 37 C up to 4 hr. XO/dehydrogenase activities were measured at 10 min intervals, then every 30 min after incubation started. Samples were also treated with 1% ME, 6 M urea, or SDS-PAGE sample buffer prior to electrophoresis. Gels were stained with either Coomassie Blue or for XO activity. The molecular weight of the fragments of proteolyzed-XO appearing in SDS-gels was estimated as previously described.

Flavin Content and XO in Milk

Fresh raw milk was obtained from a specific Holstein cow, No. 1791, in the Michigan State University dairy herd. Streptomycin (50 mg) and penicillin (5,000 unit) were added to milk immediately after milking to prevent microbial spoilage. Prior to analyzing the change in flavin content and XO activity, samples of milk were treated as follows: (1) Fresh raw milk was dialyzed against deionized water for 24 hr at 4 C and stored for an additional 1 and 2 days; (2) milk was stored at room temperature in the presence and absence of aprotinin (20 Kallikrein IU/ml), a Kallikrein inhibitor, for 0, 6, 20 and 48 hr; (3) milk was stored at 4 C in the presence and absence of aprotinin for 0, 6, 20 and 48 hr; (4) milk was stored at 70 C for 0, 6, 20 and 48 hr.

Flavin contents, e.g., free riboflavin, FMN and FAD, and XO activities were measured at every stage of the above treatments.

Stability of Milk Fat Globules

Stability of fat globules in an emulsion formed by homogenizing protein solution and butter oil were analyzed by a spectrophotometric method according to Walstra (1965). Protein samples, e.g., casein, whey protein and purified XO, and butter oil were prepared as previously described.

A portion of protein, consisting of about 64 mg, was dissolved in 100 ml of simulated milk ultrafiltrate (Jenness and Koops, 1962) and warmed to 45 C. Butter oil was added to a final oil concentration of 2.5%. THe mixture was homogenized with a Logeman laboratory homogenizer (one piston). The emulsified mixture was stored at 45 C in a water bath and the change in optical turbidity was measured at intervals of 10 min for the first hour and thereafter, every hour for eleven hour.

Measurement of turbidity was conducted by diluting the emulsion 1:50 with 0.03% EDTA (disodium form) solution, pH 8, to give a final pH on dilution between 7 and 8. The diluted solution was mixed by inverting the tube 3 to 4 times to obtain homogenity. Light scattering of the dilution was measured by absorbance at 420 nm with a Beckman DK-2A spectrophotometer. Turbidity was represented as A(420nm).

RESULTS AND DISCUSSIONS

Isolation

Purification of Xanthine Oxidase

No proteolytic digestion step was used in the isolation procedure. Data representing the yield and purification for the isolation of XO from whole milk are given in Table 1. A total 22.2 mg of final enzyme preparation was obtained from 2.8 gal of fresh milk with a 5980-fold purification. Yield of XO activity from buttermilk was 14.4% which was lower than that reported by some other groups, i.e., 70-85% with 85-95% purity (Gilbert and Bergel, 1964), 44.7% with 90% purity (Massey et al., 1969), and 24.4% with 100% purity (Hart et al., 1970). These three groups used a pancreatin digestion in the isolation procedure. However, the yield obtained in this study was in the range of the 10-21% activity obtained by Waud et al. (1975) and by Zikakis (1979a) and was higher than the 12.4% value reported by Mangino and Brunner (1976a), all of whom employed a non-proteolytic isolation procedure.

Mangino (1976) indicated that the time required for the enzyme purification by this procedure was much shorter than other methods. Seven hours were required for a small scale preparation (about 25 mg) and 14 hours for large scale preparation (e.g., 75-100 mg). He found similar specific activities in the preparations before and after the hydroxylapatite adsorption step and suggested that this step could be eliminated when operations were carefully performed throughout the procedure. However, similar results were not consistantly experienced in this study. Thus, the the employment of hydroxylapatite

Table 1. Purification of milk xanthine oxidase

Fraction	Volume (ml)	Protein (mg)	Activity (unit[a])	Specific Activity (unit/mg)	Purification (fold)	Yield (%)
Whole milk	10,900	405,500	230.0	0.0006	1	---
Washed cream	500	21,900	278.0	0.0127	21	---
Buttermilk	192	2,230	552.0	0.2480	413	100
100 S	100	1,310	365.0	0.2780	463	66
First supernatant[b]	138	391	315.6	0.8080	1,347	57
Second supernatant[c]	107	220	201.6	1.26	2,100	37
Third supernatant[d]	50	22.2	79.5	3.59	5,980	14

a. Micromoles xanthine oxidized per min. in 0.1 M pyrophosphate buffer, pH 8.3,at 23.5 C.

b. After centrifugation at 44,000xg for 40 min.

c. After centrifugation at 105,000xg for 50 min.

d. Final purified XO.

chromatography for obtaining the best enzyme preparation is recommended.

The purified XO did not contain protease activity. This was significant because proteases alter the characteristics of the enzyme. Milk contains an endogeneous protease, plasmin, which affects the enzyme like other proteases during isolation. Data reported in Table 2 show the results of incubating purified XO with 1% and 5% plasmin at 37 C for up to 24 hr. Within 10 hr, XO activity did not change significantly after treatment and showed only a slight decrease after 24 hr. Though the dehydrogenase activity was not measured here, the data indicate that the enzyme activity would not be affected by plasmin. In fact, the concentration of plasmin in milk is much less than that used here. Also, the purified XO still possessed a high specific activity with a molecular weight of 151,000 and a detectable dehydrogenase activity with a capacity of conversion to dehydrogenase (see discussion below). It was concluded that milk endogenous protease did not influence the purification of XO. Della Corte and Stirpe (1972) suggested that xanthine dehydrogenase could be converted to an intermediate form by proteolysis (see Figure A2 of Appendix). It appears that milk plasmin could affect XO only during the time milk was held in the mammary gland. Thus, the addition of protease inhibitors during the isolation procedure is not useful since proteolysis of XO-if indeed there was any-already occurred before isolation. Fortunately, the observed properties of the purified XO indicated that the time from milk secretion to the end of purification was not long enough to alter the enzyme properties. Possibly, the activity of plasmin was directed to more susceptible substrates, the

Table 2. Effect of plasmin on the xanthine oxidase activity of the purified enzyme[a]

	Activity (IU/ml)		
Time (hr)	Control	1% plasmin	5% plamin
0	0.125	0.124	0.121
0.5	0.123	0.124	0.123
1	0.125	0.124	0.121
2	0.124	0.121	0.117
3	0.128	0.121	0.119
4	0.127	0.127	0.116
5	0.123	0.125	0.117
6	0.124	0.122	0.117
7	0.123	0.129	0.119
8	0.122	0.126	0.118
9	0.124	0.126	0.118
10	0.122	0.120	0.117
24	0.112	0.109	0.100

a. Using the simulated milk ultrafiltrate as the incubation buffer (Jenness and Koops, 1962). Incubation temperature was 37 C.

milk caseins.

Criteria of Purity of XO

The specific activity of XO prepared for this study was 3.59 IU/mg. Its spectral properties were: A(280nm)/A(450)=4.84, A(1cm, 280nm, 1%)=11.9 and activity/A(450nm)=141. Compared to the published data (see Table 3), these values agree favorable with the results of Hart et al. (1970), Massey et al. (1969) and Mangino and Brunner (1976). A decrease in the concentration of non-XO protein (at 280nm) and a simultaneous increase in the concentration of XO (at 450nm) should give an increasingly lower PFR value (Zikakis, 1979a). In other words, the lower the PFR value, the higher the purity of the enzyme preparation. Thus, the enzyme preparation obtained here is purer than preparations reported by other groups.

By SDS-PAGE, purified XO revealed a single band with a molecular weight of 151,000 $\pm$ 4,000 (see Gel C in Figure 2 and Figure 3). The estimated molecular weight of XO is 302,000 since XO contains two identical subunits. This value is in the range of 270,000 to 370,000 reported for the enzyme from various sources and is in excellent agreement with the generally accepted molecular weight of 300,000 (Bray, 1975).

The purified enzyme revealed three zones in Disc-PAGE gel patterns (Figure 1, gel D). All three bands showed positive staining for enzyme activity and a single band with an estimated molecular weight of 151,000 in SDS-gel (Figure 1, gel E). Nathans and Hade (1978), employing a 7.2% (T) gel, obtained a single band by disc-PAGE. However, they suggested that the use of size as the primary criterion of homogeneity would minimize the problems encounterd when charge or

Table 3. Activity and spectral properties of purified milk xanthine oxidase

Preparation	Reference	I.U./mg	PFR[a]	AFR[b]	A(280nm, 1cm, 1%)
Pancreatin and crystallization	Avis et al. (1955, 1956a)	---	5.2	79	11.5
Pancreatin	Massey et al. (1969)	3.5	5.4	143	11.3
Salicylate denaturation	Hart et al. (1970)	3.6	5.2	140	12.3
Affinity chromatography	Edmondson et al. (1972)	5.0	---	183	----
Centrifugation & hydroxylapatite	Mangino and Brunner (1977)	3.55	5.05	---	11.42
Ultrafiltration	Nathans and Hade (1978)	2.6	4.6	---	----
Gel filtration & ion-exchange	Zikakis (1979a)	3.6[c]	4.1	---	----
Affinity chromatography	Nishino et al. (1981)	---	5.0	195	----
Centrifugation & hydroxylapatite	This study	3.59	4.84	141	11.9

a. Defined as a ratio of A(280nm)/A(450nm).

b. Defined as activity, A(295nm)/min, divided by A(450nm, 1cm) for the enzyme at the dilution of the assay.

c. Calculated from polarographic unit (Zikakis, 1979b).

Figure 2. Electropherograms of purified XO. (A) low MW protein standard in SDS-PAGE, (B) high MW protein standard in SDS-PAGE, (C) purified XO in SDS-PAGE, (D) purified XO in disc-PAGE, (E) bands cut from (C) in SDS-PAGE. SDS-gel is 9% T and 2.6% C in running gel and 5% T with 2.6% C for running gel of disc-PAGE.

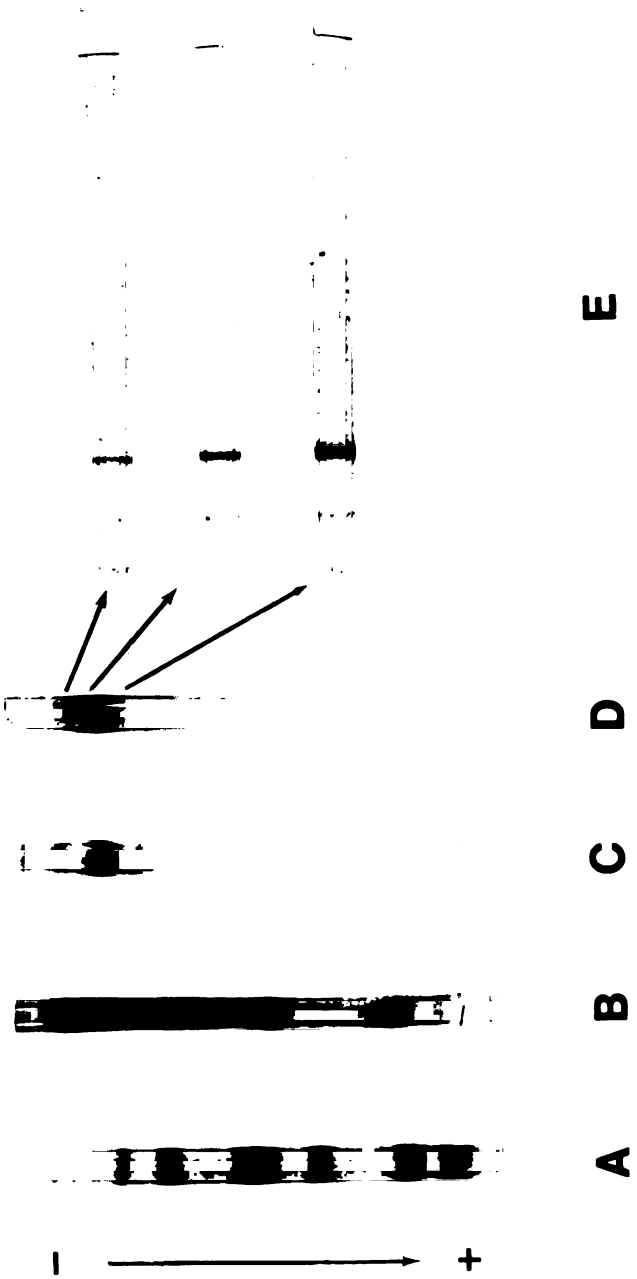

Figure 2

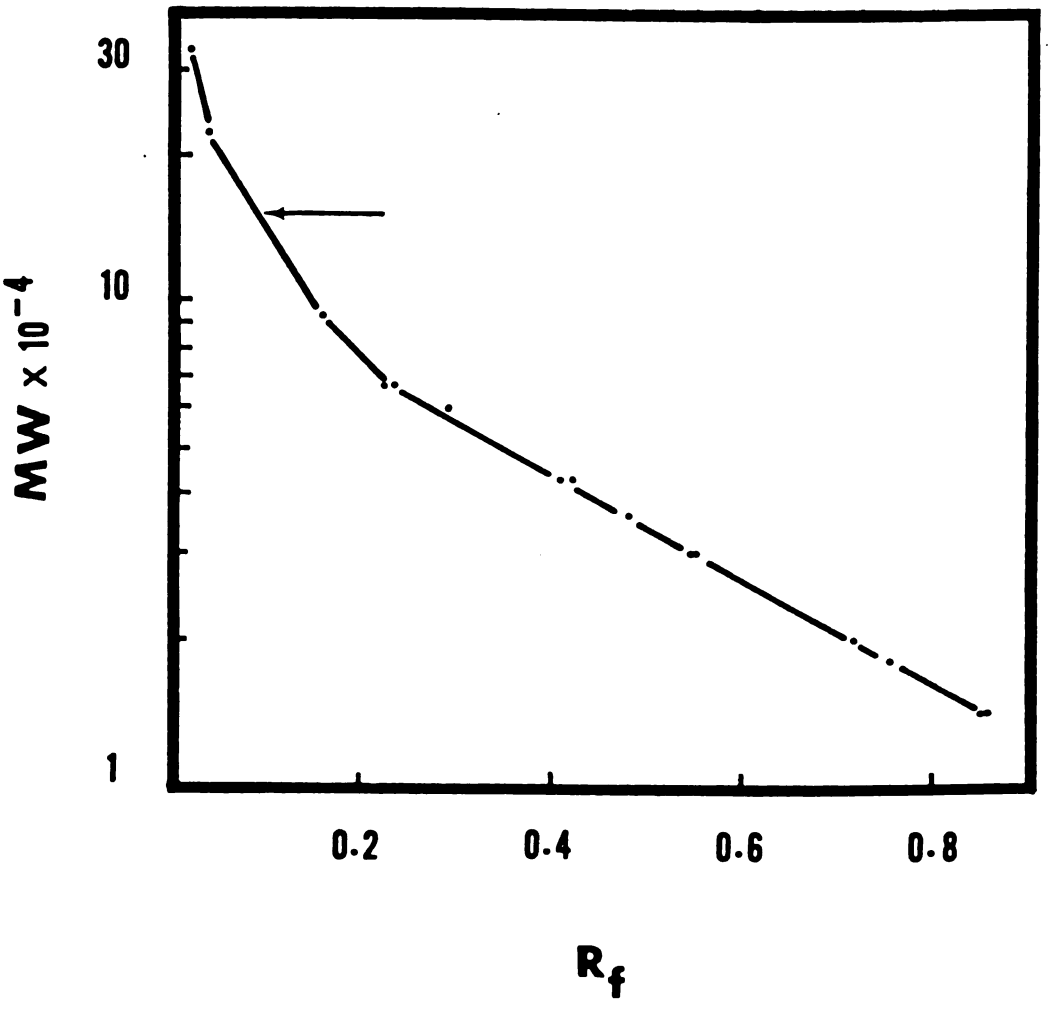

Figure 3. Standard curve for the estimation of molecular weight in SDS-PAGE. Protein standards used for this plot are: thyroglobulin (330,000), ferritin (half unit, 220,000), phosphorase b (94,000), albumin (67,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and alpha-lactoalbumin (14,400). Arrow indicates the relative mobility value of purified XO. SDS-PAGE (9%T and 2.6%C) is according to the method of Laemmli (1970).

activity were employed as criteria. Thus, the enzyme purified for this study was very close to 100% pure as estimated from spectral analyses as well as electrophoretic analyses. The three bands observed in disc-PAGE gel patterns could be explained by either genetic polymorphism or by an association-dissociation phenomenon which occurred during electrophoresis, resulting in species differing in charge but not in size. They may also be isozymes of XO. In fact, Jarasch et al. (1981) found that XO showed three isoelectric variants with the same molecular weight in two dimensioned gel electrophoresis of MFGM.

Enzyme Nature

Kinetics

Figure 4 represents the Lineweaver-Burk plot of the enzyme reaction with different xanthine concentrations. Treatment of these data by linear regression yielded a $K_m = 1.61 \pm 0.08 \times 10^{-5}$ M and $V_{max} = 3.68 \pm 0.12$ IU/mg which compared favorable with similar values obtained by Mangino and Brunner (1976a) and Massey et al. (1969).

Xanthine Dehydrogenase Properties

Purified XO possessed a detectable NAD^+-dependent reductase activity (see Table 4). After incubated the enzyme with 10 mM DTT or 1% ME at 37 C for 20 min, the dehydrogenase activity increased about 23 folds and about 20% of the XO activity was retained when oxygen was the electron acceptor. This indicated that approximately 80% of purified XO could be converted to xanthine dehydrogenase. A comparison of uric acid production by the enzyme treated as above, using oxygen and oxygen-NAD^+ as electron acceptors, indicated that NAD^+ masked or

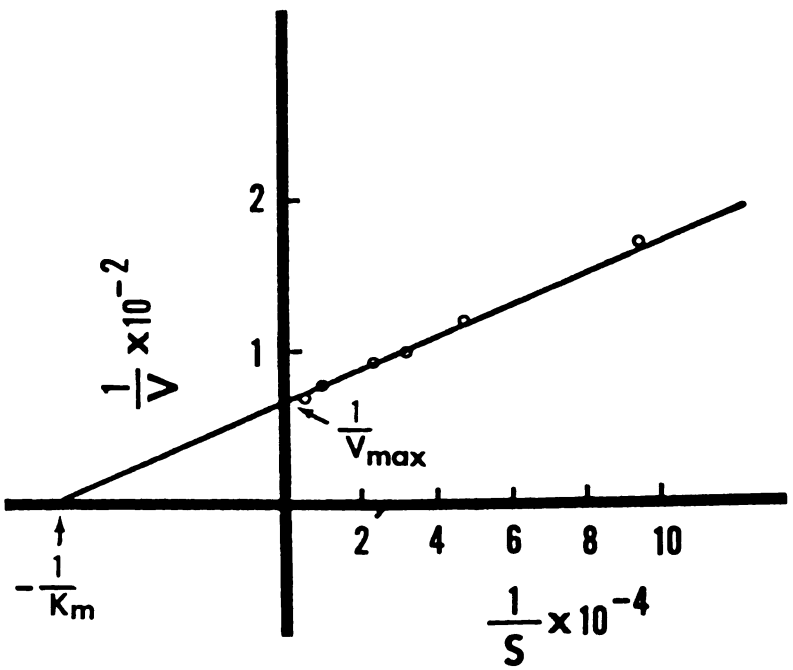

Figure 4. Lineweaver-Burk plot of xanthine oxidation by XO. Reaction mixture contained 0.1 M pyrophosphate, pH 8.3, 10.6-212 mM xanthine and 0.16 mg XO. 1/S is reciprocal units of xanthine concentration and 1/V is reciprocal units of velocity in micromole/min.

Table 4. Conversion of xanthine oxidase-dehydrogenase activities of purified XO by dithiothreitol (DTT), mercaptoethanol (ME) and chymotrypsin

Treatment	Activity (IU/ml)		
	Acceptor		
	oxygen[a]	oxygen+NAD^+[a]	NAD^+[b]
XO	0.112	0.112	0.002
XO+DTT[c]	0.023	0.072	0.050
XO+ME[c]	0.024	0.069	0.046
XO+chymotrypsin[d]	0.114	0.110	0
XO+chymotrypsin+DTT[e]	0.100	0.100	0

a. Measurement of uric acid formed at 295nm, IU/ml=micromole xanthine oxidized per ml.

b. Measurement of NADH formed at 340nm, IU/ml=micromole NADH formed per ml.

c. The enzyme was incubated at 37 C with 10mM or 1% ME of final concentration in 0.1M phosphate buffer, pH 6.8, for 20 mim.

d. XO was incubated with chymotrypsin, 10ug/ml, in 0.1M phosphate buffer, pH 6.8, at 37 C for 45 min.

e. After incubation with chymotrypsin at 37 C for 45 min, DTT was added to the final concentration of 10 mM in the mixture and proceeded the incubation for another 20 min.

influenced the reaction of FAD with oxygen. The reduction in the amount of uric acid produced by treated enzyme in the oxygen-NAD^+ assay when compared to untreated enzyme is attributable to the product, NADH, which serves as a competitor of NAD^+ for binding the FAD molecule (Bray, 1975; Battelli et al., 1973). The interconvertible properties of the enzyme was lost when it was treated with chymotrypsin.

Battelli et al. (1973) isolated a convertible XO from milk by treating fresh milk with ME. They obtained approximately an 8-fold increase in xanthine-NAD^+ reductase activity after treating the isolated enzyme with 10 mM DTT. It was suggested that XO occurs as a reversible oxidase in milk and could not be obtained as a dehydrogenase. Though no attempt was made to separate xanthine dehydrogenase from the purified XO, data from this study showed that purified XO is not only a reversible oxidase but also possesses dehydrogenase activity. Additionally, no proteases and/or reducing agents were added in the isolation procedure. Thus, it is concluded that XO in milk is capable of interconvertibility between D and O forms, as is the case for the enzyme isolated from other mammalian sources, and that the reversible O form is the major state of the enzyme in fresh milk. Also, it is postulated that XO in the mammary gland exists in the dehydrogenase form as it existes in the liver. Somehow, it is converted into a reversible and/or irreversible oxidase form. The postulated conversion may effected by (1) a redox reaction in the presence or absence of glutathione (GSSG) in the cell, (2) by endogenous protease in the cytoplasm of the secretory cells, (3) by sulfhydryl oxidase when fat globule complexes reach the apical plasma

membrane of secretory cells during the secretory process, and/or (4) all or anyone of the above possibilities during storage of milk in the mammary gland.

Active Monomer

During the investigation on the effects of dissociating agents on XO, it was observed that the enzymic activity remained after the purified XO was treated with 6 M urea in 0.1 M phosphate buffer, pH 6.8, containing 5 mM salicylate. Since urea is generally used to dissociate the quaternary structure of proteins, it is possible that the active species is the monomer (150,000) of XO rather than the dimer (300,000).

Waud et al. (1975) found that 8 M urea, 4 M urea or 0.5% Triton X-100 in pH 8.9 polyacrylamide gel electrophoresis did not dissociate the enzyme into its subunits or fragments. The enzyme released its iron moieties and was denatured after exposing to 6.25 M urea at pH 6.25 during electrophoresis. In the presence of 6 M guanidine-HCl, he found that the enzyme dissociated into a fragment possessing a molecular weight of 157,000. Since guanidine-HCl is known to be a strong protein denaturant, it is understandable why this fragment did not exhibit activity. In this study, it was determined that gel patterns of purified XO did not change when disc-PAGE was performed in the presence of several dissociating agents. However, the bands showing enzymic activity migrated further after the purified XO treated with 6 M urea. Since disc- and SDS-PAGE did not resolve the urea-dissociated enzyme into its active subunits, gel filtration chromatography was employed to examine whether the enzyme could be dissociated into an active monomer with 6 M urea.

Figure 5 represents a chromatogram of urea treated enzyme eluted from a Sephacryl S-200 column which was calibrated with standard proteins of known molecular weight. From the elution pattern and standard curve, it is apparent that the active species was eluted as a single peak close to the IgG peak, possessing an estimated molecular weight of 160,000. SDS-gel patterns of eluted active fraction revealed a single band with molecular weight of 155,000 which is similar to that of the untreated enzyme.

Table 5 summarizes the results of analysis of XO/dehydrogenase conversion of the eluted enzyme monomer. No uric acid formation was detected when the eluted enzyme samples were in the assay in the absence of xanthine. It is, therefore, confirmed that urea is not a substrate of XO and does not influence the results of subsequent treatments of XO. There was no NADH form in the urea-treated enzyme. Nevertheless, a low dehydrogenase activity was obtained after the eluted species was treated with 10 mM DTT or 1% ME. This result may be due to the presence of urea, a strong dissociating agent, which does not allow NAD^+ to reach FAD molecule while DTT or ME could mitigate this effect. The significance of the data is not so much that of detectable dehydrogenase activity, but they indicate that the active monomer retained the capacity to convert oxidase to dehydrogenase.

Although urea was present throughout the enzyme samples during analysis to maintain a dissociated state, the results provided direct evidence in support of the hypothesis that the monomer of XO is active.

Inhibition by Chloroquine

Eigel (1980) found no reduction of XO activity in MFGM prepared in

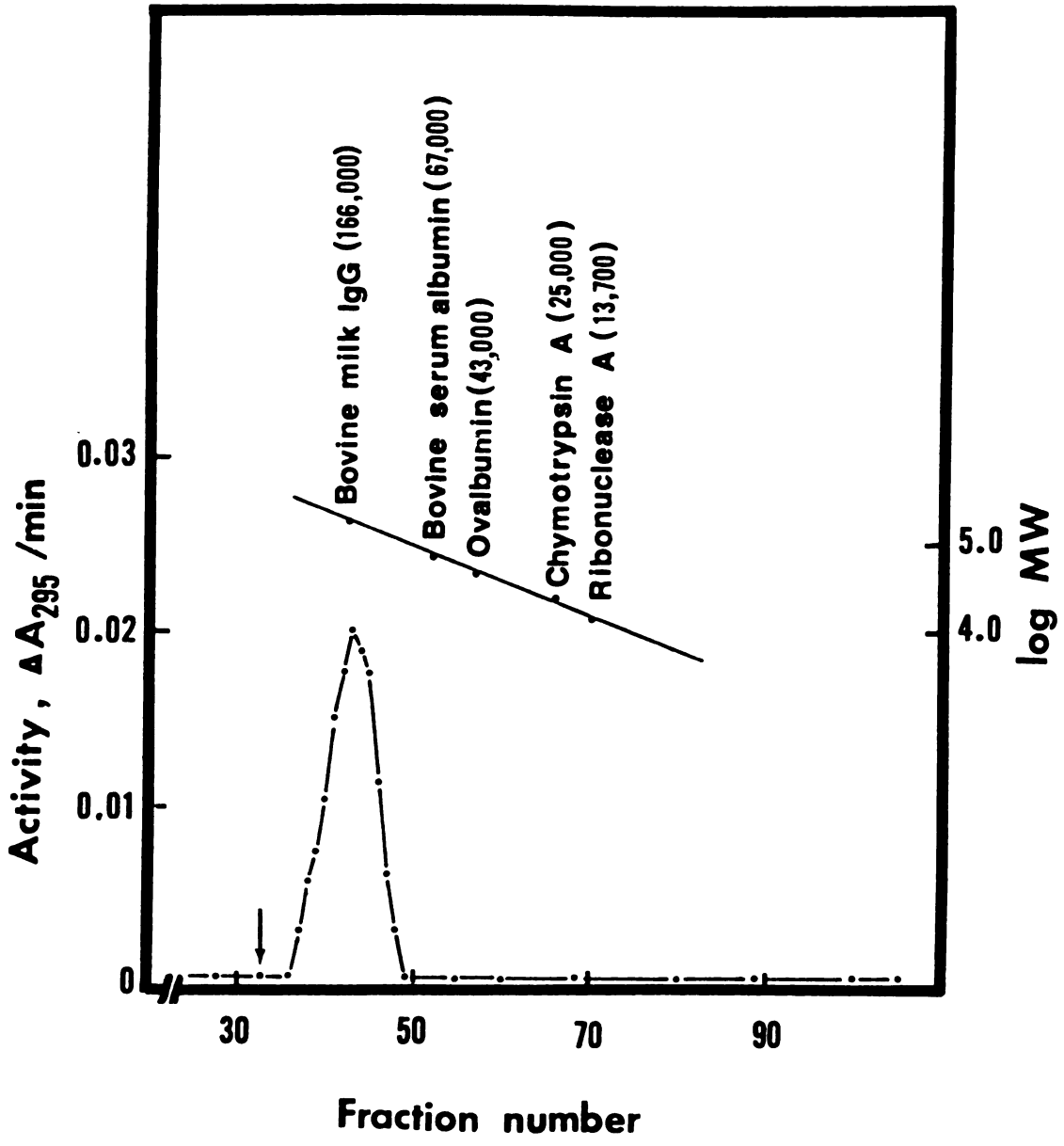

Figure 5. Gel filtration chromatogram on Sephacryl S-200 of urea-treated XO. Fractions of 4.6 ml were collected. Activity of each fraction was measured by enzyme assay at 295 nm. Arrow indicates the void volume. Upper straight line indicates calibration of the column by standard proteins.

Table 5. Conversion of xanthine oxidase-dehydrogenase activities of active XO monomer by DTT and ME

Treatment	Activity (IU/ml)		
	Acceptor		
	oxygen[a]	oxygen+NAD+ [a]	NAD+ [b]
Active monomer without xanthine[c]	0	0	0
Active monomer with xanthine[c]	0.136	0.149	0
Active monomer+DTT[d]	0.103	0.119	0.005
Active monomer+ME[d]	0.114	0.117	0.006

a. Micromole xanthine oxidized/ml, (IU/ml), by measuring uric acid at 295nm.

b. Micromole NADH formed/ml, (IU/ml), by measuring NADH at 340nm.

c. Only enzyme species to check the effect of urea in the assay.

d. Enzyme species was incubated at 37 C with 10mM DTT or 1% ME of final concentration in 0.1M phosphate buffer, pH 6.8, for 20 min.

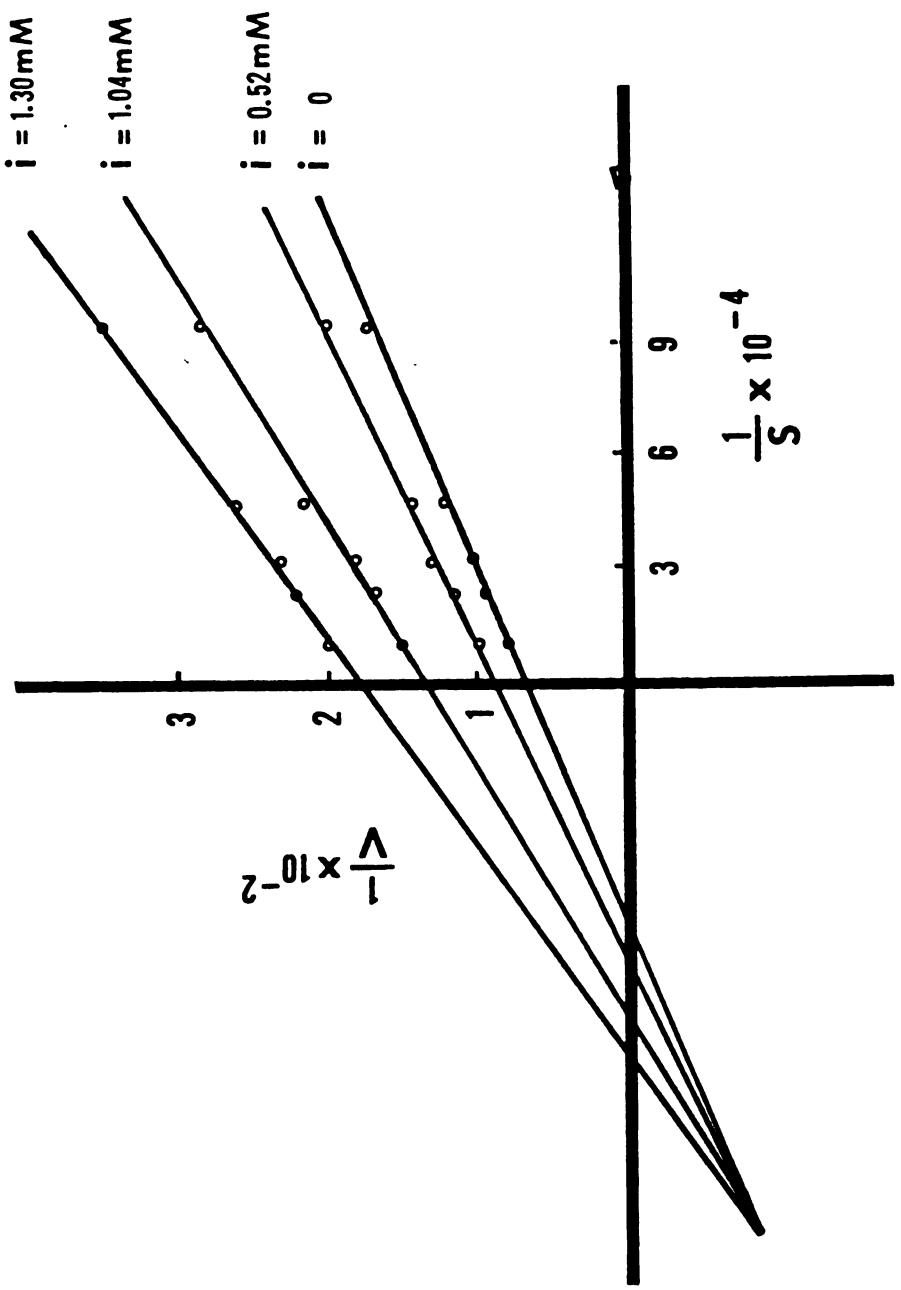

Figure 6. Lineweaver-Burk plot of xanthine oxidation by XO in the presence of chloroquine (i), ranging from 0 to 1.30 mM in the reaction mixture.

the presence of protease inhibitors such as chloroquine and Kallikrein inhibitor. During the investigations on the increase of XO activity in milk stored at different temperatures, chloroquine and/or aprotinin were added as protease inhibitors to prevent the proteolysis of XO. It was found that XO activity decreased after the addition of inhibitor(s). This observation raised the question concerning the possible effect of these inhibitors on the enzyme. The enzyme activity of purified XO did not change in the assay mixture containing aprotinin, whereas a 20% decrease in activity was found after adding chloroquine to the assay mixture. This suggests that chloroquine may inhibit the reaction to a limited extent or may be a substrate competitor of the enzyme.

Figure 6 shows the 1/V vs 1/S plot of the study of enzyme kinetics in the presence of different fixed concentrations of chloroquine. The intersection of the plotted data below the 1/S axis indicates that chloroquine acts as a hyperbolic (partial) mixed-type inhibitor in the enzyme reaction (Segel, 1975). No further analysis of this inhibition was attempted since the evidence is sufficient to indicate the influence of chloroquine on the enzyme.

Chemical Compositions

Lyophilized, purified XO contained 14.8% protein nitrogen which is equilvalent to 92.5% protein based on lyophilized weight and a factor of 6.25. No lipid was found after staining the disc-gel of purified XO with lipid staining.

Table 6 compares the amino acid composition of bovine milk XO

Table 6. Amino acid composition of four preparations of bovine milk XO

Residue	Amino Acid Content (mole %)			
	Bray and Malmstrom (1964)	Nelson and Handler (1968)	Mangino and Brunner (1977a)	this study
Lysine	6.8	6.9	6.6	6.8
Histidine	2.3	2.3	2.3	2.3
Arginine	4.4	4.7	4.9	4.3
Aspartic Acid	8.4	8.6	8.9	8.4
Threonine	7.1	7.0	6.9	6.7
Serine	6.5	6.5	6.3	5.7
Glutamic Acid	10.2	10.0	10.5	9.5
Proline	5.5	5.5	5.3	5.6
Glycine	8.2	8.2	8.2	8.0
Alanine	7.5	7.6	7.5	7.3
Half Cystine	2.6	2.7	2.7	3.5
Valine	6.9	6.8	6.7	7.0
Methionine	2.0	2.2	2.9	3.0
Isoleucine	5.0	4.8	5.2	5.0
Leucine	8.7	8.9	8.8	9.1
Tyrosine	2.6	2.4	2.5	2.1
Phenylalanine	5.0	4.9	4.1	5.0
Tryptophan	0.4	0.4	0.4	0.6
S▲Q(this study vs)	3.66	2.87	3.90	

prepared in this study with values reported by Bray and Malmstrom (1964), Nelson and Handler (1968) and Mangino and Brunner (1977a). The values of S▲Q calculated by comparing the data of this study with that of the latter three groups were 3.66, 2.87 and 3.90, repectively. These values indicate that the analyses are in excellent agreement since a value of 4 was considered by Weltman and Dowben (1973) as the limit of analytical precision between different laboratories for the same protein. Comparing the data of the individual amino acid residues showed a general agreement, except a high value of half-cystine for the enzyme preparation in this study. Common to all is the extreme low value for tryptophan which is characteristics of the enzyme (Bray, 1975).

Based on tne data of mole percentage of each amino acid residue, the ratio of the acidic residues, Asp and Glu, to the basic residues, Arg, Lys and His, is approximate 1.3. The ratio of the hydrophobic residues, Leu, Ileu, Val, Pro, Phe and Met, to the hydrophilic residues, Tyr, Asp, Glu, Lys and His, is 1.2. This illustrates that bovine milk XO possesses an acidic but hydrophobic nature. The average hydrophobicity of purified enzyme calculated by the method of Bigelow (1967) is 1135 cal which is 5% higher than 1074 cal obtained by Mangino and Brunner (1977b).

Values of S▲Q and average hydrophobicity is commonly used to examine the relatedness of various proteins. Generally, the discrimination of the S▲Q method is better than that of the average hydrophobicity procedure. Mangino and Brunner (1977b) employed both methods to demonstrate the relatedness among selected proteins including XO according to the following criteria: (1) related

proteins, in general, have similar average hydrophobicities (Bigelow, 1967), (2) value of S▲Q less than 50 for paired comparison indicates a high degree of primary structural homology of proteins (Marchalonis and Weltman, 1971). They deduced that bovine milk XO exhibits a high degree of compositional relatedness to membrane ATPase, actin and other contractile proteins. Although many of these proteins can be related to purine metabolism or recognize the purine ring, some of them such as acetylcholinesterase and phosphatase have no connection with purine metabolism. However, all of the proteins were either membrane-associated, or form an insoluble complex in water. Thus, they suggested that these proteins may be "actin-like" in composition but not in primary sequence or function. Their relatedness may be due to the evolutionary convergence toward similar amino acid compositions capable of interacting with the environment such as a lipodial membrane.

It is more important to consider here the hydrophobic nature of XO and its compositional similarity to the membrane-associated proteins than to compare the homology of the primary sequence between proteins. This characteristic could offer logical explanation why the enzyme is concentrated in the cream phase and MFGM in milk and can be entrapped in liposomes for intestinal absorption.

The specific volume of the enzyme calculated from its amino acid composition was 0.736 which is in excellent agreement with values of 0.737 and 0.74 reported by Avis et al. (1956a) and Andrews et al. (1964), respectively.

The data in Table 7 represent the results of analyses for available (exposed), total (unexposed and exposed) and reduction-

Table 7. Sulfhydryl content of xanthine oxidase

Component	No. of -SH per mole of XO[a]
Available -SH	4
Total -SH	38
Total -SH after reduction of S-S	82
Total half-cystine content (-SH) from amino acid composition:	
This study	88
Bray and Malmstrom (1964)	73
Nelsons and Handler (1967)	76
Mangino and Brunner (1977a)	75
Nalger and Vartanyan (1973)	120

a. Based on molecular weight of 302,000.

induced sulfhydryl groups in the purified XO. There were 4 available sulfhydryl groups per mole of the enzyme (300,000) in the native comformation. The enzyme, after exposure to 0.2% SDS, revealed a total 38 detectable sulfhydryl groups which indicated that 34 sulfhydryl groups were buried inside the molecule. After treating XO with a strong reducing agent, sodium borohydride, a total of approximate 82 sulfhydryl groups per enzyme molecule was determined. Difference between the number of sulfhydryl groups before and after reduction of the enzyme suggested that there were 22 disulfide bridges distributed throughout the interior of the molecule (subunits). From the ratio of moles of half-cystine/moles protein, using a molecular weight of 302,000, it was calculated that the purified XO contained 88 total half cystine residues (-SH groups) per mole which is close to 82 obtained by chemical analysis.

Only the total half-cystine content has been reported which was based on amino acid composition of XO (see Table 7). The data of the total half-cystine residue or sulfhydryl groups obtained in this study, either 88 or 82, is higher than the 73, 76 and 75 residues calculated from the data of amino acid compositions of Bray and Malmstrom (1964), Nelson and Handler (1968) and Mangino and Brunner (1977a), respectively. Nalger and Vartanyan (1973) obtained a value of 120 semi-cystine residues which is higher than any one of the above values. They also studied the kinetics of the reaction of PCMB with SH groups of XO in the pH range of 6.9-12.9 at 22 C. Approximately 30% of all SH groups were found to react slowly with PCMB only at pH>11.7. Since at high alkalinity disulfide bond-splitting is accompanied by degradation of protein, the possibility exists that the reactive SH

groups were generated by the dissociation of disulfide bonds at pH>11.7. Approximately 19 disulfide bonds could be calculated from their data which approximates the 22 disulfide bridges obtained in this study.

The significance of these data is not so much the numbers of sulfhydryl and disulfide groups found but the observation that disulfide bonds exist in the enzyme molecule. Waud and Rajagopalan (1976) and Coughlan (1980) suggested that many of the free sulfhydryl groups of the dehydrogenase form of XO are oxidized to disulfide bonds during the conversion of the dehydrogenase form to oxidase form. Battelli (1980) converted Wistar rat liver xanthine dehydrogenase to the oxidase form in the presence of glutathione (GSSG) and a crude fraction obtained from rat liver homogenates. He reported that bovine milk sulfhydryl oxidase failed to catalyze the conversion and suggested that the conversion is via a thio-disulfide interchange mechanism. In contrast, Clare et al. (1981) found that purified milk XO form D could be converted to the O form when catalyzed by purified milk sulfhydryl oxidase. They suggested that the conversion of dehydrogenase to oxidase of milk XO could be via a de novo disulfide bond generation catalyzed by sulfhydryl oxidase. Regardless whether by thio-disulfide interchange or de novo disulfide formation, disulfide bonds were formed from sulfhydryl groups during the conversion. Clare et al. (1981) reported the disappearance of the protein's sulfhydryl groups concomitant with the conversion of enzyme activity. Thus, the content of disulfide groups could be a special characteristics of the enzyme and may be used as a tool to estimate the purity, enzymic state and/or degree of the conversion of the enzyme.

The techniques applied here could not distinguish between intra- and inter-molecular disulfide bonds. However, results and discussion with regards to effects of dissociating agents on purified XO indicate that the disulfide bonds could be considered to be the intrachain type because treatment with ME did not change the electrophoretic patterns of XO.

Data presented in Figure 7 show the results of chromatography of the N-terminal amino acid of the purified enzyme. Four solvents were used as described in the EXPERIMENTAL section. Solvent 3 and 4 were run in the same direction as solvent 2 and were designated as dimension 3 and 4, respectively. The first two dimensions separated most amino acids but not lysine, histidine and arginine which remained in the same position. Dimension 3 separated histidine from lysine and arginine while dimension 4 distingished arginine from lysine and histidine (Weiner et al., 1972). Thus, the four dimensioned chromatography run can resolve lysine, histidine and arginine (Figure 6A). The acid hydrolysate of dansylated purified XO showed the same chromatogram as that of the dansylated lysine standard run on the reverse side of the micropolyamine sheet (Figure 6B). Therefore, lysine appears to be the N-terminal amino acid of milk XO.

Effects of Dissociating Agents

The effects of five dissociation conditions-(1) ME, (2) urea, (3) urea plus ME, (4) SDS and (5) Triton X-100 in combination with each of former four conditions-on the enzyme was studied by PAGE. Gels for SDS-PAGE contained 9% (T) with 2.6% (C) while gels with 5% (T) and

Figure 7. Chromatograms of dansylated amino acids on micropolyamide sheet. (A) dansylated standard amino acids, (B) dansylated N-terminal amino acid of XO. Legend: A-arginine, H-histidine, NH_3-dansyl NH_3, OH-dansyl OH, L-lysine. See text for description of solvent system.

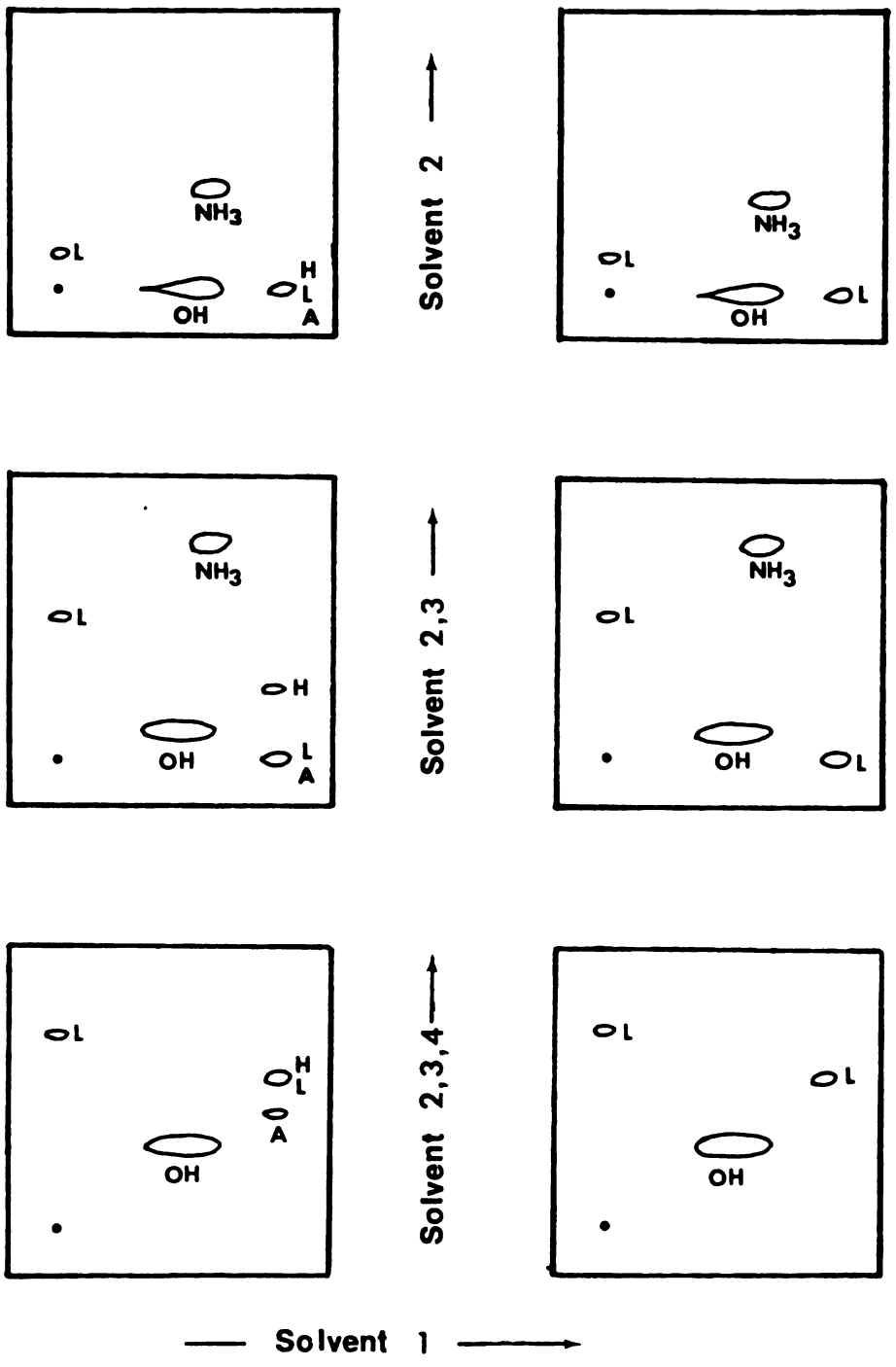

Figure 7

Figure 8. Electrophoretic patterns of purified milk XO in the presence and absence of various dissociating agents: A, no reagents (5%T); B, sample equilibrated against 10 mM (1%) ME (5%T); C, sample equilibrated against 6 M urea (5%T); D, sample equilibrated against 6 M urea plus 10 mM ME (5%T); E, with SDS according to Laemmli (1970) (9%T).

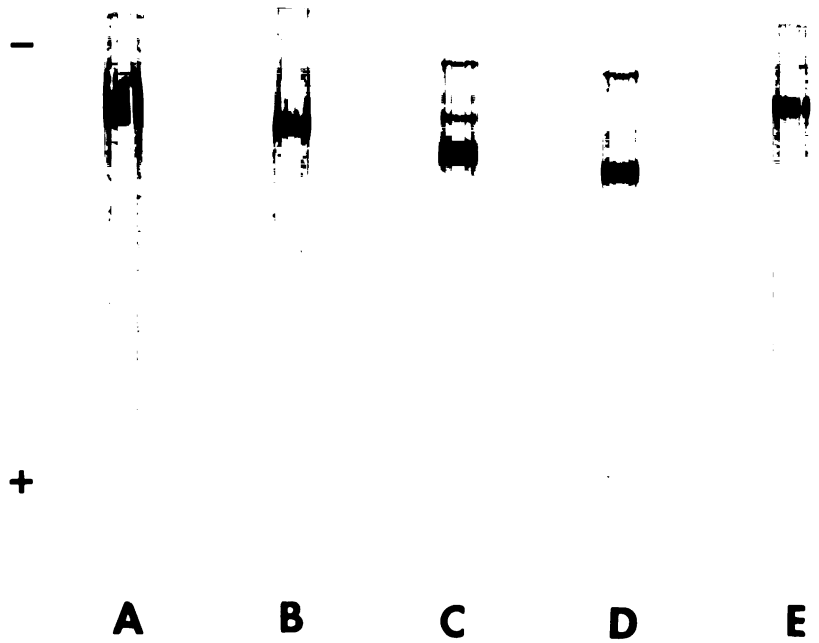

Figure 8

2.6% (C) were used for other electrophoreses. The results are shown in Figure 8. Except for SDS, the treated enzyme showed a positive reaction to enzyme activity-staining after electrophoresis. Gel 1 represents the undissociated XO with three enzymically active bands-one major and two minor zones. After purified XO was exposed to 10 mM (1%) ME for 20 min, disc-gel patterns revealed a single active band (Gel 2). ME can convert the reversible O form of XO to D form which possessed different electrophoretic mobilities than the irreversible O form (Battelli et al., 1973). It appears that the three bands in gel 1 indicate that the enzyme preparation is a mixture of these two forms as was evident from previous studies on the nature of its activities. Gel 3 represents the pattern of purified XO equilibrated with 6 M urea and subsequently applied to the gel system containing 6 M urea. Three positive activity-staining bands were observed which migrated further than those in gel 1. The addition of ME consolidated these bands into a single enzymic active band (Gel 4) with higher electrophoretic mobility than observed in the gel 2 pattern. The faster migration of protein bands in urea-gel may be due to the differences of size of the protein since urea could dissociate the enzyme into active monomer, resulting in a high charge to size ratio-electrophoretic mobility.

SDS-gel patterns of the enzyme sample revealed a single polypeptide band (Gel 5 in Figure 8). A comparison of the relative mobility of the band with those in the standard curve (see Figure 2) revealed a molecular weight of approximate 151,000. The value falls in the range of 150,000-155,000 reported from SDS-PAGE estimation for milk XO (Nalger and Vartanyan, 1973; Waud et al., 1975; Mangino and Brunner, 1977a; Frendenstein et al., 1979). It is in excellent

agreement with the value of 150,000 commonly recognized as a monomer of XO from various sources (Bray, 1975).

Addition of 1% Triton X-100 to each of the treated specimen had no effect on enzyme activity. Results of PAGE following the addition of Triton X-100 showed similar gel patterns to that of corresponding treatments without Triton X-100, see Figure 8. These data indicate that Triton X-100 does not affect the enzymic activity or physical characteristics.

Effects of Limited Proteolysis

Results showing effects on enzyme activities by limited proteolysis with trypsin, chymotrypsin, plasmin, pancreatin, pepsin and papain are presented in Figure 9. Oxidase activity was resistant to proteolysis. The six proteases investigated did not change the ability of purified XO to convert xanthine to uric acid with oxygen or neotetrazolium as an electron acceptor. However, following proteolysis purified XO lost its xanthine dehydrogenase activity with NAD^+ as an electron acceptor. Furthermore no NADH formtion could be detected in the dehydrogenase assay when proteolyzed XO was treated with 1% ME or 10 mM DTT.

Nalger and Vartanyan (1976) treated milk XO with trypsin, chymotrypsin and subtilisin at pH 8. They found that the enzyme retained its oxidase/dehydrogenase activities and the ability to transfer electrons from xanthine to other acceptors such as tetranitroblue tetrazolium, phenazine methosulfate and indophenol. They also found that there was no effect on the catalytic properties

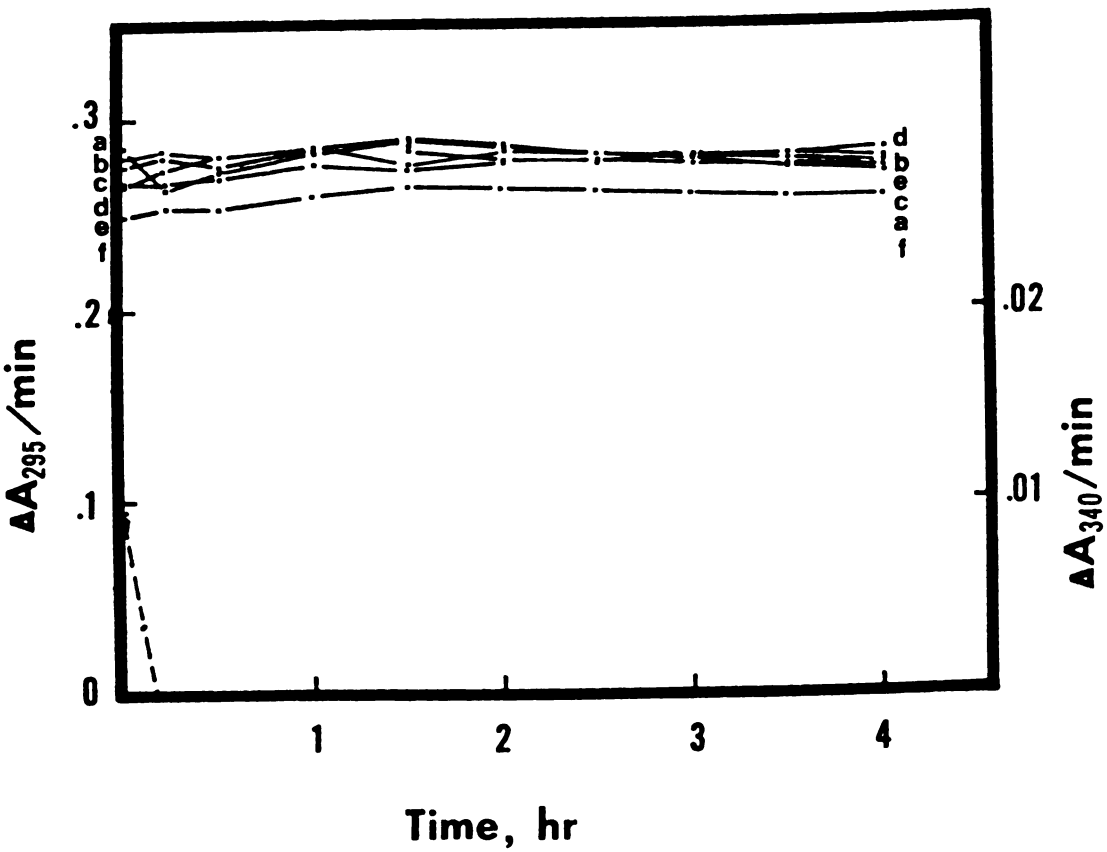

Figure 9. Effect of limited proteolysis by various proteases on the oxidase/dehydrogenase activities of XO. Solid lines indicate oxidase activity after treatment of XO with: a-trypsin, b-papain, c-plasmin, d-chymotrypsin, e-pancreatin, and f-pepsin. Dotted line indicates the dehydrogenase activity of protease-treated XO.

of the enzyme at pH 10.7, whereas the activity reduced gradually and remained at 5-6% of initial activity after incubating XO at pH 11.0 to 11.2 for 24 hr. The ability of the enzyme to transfer electrons to other acceptors mentioned above was reduced three folds. This behavior is attributed to the dissociation of FAD from the enzyme at high pH. The enzyme specimen digested by subtilisin at pH 11 retained the ability to oxidize xanthine and to transfer electron to the above mentioned acceptors. This characteristic was completely lost when XO was digested by subtilisin at pH values higher than 11.3.

Thus, the above evidences led to the conclusion that XO activity is very resistant to proteolysis. This characteristic may be a ramification of compact molecular configuration rendering enzymic active sites stable in the condition of proteolytic digestion.

Figure 10 and 11 represent electrophoretic results of the digested enzyme with and without treatments of ME, urea and ME plus urea. Gel patterns of XO which were digested individually by six different proteases were similar to that of an undigested enzyme sample, except that the protein band in some gels migrated slightly faster as apparent in Figure 10A. Mobilities of the band of trypsin-, chymotrypsin- and pancreatin-treated samples, designated gel 2, 3, and 5, repectively, were slightly higher than that of the control XO specimen. Waud et al. (1975) and Nalger and Vartanyan (1976) obtained similar results, noting that treatment of XO with trypsin, chymotrypsin and pancreatin resulted in an increase in the electrophoretic mobilities of the protein bands in disc-PAGE. Figure 10B, 11A and 11B show the gel patterns of proteolyzed XO further treated with 1% ME, 6 M urea and 1% ME plus 6 M urea, respectively.

Figure 10. Disc-PAGE electropherograms of purified XO subjected to proteolytic digestion by the following enzymes: 1-control (no enzyme), 2-trypsin, 3-chymotrypsin, 4-plasmin, 5-pancreatin, 6-pepsin, 7-papain. Group A represents data from 4 hr digestion. Group B illustrates effect of 1% ME added subsequent to digestion.

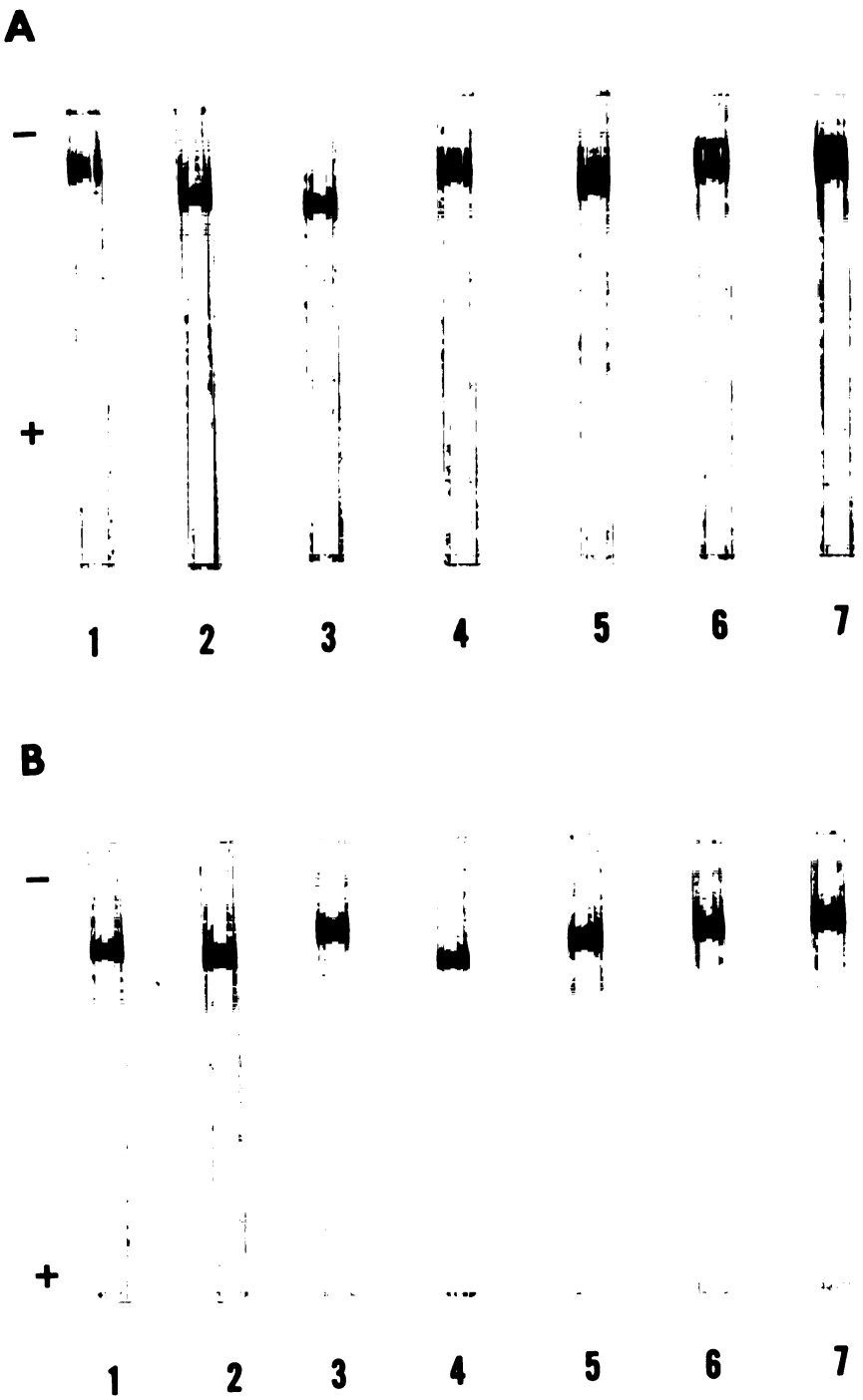

Figure 10

Figure 11. Gel electropherograms of proteolyzed XO treated with 6M urea (A) and 1% ME plus 6 M urea (B) in disc-PAGE (5%T). Legend for gel patterns: 1-no protease, 2-trypsin, 3-chymotrypsin, 4-plasmin, 5-pancreatin, 6-pepsin, 7-papain.

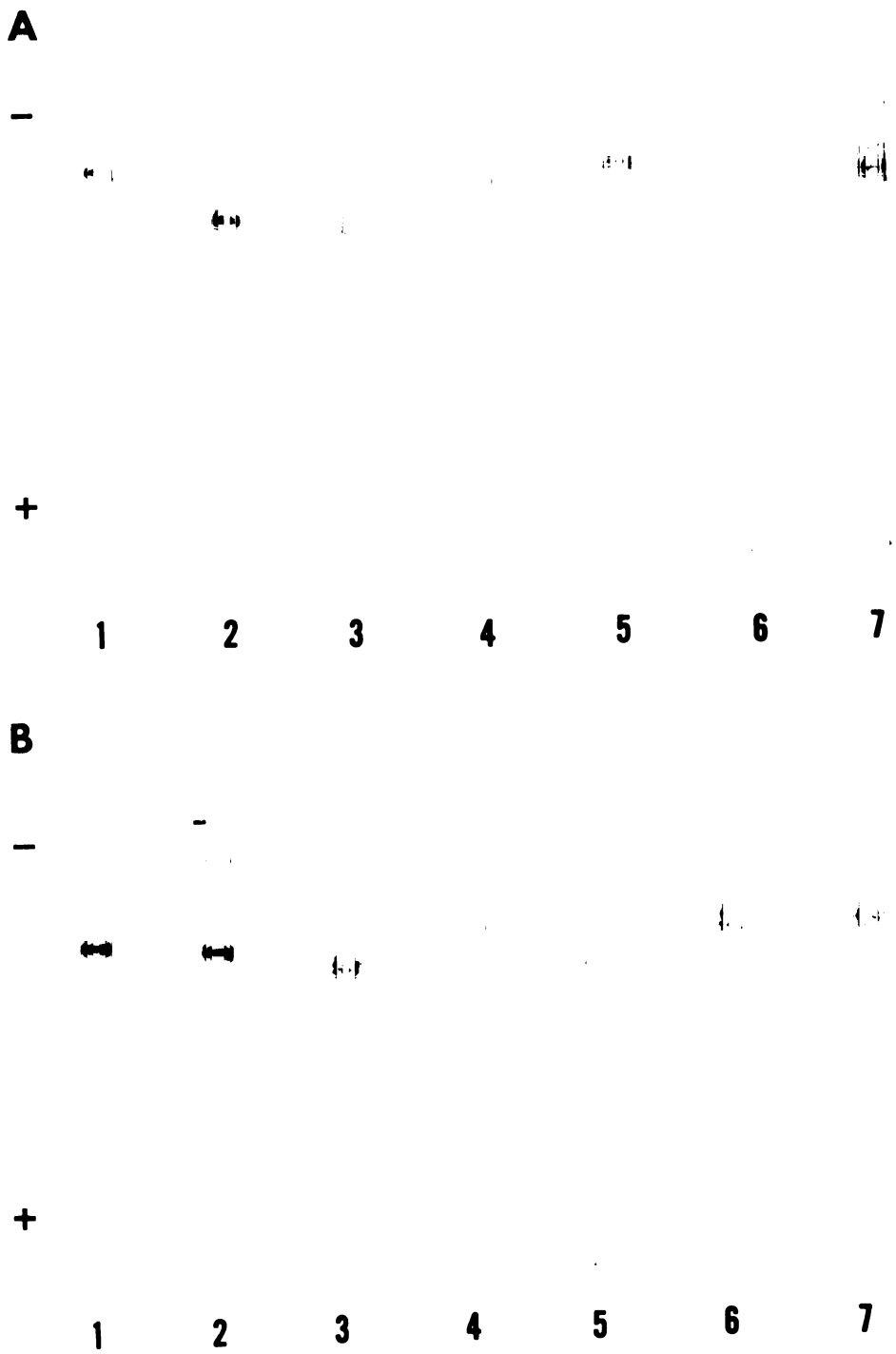

Figure 11

The numbers of bands representing the digested enzyme samples were similar to those of undigested XO with the same treatment, whereas their electrophoretic mobilities were different. All the bands visulized by Coomassie blue staining also revealed positive staining for enzyme activity. These studies indicate that, under the above conditions of treatment, proteolyzed XO could not be electrophoretically resolved. Thus, it is apparent that the enzyme is very resistant to digestion by proteases at pH 6.8 and that integrity of its activity and molecular structure are conserved. The data of Nalger and Vartanyan (1976) represent support for this conclusion. They found that proteolyzed polypeptides of XO were resistant to further digestion by proteases at pH 8. They, too, failed to separate the proteolytic fragments under conditions at which the enzyme remained enzymically active. They found that separation of these fragments was possible only in an electrophoretic environment containing 1% SDS or 5 M guanidine-HCl. Under these conditions, enzymic activity was lost.

Nalger and Vartanyan (1976) also studied the limited proteolysis of XO under partially denaturing conditions such as high pH. They reported that the oxidase activity of the enzyme remained unchanged before and after the addition of subtilisin A at pH 11.2. SDS-gel patterns of the enzyme after partial denaturation and proteolysis indicated that a selective digestion occurred. They suggested that the protein molecule of XO may be a bi- or poly-globular type, since a protein made up of only one type of globule would be unfolded under the conditions employed resulting in a non-selective and/or complete digestion of the polypeptide chain. However, the FAD molecule of the

enzyme is dissociated from the enzyme at high pH which may result in a partial alteration of the protein structure. Subtilisin A is a serine protease similar to chymotrypsin which has a limited substrate specificity. Investigation of the hypothesis of poly-globular structure of the enzyme is best performed with the intact protein and a protease capable of performing random cleavage. If the XO molecule is made up of only one type of globule, proteolysis should consist of a non-selective digestion of polypeptide chains. Papain was chosen here for this purpose since papain has broader substrate specificity than serine proteases.

The results obtained with papain digestion showed that the oxidase activity of purified XO did not change (see Figure 9) and its protein molecule remained compact and could not be further dissociated by ME and urea (see gel 7 in Figure 11A and B). The SDS-gel pattern of papain digested-XO (Gel I in Figure 12) indicated that a restricted digestion occurred. Although papain is not an ideal protease to use for this investigation, the results obtained, when considered with the results obtained from the limited proteolysis of XO by the other five proteolytic enzymes and the work of Nalger and Vartanyan (1976), provide sufficient evidence to conclude that the molecular structure of XO is a polyglobular type.

SDS-gel patterns shown in Figure 12 represent the purified XO specimen digested by six proteases at 37 C for 4 hr. Molecular weights of the polypeptide bands revealed in all gels were estimated by referring their relative mobilities to the standard curve shown in Figure 2.

Gel C represents the undigested XO, revealing a single band with

Figure 12. SDS-gel electropherograms of purified XO digested by various proteases. Legend: A-low MW protein standard, B-high MW protein standard, C-undigested XO, and the enzyme treated with D-trypsin, E-chymotrypsin, F-plasmin, G-pancreatin, H-pepsin, and I-papain. SDS-PAGE (9%T) is according to Laemmli's method (1970).

Figure 12

molecular weight of 149,000. As discussed previously, this polypeptide is the monomer of XO. Gel D shows the dissociation of trypsinized XO after a 4 hr digestion. Four major bands were observed with molecular weights of 136,000, 85,000, 35,000 and 18,000 (from top to bottom of the gel, respectively). Degradation of XO by trypsin digestion was also examined by SDS-PAGE. Sample were removed from the digestion mixture after 10 min and at subsequent intervals of 30 min for 4 hr. Only the 136,000 dalton species and several very faint bands migrating close to the marker dye were observed after 30 min of digestion. Thirty minutes later, the SDS-gel pattern revealed four major bands of molecular weight 136,000, 95,000, 33,000 and 20,000. Afetr 90 min an additional 85,000 dalton band appeared in the gel pattern accompanied by a minor band of 95,000 daltons. This minor band disappeared after two hours digestion and the remaining four species were resistant to further breakdown. The results indicate that trypsin rapidly converted the subunit (150,000) of XO into a 136,000 dalton species with a subsequence breakdown into fragments. The 85,000 dalton species was derived from either the 95,000 or the 136,000 dalton species. Waud et al. (1975) obtained one major band of approximately 130,000 molecular weight after treating XO with trypsin for 30 min. They suggested that trypsinization caused a single cleavage in the subunit at one of the termini resulting in 130,000 and 20,000 molecular weight fragments. Mangino (1976) also observed a limited breakdown of the enzyme by digestion with trypsin. Two major bands with molecular weights of 153,000 and 90,000 were found after 50 min of digestion. Only the 90,000 dalton band and several trace bands were seen in SDS-gel after the digestion proceeded 24 hr. He found the only 13% of the specific

activity of the original enzyme was present in the 90,000 dalton species. Nalger and Vartanyan (1976) observed that the subunit (150,000) of XO was rapidly converted into a 135,000 dalton species during trypsin digestion. Three major bands in SDS-gel, possessing molecular weights of 135,000, 92,000 and 20,000, were observed after trypsinization of XO for 24 hr at pH 8. They suggested that the 92,000 band was formed from the 150,000 and 135,000 dalton species by tryptic activity. They also found that a high concentration of trypsin split the 92,000 dalton polypeptide into a 84,000 dalton species.

Gel E shows the results of incubating purified XO with chymotrypsin at 37 C for 4 hr. Three major XO-derived bands with molecular weights of 80,000, 29,000 and 18,000 and, just above the 18,000 species, a band representing chymotrypsin. The location of the chymotrypsin band was identified by running SDS-PAGE with a sample containing chymotrypsin only carried through the incubation. Two minor bands of molecular weight of 63,000 and 33,000 were also seen in the gel pattern. After the first 30 min of digestion, a 110,000 band was observed in addition to above bands. The staining of band 33,000 was more intense than that of band 29,000. After 1.5 hr the disappearance of band 110,000 and a reduction in color density of band 33,000 accompanied by an enhanced color density for band 29,000 were recorded. The gel pattern remained unchanged after further digestion. Nalger and Vartanyan (1976) reported that treatment of XO with chymotrypsin and subtilisin for 24 hr gave rise to three major fragments with molecular weights of 92,000, 42,000 and 20,000. They found that high concentration of the proteases could further digest the 92,000 and 42,000 dalton species to 84,000 and 35,000 dalton

species, respectively.

Gel F represents the result plasmin treated XO after 4 hr at 37 C. Molecular weights of the observed three major zones were 136,000, 115,000 and 18,000. Degradation of XO by plasmin was slower than that by the other five proteases. After the first 2 hr, the SDS-gel pattern revealed a single band of molecular weight 146,000. This species was converted to a 136,000 dalton species after 2.5 hr. After 3 hr of digestion an additional minor zone of 115,000 molecular weight and a trace band of 18,000 daltons were observed. The latter two bands became slightly darker when digestion was terminated. Since plasmin is a endogenous protease in milk and its concentration in milk is much less than that used in this experiment, the results allow the suggestion that milk endogenous plasmin would not influence the purification of XO if started with fresh milk.

Degradation of XO by pancreatin was observed in the first 10 min. The subunit (150,000) completely disappeared and was replaced by a 134,000 dalton band. This molecular species was further degraded into three major fragments with molecular weights of 85,000, 33,000 and 18,000 after 30 min of digestion. These three species did not show further breakdown, constituting the SDS-gel patterns to the conclusion of digestion (see Gel G in Figure 12). Waud et al. (1975) obtained similar results when XO purified by a non-proteolytic method was degraded into 89,000 and 38,000 dalton polypeptides by digestion with pancreatin for 30 min. An additional three bands of 80,000, 75,000 and 19,000 molecular weight were observed after 6 hr digestion. Data obtained here indicate that pancreatin affects the molecular properties of XO and its dehydrogenase characteristics. Addition of

pancreatin in the isolation procedure would promote the degradation of XO molecule although its catalytic properties would be retained. This conclusion was supported by Waud et al. (1975), Nalger and Vartanyan (1976) and Battelli et al. (1973) who showed that XO purified in the presence of pancreatin was dissociated into 92,000, 35,000 and 15,000 dalton species when assayed by SDS-PAGE.

Pepsin and papain effected a fast digestion of XO. The degraded products after 10 min were resistant to further cleavage. XO was degraded into 136,000 dalton species by pepsin, whereas fragments of 130,000, 105,000, 35,000, 29,000 and 18,000 molecular weight were obtained after 10 min of digestion with papain. The gel patterns remained unchanged for up to 4 hr of digestion (see Gel H and I in Figure 12). Since the optimum pH for pepsin activity is 2, the digestion pH of 6.8 used here-close to the tail of its pH-activity profile, was too far removed from its optimum to achieve a full catalytic reaction. Whether or not XO is resistant to proteolysis by fully active pepsin remained unanswered. However, it may be suggested that XO in milk does not degrade extensively when pepsin is added in fresh milk since the pH of milk is about 6.7. The importance of degradation of XO by papain is not merely the characteristics of the degradation products but also the resistance these products exhibit to further digestion.

From the above data, it was apparent that three polypeptides of molecular weights ranging 85,000-100,000, 30,000-35,000 and 18,000-20,000 were commonly found after cleavage of XO by proteolysis. Nalger and Vartanyan (1976) obtained the same results noting that XO was converted to three fragments with molecular weight of 92,000, 42,000

and 20,000 by proteolytic enzymes and that the 92,000 and 42,000 dalton fragments could be further converted to 84,000 and 35,000 dalton species, respectively. The results obtained in the present study were essentially similar and support their postulation of a subunit molecule (150,000) of XO composed of three globular fragments. Furthermore, the results further suggest that a strong hydrophobic binding force is required to hold the three globules in a tight configuration. Hence, the intact molecule could not be separated under the dissociating conditions afforded by ME and urea, retaining its catalytic properties after proteolysis.

Amino Acids Contents of the Fragments of Trypsinized XO

All major fragments of trypsinized XO, e.g., 136,000, 85,000, 35,000, and 18,000 contain lysine as their N-terminal amino acid. Figure 13A shows typical results of four dimensional chromatography of dansylated N-terminal amino acid of the 85,000 fragment on micropolyamide sheet. Two spots were observed in the sample plate which were absent in the chromatogram developed from the blank gel after third dimension development (Column B in Figure 13). After running the dansylated lysine standard on the reversed side of the sample sheet, the two spots coincided with the spots of lysine standard in all 4 dimension runs. The chromatogram of a lysine standard is shown in Figure 7A. Chromatograms of dansylated N-amino terminal of the remaining three fragments were similar to that shown in Figure 13A. It is unusual that all fragments from trypsinized XO and the intact enzyme showed lysine to be the N-terminal amino acid.

Figure 13. Chromatograms of dansylated N-amino terminal residues of trypsin-derived fragments of XO. Column A indicates results from four dimension chromatography of acid hydrolysate of the dansylated 85,000-dalton species. Chromatograms of dansylated 136,000-, 35,000-, 18,000-dalton species were similar to column A. Column B indicates chromatograms of a blank gel. Legend: G-glysine, NH_3-dansyl NH_3, OH-dansyl OH, L-lysine. Solvent system was described in EXPERIMENTAL.

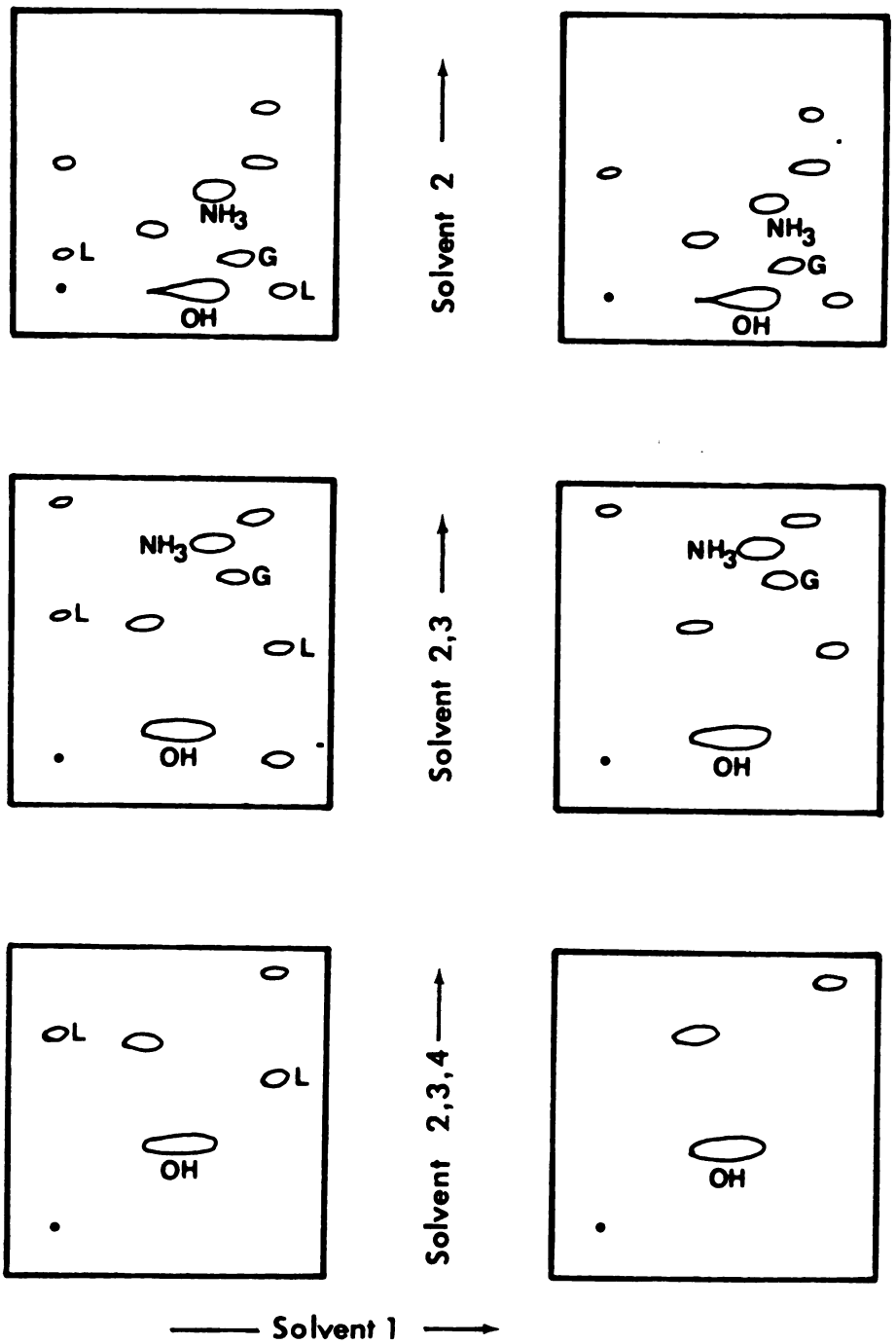
NH3
G
L
L
OH
Solvent 2
NH3
G
OH
NH3
G
L
L
OH
Solvent 2,3
NH3
G
OH
L
L
OH
Solvent 2,3,4
OH
Solvent 1
A
B

Figure 13

However, the lysine content of XO is, in fact, relatively high compared to other residues, e.g., 172 lysine residues per mole XO calculated from 6.8 mole percent (see Table 6). Trypsin cleaves the peptide bond between the COOH group of lysine or arginine and the amino group of the adjacent amino acid except when proline is the adjacent amino acid. It is suggested that the first N-terminal peptide bond of the fragments as well as the enzyme is a lys-pro bond.

Only a 60% recovery was obtained from the electrophoretic elution of trypsin-derived fragments based on a comparison of the amount of XO subjected to SDS-PAGE and the total amount of polypeptides eluted from the agarose gel system. The amount of each eluted fragment was not sufficient to determine the content of sulfur-amino acids and tryptophan. Table 8 represents the amino acid composition without methionine, half-cystine and tryptophan of the major polypeptide fragments of trypsinized XO. All fragments have low histidine, tyrosine and arginine contents and high aspartic acid and glutamic acid. A comparison of the results of each polypeptide revealed that the 136,000 dalton species contained relative high lysine, glutamic acid and leucine, whereas high contents of glycine and arginine were found in the 85,000 polypetide. Relative high contents of serine, lysine and glycine were found in the 35,000 dalton fragment, whereas the 18,000 dalton polypeptide contained a relative high leucine content.

Although performic acid oxidation of fragment species was not performed, the amino acid chromatogram of the HCl-hydrolysate of the 18,000 dalton sample showed a peak at the half-cystine position. The chromatograms of the other fragments showed only a small or no peak at

Table 8. Amino acid compositions of the major fragments of trypsinized XO

Residue	Amino Acid Content (mole%)			
	136,000 dalton species	85,000 dalton species	35,000 dalton species	18,000 dalton species
Lysine	8.7	5.3	6.8	3.9
Histidine	1.6	2.3	1.9	2.2
Arginine	1.8	3.5	2.6	2.9
Aspartic acid	12.8	10.0	8.4	12.1
Threonine	5.8	5.6	6.4	6.5
Serine	4.6	6.2	11.5	7.7
Glutamic acid	16.7	10.7	12.2	12.2
Proline	5.9	5.9	6.0	7.5
Glycine	5.0	15.8	12.8	5.1
Alanine	6.9	8.0	7.7	8.5
Half cystine	---	---	---	---
Valine	7.2	7.1	6.0	8.0
Methionine	---	---	---	---
Isoleucine	5.6	5.1	3.8	5.9
Leucine	12.4	7.7	7.9	10.2
Tyrosine	1.8	2.6	1.9	2.4
Phenylalanine	3.3	4.3	4.2	5.0
Tryptophan	---	---	---	---
Average hydrophobicity	1272	1116	1062	1272

this position, indicating that the 18,000 dalton polypeptide contained significantly more free SH groups than the other three polypeptides. Coughlan (1980) suggested that a peptide with molecular weight of 20,000 containing about ten free sulfhydryl groups which possibly function to stabilize the dehydrogenase conformation of XO. This peptide could be removed by proteolysis. Since XO lost its dehydrogenase activity after proteolysis by six proteases and the 18,000 dalton band was observed in all SDS-gel of proteolyzed XO (except pepsin treatment), it is suggested that the 18,000 dalton species is the peptide described by Coughlan (1980).

Average hydrophobicities calculated according to Bigelow (1967) were 1,272, 1,116, 1,062 and 1,272 cal for 136,000-, 85,000-, 35,000- and 18,000-dalton fragments, respectively. Average hydrophobicities of the 136,000 and 18,000 polypeptides are higher than the 1,135 cal calculated for intact XO, whereas the 85,000 and 35,000 polypeptides revealed slightly lower average hydrophobicities than that of XO. These results led to the conclusion that the fragments of trypsinized XO possess hydrophobic characteristics which account for their association in dissociating environments.

Photometric scanning of stained SDS-gels of trypsin-digested XO revealed peak area percentages of 6.89, 57.54, 25.59 and 13.22 for bands corresponding to the 136,000, 85,000, 35,000 and 18,000 dalton species, respectively. Assuming equal dye binding capacities for each of the fragments, their relative peak area distributions represent the relative weight concentrations. Therefore, the sum of the amino acid contents for each fragment should represent the amino acid composition of XO. The results of these calculations are given in Table 9. Column

Table 9. Comparison of amino acid composition of the purified XO and its amino acid composition calculated from its trypsin-derived fragments

Residue	Amino Acid Cotent (mole%)			
	A	B	C	D
Lysine	7.3	5.5	5.3	5.6
Histidine	2.5	1.9	2.0	2.0
Arginine	4.6	3.0	3.0	2.5
Aspartic acid	9.1	9.8	9.3	12.3
Threonine	7.2	5.7	5.6	6.3
Serine	6.1	7.3	7.3	6.6
Glutamic acid	10.2	11.3	10.6	13.7
Proline	6.0	5.9	5.8	6.9
Glycine	8.6	15.8	12.7	5.1
Alanine	7.9	7.7	7.5	7.9
Valine	7.5	6.8	6.5	7.7
Isoleucine	5.4	4.8	4.5	5.8
Leucine	9.8	8.2	7.6	10.9
Tyrosine	2.3	2.3	2.3	2.2
Phenylalanine	5.4	4.1	4.1	4.4

A; Amino acid composition of the purified XO recalculated the data from Table 6 without half cystine, methionine and tryptophan.

B; Calculated from data in Table 8. Each amino acid content is the sum of 6.89%, 57.54%, 25.59% and 13.22% of its content of 136,000, 85,000, 35,000 and 18,000 dalton species, respectively.

C; Same calculation as B with 59.75%, 26.52% and 13.73% for 85,000, 35,000 and 18,000 dalton species, respectively.

D; Same calculation as B with 34.26% and 65.74% for 136,000 and 18,000 dalton species, respectively.

B represents an amino acid composition calculated from all four major fragments. Data in column C was obtained by assuming that XO is constituted from 85,000, 35,000 and 18,000 dalton species in weight ratio of 59.75%, 26.52% and 13.73%, respectively, recalculated from the peak-area percentage of the scanned gel. Data in column D were based on weight ratios of 34.24% and 65.74% for the 136,000 and 18,000 species, respectively. Here, it was assumed that these two polypeptides constitute the XO molecule. When compared to the data for the original XO (Column A), all three calculated results show differences in distribution of amino acids. The discrepancy may be due to the ignored small peptides produced by trypsin digestion. In fact, trace bands, of 85,000 and 35,000 dalton, derived from the 95,000 and 42,000 dalton bands, respectively, were observed in SDS-PAGE gels. Similar species were reported by Nalger and Vartanyan (1976).

Quaternary Structure of Milk XO

Based on the above results from analyses of trypsinized XO, the model of polyglobular structure of milk XO proposed by Nalger and Vartanyan (1976) can be further enhanced in Figure 14. Each subunit (150,000) of XO is composed of three globulars segments, A, B and C, with molecular weights of 92,000 (85,000), 42,000 (35,000) and 20,000 (18,000), respectively. The unparenthesized numbers were suggested by Nalger and Vartanyan (1976) while numbers in parentheses were obtained in this study. A peptide bond between the amino group of lysine and the COOH group of other amino acid, possibly lysine or arginine (due to trypsin substrate specificity), linked each globule. In addition to

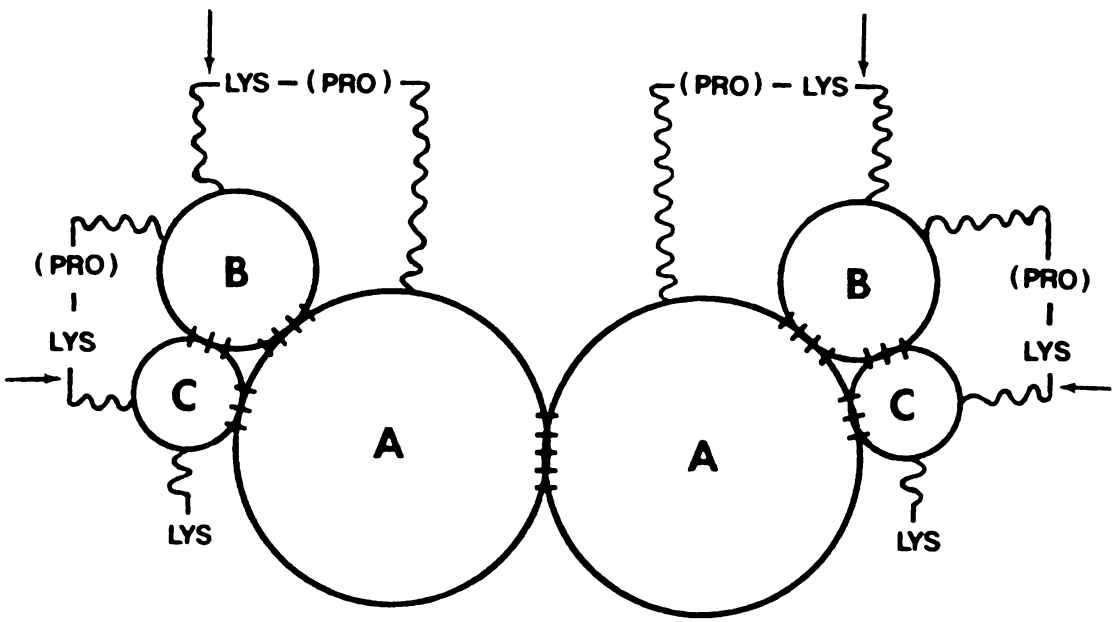

Figure 14. Schematic representation of a suggested modification of the polyglobular structure of native milk XO as proposed by Nalger and Vartanyan (1976). Molecular weights of each globule are: A-92,000 (85,000), B-42,000 (35,000), C-20,000 (18,000). Numbers in parentheses indicate molecular weight values obtained in this study. Legend: PRO=proline, LYS=lysine, ⊣⊢⊣⊢-hydrophobic binding, and arrows indicating the peptide bonds cleaved by trypsin.

the peptide bond between each globule, hydrophobic binding is probably involved in stabilizing the three globules, preventing their dissociation in urea and/or ME environments after proteolytic cleavage of peptide bonds.

Nalger and Vartanyan (1976) suggested that globule C was the first released followed by the release of globule B during proteolysis. Attempts to prove this hypothesis failed since lysine was found to be N-terminal amino acid for all three globules and the intact enzyme in this study. It is not clear whether the N-amino terminal exists in globule A or C because lysine is N-terminal in the intact XO. Further research is required to provide evidence in support of this model.

Free Riboflavin, FMN and FAD Contents and XO Activity in Cow's Milk

Swope et al. (1965) found a fluorescent material in aqueous suspensions of fat globule membrane preparations which contained riboflavin and its natural derivatives such as FMN and FAD. About 92-96% of the total riboflavin in the fluorescent substance is FAD. They suggested that FAD is contributed by dissociation from XO in the preparation of MFGM. Milk contains a system of enzymes including alkaline phosphatase capable of converting FAD to FMN and riboflavin (Manson and Modi, 1957). The FAD moiety released from XO, if it occurrs, can be converted to FMN and riboflavin, resulting in an increase in free riboflavin in milk.

Table 10 shows the contents of FAD and its components and XO activity in milk samples stored at room temperature for 0, 6, 20 and

Table 10. Flavins content and XO activity in milk stored at room temperature

Hour	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
0	0.037	0.178	0.252	1.018	1.448
6	0.046	0.161	0.211	0.932	1.304
20	0.080	0.244	0.141	0.920	1.305
48	0.125	0.260	0.336	0.879	1.475

Table 11. Flavins content and XO activity in milk stored at 4 C

Hour	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
0	0.037	0.178	0.252	1.018	1.448
6	0.152	0.120	0.217	1.041	1.378
20	0.152	0.164	0.309	0.9	1.417
48	0.132	0.250	0.378	0.812	1.440

Table 12. Flavins content and XO activity in milk stored at room temperature in the presence of aprotinin (protease inhibitor)

Hour	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
0	0.010	0.081	0.197	0.456	0.733
6	0.013	0.062	0.159	0.462	0.683
20	0.032	0.079	0.176	0.470	0.722
48	0.065	0.108	0.128	0.585	0.820

Table 13. Flavins content and XO activity in milk stored at 4 C in the presence of aprotinin.

Hour	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
0	0.010	0.081	0.197	0.456	0.733
6	0.158	0.134	0.105	0.431	0.670
20	0.162	0.084	0.155	0.471	0.710
48	0.151	0.091	0.143	0.474	0.708

Table 14. Flavins content and XO activity in milk dialyzed at 4 C for 24hr and followed by stored at 4 C

Treatment	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
After dialyzed 24 hr at 4 C	0.107	0.100	0.034	0.082	0.216
1 day storage	0.100	0.101	0.034	0.083	0.218
2 days storage	0.085	0.101	0.037	0.083	0.221

Table 15. Flavins content and XO activity in milk stored at 70 C

Hour	XO activity (IU/ml)	FAD (ug/ml)	FMN (ug/ml)	Free riboflavin (ug/ml)	Total riboflavin (ug/ml)
0	0.037	0.178	0.252	1.018	1.448
6	0.070	0.110	0.173	1.141	1.424
20	0.022	0.150	0.338	0.896	1.384
48	0.002	0.141	0.401	0.869	1.411

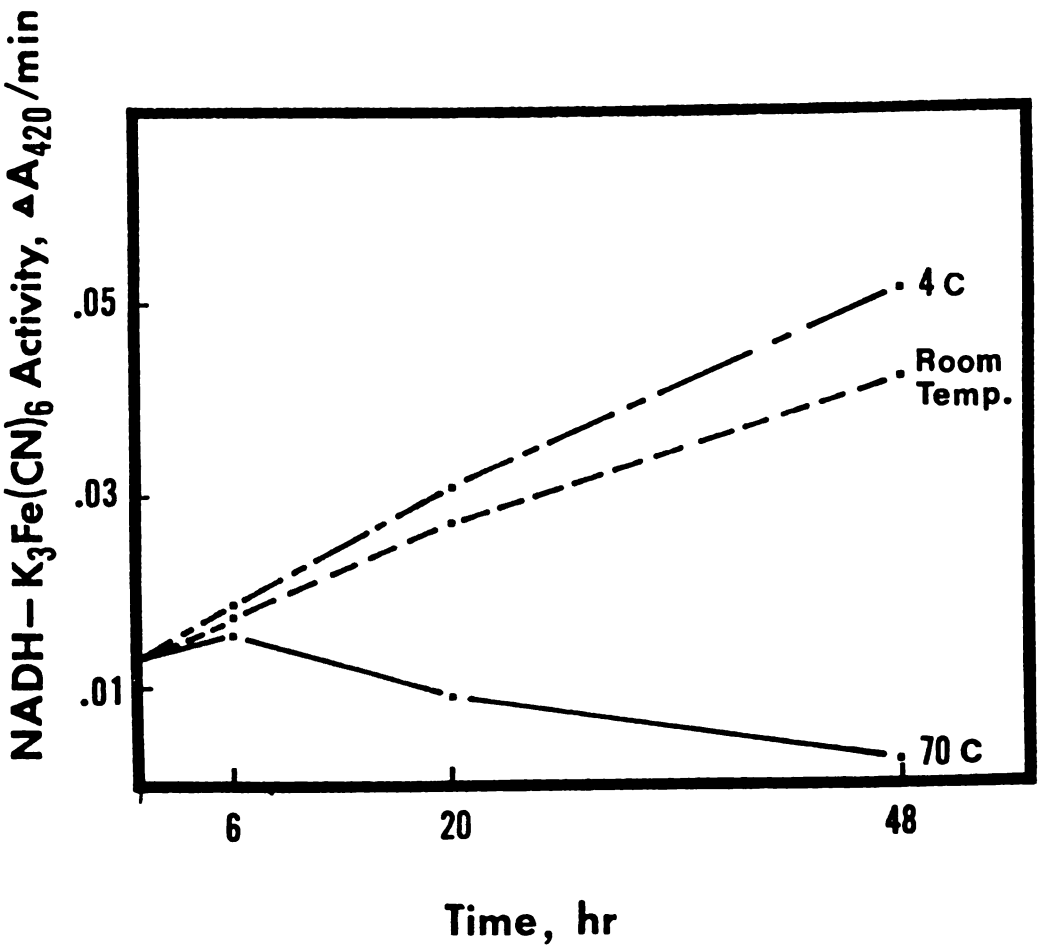

Figure 15. NADH-ferricyanide reductase activity of XO in milk stored for 0, 6, 20 and 48 hr at different temperatures.

48 hr. XO activity increased gradually and reached about 3-fold of its original activity after 48 hr. Amounts of FAD and FMN decreased at first (6 hr), then increased after 48 hr, whereas the amount of free riboflavin decreased during the storage period. Assuming that free riboflavin is not further degraded into non-fluorescent products by environmental effects, the results indicate that increased XO activity is accompanied by an increase in FAD content and a decrease in free riboflavin. It is suggested that the conversion of FAD to riboflavin by the enzymes in milk acted in a reverse direction to form FAD from free riboflavin. The FAD product, could have been picked up by inactive XO, which lacks FAD, resulting in its activation. However, this hypothesis was not supported by the data obtained from milk stored at 4 C and by other evidence to be discussed.

Table 11 presents the data characterizing the flavin contents and XO activities in milk stored for 0, 6, 20 and 48 hr at 4 C. XO activity increased rapidly and reached the highest level after 6 hr with a slight decrease after 48 hr. Changes in contents of FAD, FMN and free riboflavin were random during the storage of milk. After 48 hr the contents of FAD and FMN were the highest and free riboflavin was the lowest when compared to different storage times. Changes in the flavin content were similar to that observed in the milk sample stored at room temperature. It is suggested that the interconversion of flavins in milk also occurred at low temperature. However, the increase of XO activity at 4 C did not correspond to the change in flavin content.

Since protease can degrade the enzyme without affecting enzymic activity, the experiments described above were repeated in the

presence of protease inhibitor(s). Chloroquine and aprotinin have been used as protease inhibitors to eliminate the protease effect on XO in MFGM preparations (Eigel, 1980). Only aprotinin was used here since chloroquine was found to be a mixed-type inhibitor of XO (see previous discussion in section on Enzyme Nature). Table 12 and 13 report results obtained with milk stored at room temperature and 4 C in the presence of aprotinin. Increased XO activity similar to that of milk in the absence of inhibiotr was observed, whereas the contents of free riboflavin and its natural derivatives showed a random change. FAD and FMN contents did not increase to the extent observed in milk without the aprotinin. The results suggest that the increase in XO activity was not related to the changes in FAD and its components.

Milk samples were dialyzed for 24 hr to diminsh free riboflavin in milk and subsequently stored for an additional 1 and 2 days. It was found that XO activity decreased with a very small increase of FAD, FMN and free riboflavin (see Table 14). The small change noted in the flavin content was attributed to the experimental error in fluorescence measurements. Therefore, the changes of FAD, FMN and free riboflavin are independent of the increase in XO activity.

Table 15 represents results for milk samples stored at 70 C for 0, 6, 20 and 48 hr. Decreased FAD and FMN contents accompanied by an increase in free riboflavin at 6 hr were due to the enzymes system promoting the conversion of FAD to FMN and free riboflavin in heated milk. The enzyme system was destroyed in milk subjected to prolonged heating at 70 C. Therefore, it is postulated that the FAD molecule released from XO, if, indeed, it occurs, increases the FAD content only and does not contribute to the increase in FMN and free

riboflavin. Based on this theory, the increased FAD content and decreased XO activity in milk stored for 20 hr suggests that FAD is released from XO. However, the theory does not explain why the FMN and free riboflavin contents decreased. Also, the large decrease in XO activity and decrease of FAD content in milk after heating for 48 hr could not be explained. Thus, the release of FAD from XO during storage of milk at 70 C is inconclusive.

The XO activity obtained by measuring the xanthine-uric acid reaction may not reflect the presence or absence of its FAD moiety since the active enzyme retains its reducibility by xanthine after removal of the flavin moiety. Therefore, to determine if FAD remains as a part of the active enzyme, its activity was measured, using NADH as a reducing substrate and potassium ferricyanide as an electron acceptor (Komai et al., 1969). An increase in the oxidation of ferricyanide was observed and the increasing rate of NADH-ferricyanide reductase activity of XO was similar to that calculated from xanthine-uric acid measurements (see Figure 15). Thus, it appears that the prosthetic FAD group was not dissociated from the enzyme when employing xanthine-uric acid activity measurements.

From the above results it was concluded that XO is incapable of releasing its FAD molecule as a source of free riboflavin in normal cow's milk. Although it is possible that FAD is released from the enzyme as a consequence of heating milk, evidence supporting this proposition is inconclusive. Ho et al. (1978) studied the availability of riboflavin from bovine milk XO. They found that the rate of weight gain for chicks fed with 19.4 ug of riboflavin/day in the form of XO was equivalent to that of chicks consuming 5 ug riboflavin/day. Based

on this observation, they concluded that the availablity of riboflavin in XO for growth of chicks was approximately 25% of the potential riboflavin and suggested that only 4% of milk riboflavin is XO-associated available riboflavin. They concluded that XO is not a good dietary source of riboflavin.

Stabilization of Fat Globules by XO

Figure 16 illustrates the relationship between surface tension and concentration of XO, casein and whey proteins. Increasing protein concentrations up to 0.02 mg/ml resulted in a rapid decrease in surface tension of the solution whereas further increase in concentration produced only slight change in surface tension. These results indicate that the correlation between surface tension and protein concentration is typical of surface-active systems. The data reported herein show that XO is more surface active than casein and whey proteins.

All three protein samples demonstrated interfacial activity at a butter oil-simulated milk ultrafiltrate interface (see Figure 16). Rapid reduction of interfacial tension at low protein concentrations occurred, whereas the interfacial tension did not change at high concentration of proteins. The change of interfacial tension by casein was similar to results reported by Jackson and Pallansch (1961). The reduction of interfacial tension by whey proteins was less than that by casein. Major whey protein components may be less effective at reducing the free energy at the interface than casein. This explanation is supported by the work of Jackson and Pallansch (1961)

Figure 16. Surface tension of whey proteins, casein and purified XO at room temperature.

Figure 17. Interfacial tension of whey proteins, casein and purified XO at butter oil/simulated milk ultrafiltrate interface at 45 C.

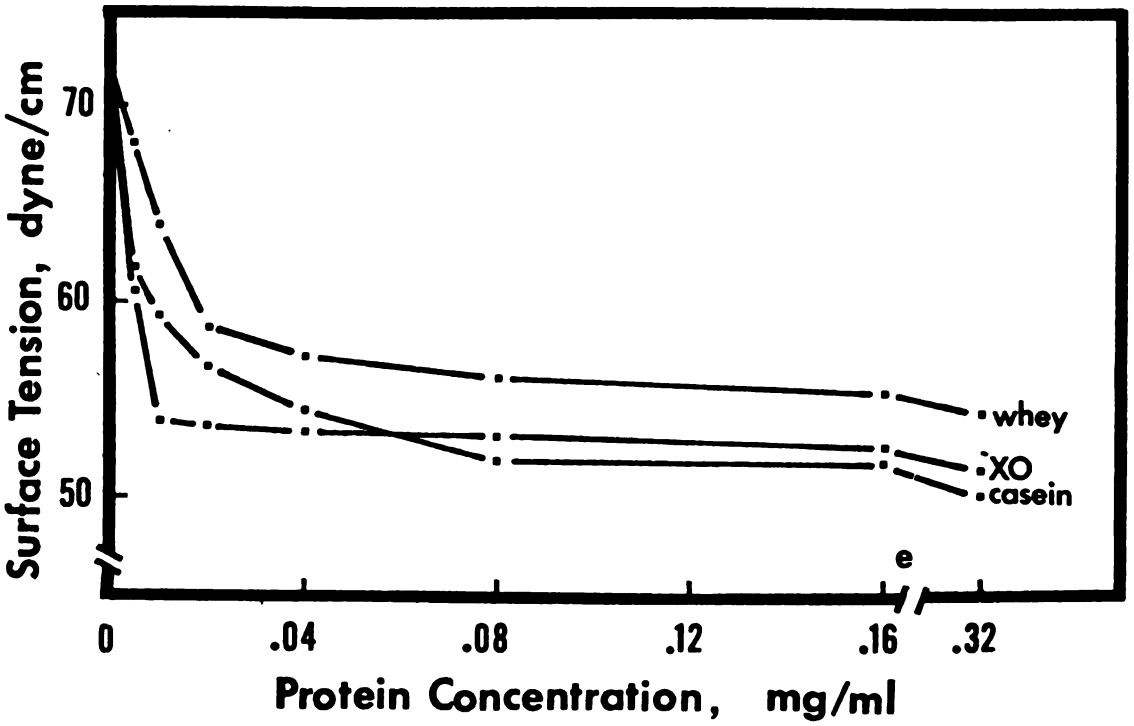

Figure 16

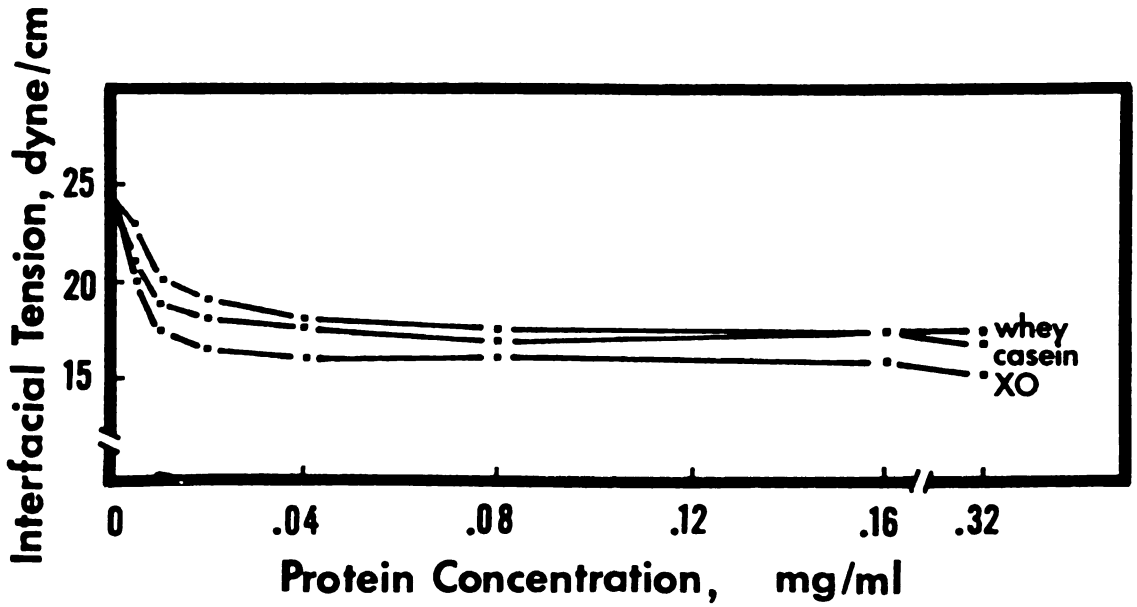

Figure 17

Figure 18. Measurement of the size distribution stability of fat droplets in protein-butter oil emulsions dispersed in simulated milk ultrafiltrate at 45 C. Proteins employed were: casein, whey proteins, purified XO and a mixture of each in a ratio of 80:19.9:0.1, repectively. SMU indicates the emulsion formed with butter oil and simulated milk ultrafiltrate only.

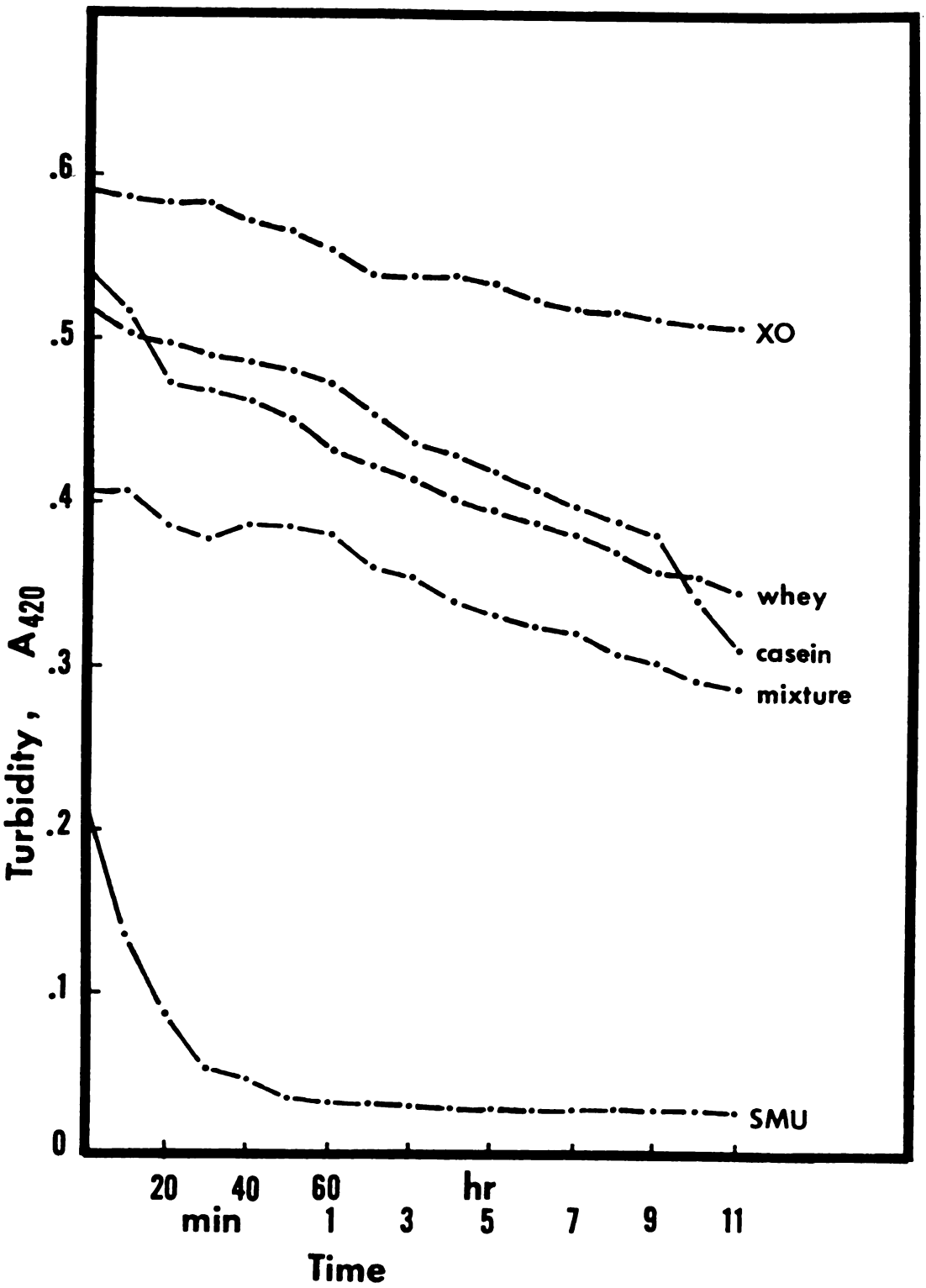
.6
.5
.4
.3
.2
.1
0
Turbidity, A_{420}
XO
whey
casein
mixture
SMU
20
40
60
hr
min
1
3
5
7
9
11
Time

Figure 18

who found that bovine serum albumin, alpha-lactalbumin, and beta-lactoglobulin showed poor interfacial activities in a butter oil-protein-free plasma system. The experimental data indicate that XO is the most interfacially active of the three proteins studied and that the amount of XO adsorbed on the butter oil surface exceeded that of casein and whey proteins.

The surface active properties of XO as well as its hydrophobic nature indicate that XO is more lipophilic than most of the other proteins in milk. This property of XO may explain why XO is concentrated in cream phase. Also, it has been postulated that XO may involve the stabilization of milk fat globules (Mangino and Brunner, 1977a).

Walstra (1965) introduced a turbidimetric technique to determine the size distribution of fat globule in milk. He suggested that the change in turbidity could be used to monitor small changes in fat globule size and the stability of fat emulsion. Based on his theory, turbidimetry was adopted to examine XO-stabilized fat globules.

Figure 18 represents the results of studies with emulsions formed by homogenizing XO, casein and whey proteins with butter oil at 45 C. A decrease in absorbance of less than 0.1 units at 420 nm was noted for the XO-stabilized emulsion after 11 hr of storage at 45 C. The casein-emulsion showed a slight decrease in A after the first hour following homogenization which was followed by a drastic decrease after further aging. The whey protein-emulsion showed a large decrease of absorbance after 11 hr. These results indicate that the size of fat globules in the XO-emulsion was more stable than that in casein- and whey protein-emulsion. Although only a slight decrease in the

absorbance of the casein emulsion occurred during first hour, subsequent decreases in absorbance reflect the coalescence of small fat globules to form large globules. In fact, an oil layer was observed on the surface of casein- and whey protein-emulsion after 6 hr, whereas no oil film was found on the XO sample. Obviously, the stabilization of fat globules by XO is more effective than that by casein and whey proteins.

The above evidence not only supports the hypothesis of stabilzation of milk fat globules by XO but also indicates that XO could be one of the first proteins to associate with naked, intracellular fat globules. Thus, the following hypothesis is suggested. Nascent fat globules in secretory cells are coated with XO (may be coated concomitantly with a phospholipid/cholesterol film, Hood and Patton, 1973) since XO is distributed throughout the cytoplasm of mammary epithelial cells which line the alveolar lumina (Jarasch et al., 1981). This coating prevents the coalesence of fat globules as they migrate from the basal to the apical region of secretory cells. Also, this XO-coat may prevent lipid oxidation in fat globules. After the XO-coated fat globules reach the inner surface of the apical cell membrane, butyrophilin and a layer of plasma membrane are added to the globules during exocytosis. Therefore, milk fat globules are surrounded by an outer layer of MFGM and an inner coat of XO. Evidence of two layers, an outside true membrane layer and a firmly bound inner layer enriched in XO, surrounding milk fat globules has been reported by several researchers (Freudenstein et al., 1979; Franke et al., 1981; Buchheim, 1982). Recently, Phipps and Temple (1982) reported that the inner coat possesses overall high interfacial

activity in milk system. Also, Keenan et al. (1982) found that fatty acids in milk lipid, such as palmitic, stearic and oleic acids, could bind XO in alkali-labile ester linkages. They did not indicated whether active XO was similarly involved in lipid binding since the enzyme was isolated from SDS-gels of MFGM and was enzymically inactive. However, their results suggested that XO possessed a strong affinity to lipid which supports the above hypothesis of XO layered on the naked fat globules. Their results and observations from this study suggest that the XO coating on nascent fat globules involves hydrophobic binding and possible alkali-labile linkages.

Since XO functions as a redox reaction, the prevention of lipid oxidation in XO-coated fat globules is disputable. However, Bruder et al. (1982) found that no significant increase in lipid peroxidation was detected by adding hypoxanthine to MFGM preparations, supernatant fractions from butter milk and tissues fractions containing native active XO including a specimen from the mammary gland. Malondialdehyde production was not measurable with NADH or NADPH as substrate unless EDTA, ADP and ferric ions were added to the lipid peroxidation assay. In the latter case promotion of lipid peroxidation was inhibited by cyanide or peroxide dismutase. They concluded that lipid peroxidation was not a biological function of XO which is in contrast to the hypoxanthine-XO system in vitro. The cell must provide an additional mechanism for preventing the tendency of XO to promote lipid oxidation to ensure the secretion of undamaged lipid components. XO may stabilize fat globules in the milk system since milk lipolysis concides with a release of membrane-bound XO into skim milk (Bhavadasan et al., 1982).

The release of XO from the fat globule complex during homogenization of milk and the stability of homogenized fat globules in homogenized milk seem to conflict with the proposed working hypothesis. It is known that the layers surrounding fat globules are disrupted and replaced partially with casein and whey proteins during homogenization. Whey proteins and casein were found to be less effective in stabilizing butter oil emulsions than was XO, see Figure 18. In fact, the stability of the fat globule size distribution in an emulsion formed by homogenizing milk fat with a protein mixture of casein, whey proteins and XO (with ratio of 80:19.9:0.1) was superior to that for either casein- or whey protein-fat emulsion (see Figure 18, curve of mixture). Decrease in the absorbance reading for the mixture-emulsion sample was only 0.12 after standing for 11 hr at 45 C which was close to that observed for the XO-emulsion. Thus, although homogenization result in a change of components surrounding milk fat globules, the newly adsorbed casein and whey proteins and the remained coat material including XO serve to stabilize the fat dispersion. Recently, Keenan et al. (1983) reported that most of the original MFGM materials, especially the inner coat which contains abundant XO and butyrophilin, were retained on the fat globule surfaces after homogenization. This evidence not only indicates the strong affinity between inner coat and fat globules but supports the hypothesis that XO plays a prominent role in the stabilization of the milk fat emulsion in normal milk and, possibly, homogenized milk.

CONCLUSIONS

1. The spectral results, specific activity and electrophoretic properties of the final enzyme preparation indicated that the purified XO was close to 100% pure.
2. A reversible oxidase form, possessing the detectable dehydrogenase activity of XO, was isolated without exposure to proteolytic enzymes and reducing agents.
3. The dehydrogenase activity in the enzyme was enhanced by 1% ME or 10 mM DTT but was completely lost after treatment with 6M urea.
4. The enzyme performed as an active monomer in 6M urea condition.
5. Chloroquine is a mixed-type inhibitor of XO.
6. The enzyme preparation contained 14.8% protein nitrogen, no lipid and 82 sulfhydryl groups/mole with 44 of these constituting disulfide bonds.
7. Proteases did not change the oxidase activity of XO.
8. Lysine was determined as the N-terminal amino acid of the intact XO and its trypsin-derived fragments.
9. Amino acid compositions of XO and its trypsin-derived fragments indicated their hydrophobic character.
10. A previous hypothesis that the XO monomer consists of three globular subunits held tightly by hydrophobic binding in addition to peptide bonds was supported.
11. XO is not a source of milk riboflavin.
12. XO is a surface and butter-oil interfacially active protein.
13. Stabilization of fat globules by XO is superior to that by

casein and whey protein.

14. A theory for the stabilization of fat globules by XO during secretion and in milk was advanced.

RECOMMENDATIONS

Questions raised by this study requiring further investigation are:

1. What is the original form of milk XO? Is the enzyme synthesized in mammary gland? If so, is it synthesized as dehydrogenase similar to liver xanthine dehydrogenase? What is the mechanism involving the conversion of this enzyme to the reversible type O form of XO found in this study?

2. What is butyrophilin? Is it possible that butyrophilin is a product of degraded XO/dehydrogenase?

3. What is the physiological evidence to support the theory of stabilization of fat globule by XO in secretory cells?

4. What is the actual binding order of the three globular subunits of XO? Which globular subunit possesses the active site(s)- Mo, FAD, or both?

5. Does the 20,000 (18,000) dalton subunit contain more free sulfhydryl groups than the other two subunits? Do these sulfhydryl groups contribute to the stabilization of dehydrogenase activity of the enzyme as suggested by Coughlan (1980)?

6. If milk XO originates from a dehydrogenase form, is the tertiary structure of this form the same as that of the liver enzyme described by Coughlan (1980), or a three globular type described in this study, or do both forms exist due to the interconvertability of the enzyme?

7. Why does XO activity increase after milk is subjected to environmental stress? Is it possible that XO molecules surrounding fat

globules orient as a polymer which results in a restriction of the enzyme activity?

8. Is it possible that XO reaches the intestinal tract as individual subunits rather than as an intact molecule? If so, is the subunit containing the Mo site still active before and after intestinal absorption? Is it possible that absorbed subunits resemble somewhere in the body and regain complete enzymic characteristics?

9. Is reconstituted XO and/or the active subunit containing the Mo site involved in atherogensis?

10. Is the reaction of XO-antiserum to its globular subunits the same? Answer may provide a tool for investigating question 8 and 9.

APPENDIX

Table A1. Chemicals used in this study and their sources

Chemical	Company
Ammonium persulfate	J. T. Baker Chemical Co.
Potassium ferricyanide	
Selenium dioxide	
Sodium phosphate, tribasic	
Sodium pyrophosphate	
Sodium deoxycholate	Baltimore Biological Laboratory, Inc.
Citrate-HCl buffer, pH 2.2, pH 3.28	Beckman Instruments
Acrylamide	Bio-Rad Laboratories
Ammonium persulfate	
Bisacrylamide	
Glycine	
Hydroxylapatite	
Sodium dodecyl sulfate	
Urea	
Ethyl acetate	Burdick & Jackson Laboratories, Inc.
Photo-flo 200	Eastman Kodak Co.
Riboflavin	
N,N,N -N -Tetramethyl-ethylenediamine	
Benzyl alcohol	Fisher Scientific Co.
Boric acid	
Bromophenol blue	
Phosphoric acid	
Potassium citrate monohydrous	
Sodium citrate-$5H_2O$	
Sodium phosphate, dibasic	
Sodium phosphate, monobasic	
Agarose	FMC Corp.
Folin-Ciocalteu phenol reagent	Harleco
Chymotrypsin	ICN Nutritional Biochemicals
Papain	
Pepsin	
Trypsin	
Xanthine	
Ammonium sulfate	Mallinckrodt
Bartital	
Chloroform	
Copper sulfate	
Formic acid	
Glacial acetic acid	
Hydrochloric acid	
Hydrogen bromic acid	
Hydrogen peroxide	
2-Mercaptoethanol	
Methanol	

Table A1. (continued)

Chemical	Company
Potassium chloride	
Potassium phosphate, dibasic	
Potassium phosphate, monobasic	
Potassium sulfate	
Sodium bicarbonate	
Sodium chloride	
Sodium hydroxide	
Sodium nitrite	
Sodium phosphate, dibasic	
Sodium phosphate, monobasic	
Sucrose	
Sulfuric acid	
Trichloroacetic acid	
Ammonium sulfamate	Matheson, Coleman & Bell (MCB)
Bromocresol green	
Calcium carbonate	
Magnesium carbonate	
Methyl green	
Sodium salicylate	Merck & Co. Inc.
Blue dextran	Pharmacia Fine Chemicals
Low MW protein calibrate kit	
High MW protein calibrate kit	
Sephacryl S-200	
Ninhydrin	Pierce Chemical Co.
Dansyl amino acids	
Dansyl chloride	
Aprotinin	Sigma Chemical Co.
Bovine serum albumin	
Chloroquine	
Coomassie blue R-250	
Dithionitrobenzoic acid	
Ethylenediamine Tetraacetic acid	
Fibrinolysin (Plasmin)	
N-Leucine	
N-1-(naphthyl)ethylenediamine dihydrochloride	
Neotetrazolium chloride	
Nicotinamide adenine dinucleotide	
Pancreatin	
Tryptophan	
Tris (hydroxymethyl)aminomethan (Sigma 7-9)	

Table A2. Equipment routinely used in this study

Equipment	Company
Analytical balance, type 2463	Satorius Balance
Top-loading balance, type K7T	Mattler Instrument Corp.
Carmera, MP-3 Land Camera	Polaroid Corp.
Electrophoresis set	Bio-Rad Laboratories
Preparative refrigerated centrifuge, Model RC2-B, type SS-34 and GSA rotors	Sorvall Instruments
Preparative refrigerated ultracentrifuge, Model L-2-65, type 21, 30 and 65 rotors	Beckman Instruments
Dialyzing tubing	Union Carbide Corp.
Disk milk separator, type LWA 205	Westfalia Separator
Hand-operated homogenizer	C. W. Logeman Co.
Lyophilizer	Laboratory-constructed, Dr. J. R. Brunner
Power supple M158	MRA Corp.
Research pH meter, Model 12	Coring Scientific Instruments
Rotary evaporator	Buchler Instruments
Thermo-lift water bath	Buchler Instruments

Table A3. Total riboflavin content and XO activity in fresh raw milk from a specific cow at different milking dates during 1981

Date	Total riboflavin (ug/ml)	XO activity (IU/ml)
8 - 24	1.713	0.020
9 - 2	0.976	0.022
9 - 14	1.011	0.015
10 - 5	1.448	0.037
11 - 3	0.878	0.010
11 - 9	1.411	0.016
11 - 17	1.127	0.011

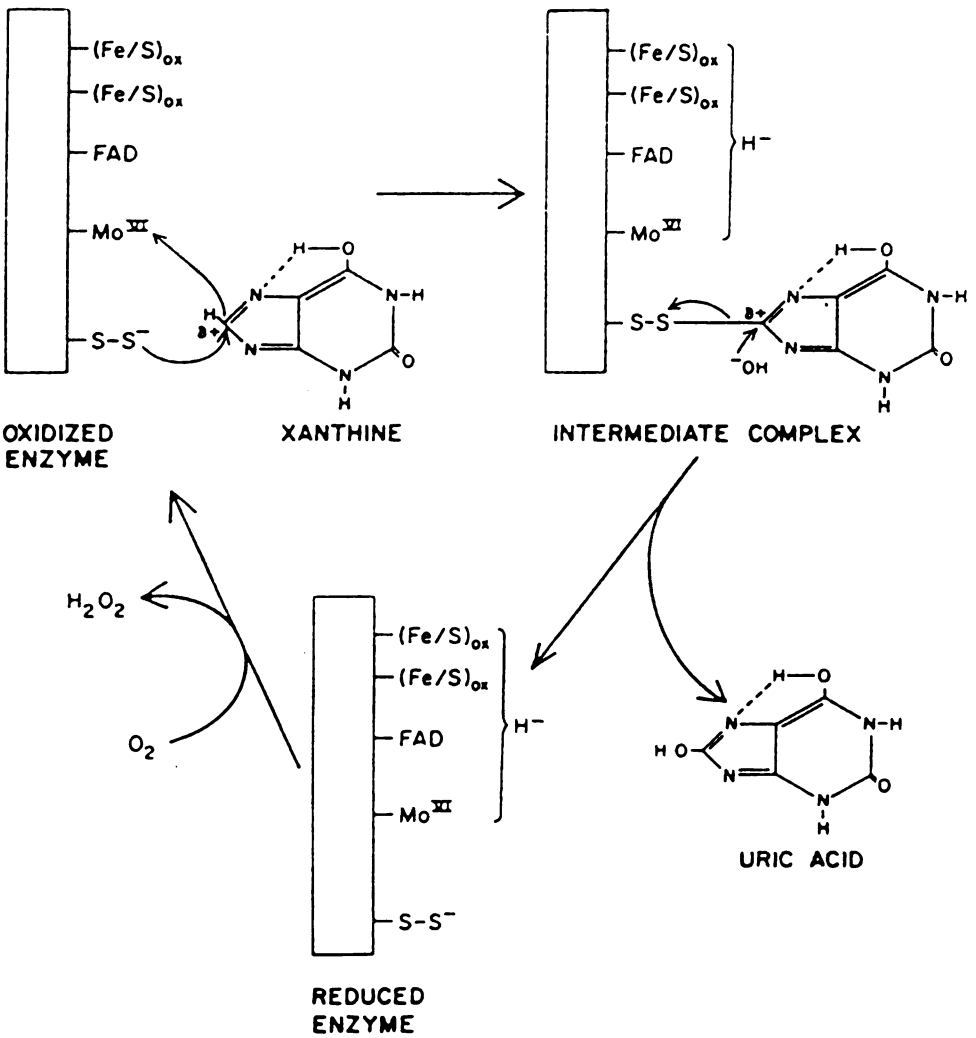

Figure A1. Proposed mechanism for the catalytic role of the persulfide in XO (Edmondson et al., 1972).

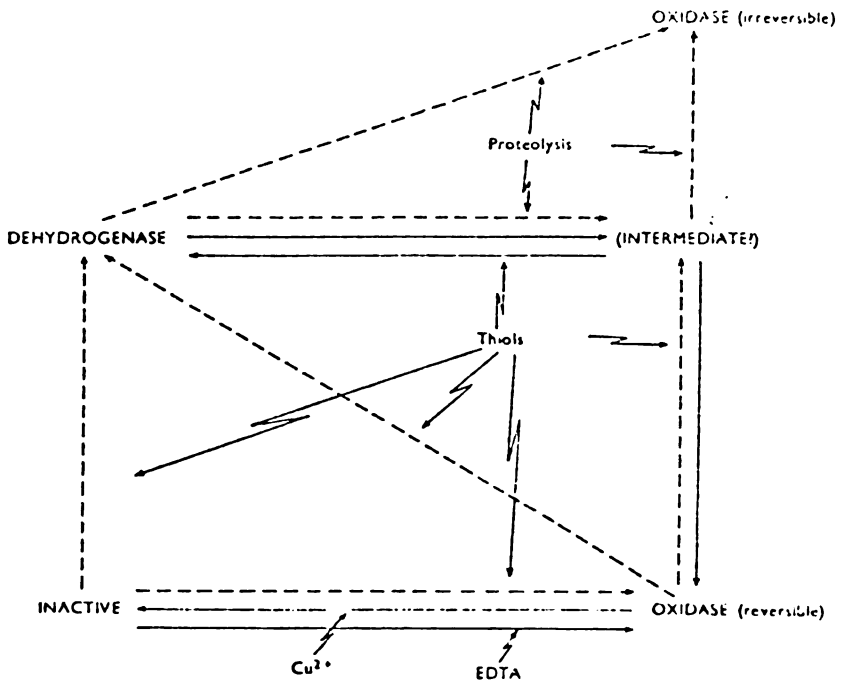

Figure A2. Interconversion of XO among its various possible forms (Della Corte and Stirpe, 1972).

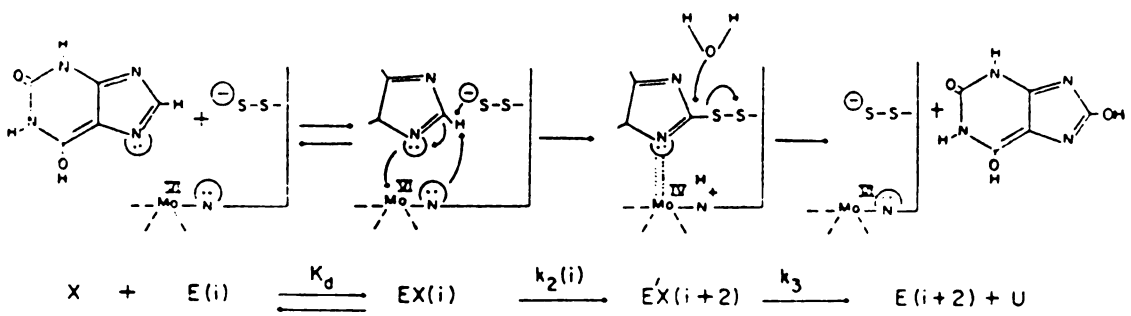

Figure A3. Chemical interpretation of the intermediates of the reaction of XO (Olson et al., 1974).

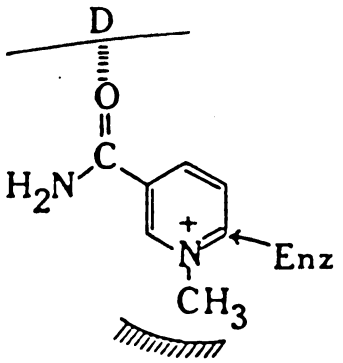

Figure A4. Mode of productive binding to XO od 1-methylnicotinamide cation. D represents the postulated enzymic hydrogen-bond donor (Bunting et al., 1980).

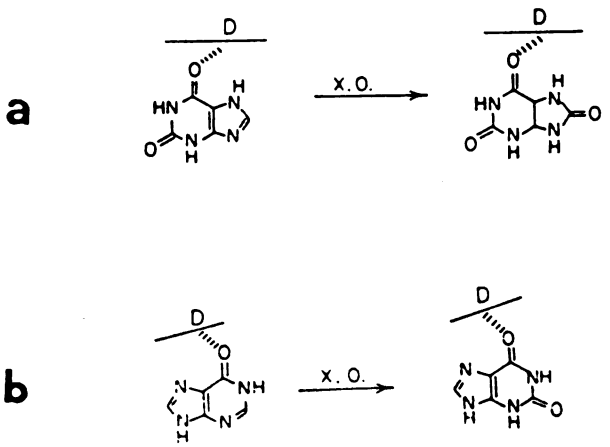

Figure A5. Productive binding of xanthine (a) and hypoxanthine (b) to XO (Bunting and Gunaskara, 1982).

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