CHARACTERIZATION OF XANTHINE OXIDASE IN THE FAT GLOBULE MEMBRANE

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ABSTRACT

CHARACTERIZATION OF XANTHINE OXIDASE

IN THE FAT GLOBULE MEMBRANE

by

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A procedure has been developed that allows for the direct isolation of xanthine oxidase from fat globule membrane material. This method employs deoxycholate as a dissociating agent and obviates the necessity of utilizing proteolytic enzymes.

Kinetic parameters of the enzyme prepared by this procedure are in agreement with published data for xanthine oxidase prepared by methods using proteolytic enzymes. The enzyme was judged to be homogenous on the basis of spectral, kinetic and electrophoretic analysis.

The freshly prepared enzyme yielded one zone on sodium dodecylsulfate containing polyacrylamide gels with a molecular weight of 153,000. Upon storage at 4°C for 30 days the electrophoretic pattern yielded 3 zones. Membrane sterilization of the freshly prepared enzyme did not halt this process and the degree of breakdown could not be correlated with the presence of microorganisms. An endogenous milk protease was suggested as the cause

of the breakdown of xanthine oxidase with time. The proteolytic activity was found to be 37 times more concentrated in membrane enriched fractions than in whole milk. A membrane origin was hypothesized as the source of the milk protease.

Exposure of purified xanthine oxidase to trypsin at 37°C at a weight ratio of 5:1 demonstrated that xanthine oxidase is extremely resistant to generalized tryptic digestion. After one hour of reaction the xanthine oxidase possessed full enzymatic activity while selected cleavage of the molecule was taking place as evidenced by gel electrophoresis. After 24 h digestion the xanthine oxidase still maintained 14% of its original activity. The 153,000 molecular weight species had been completely degraded and the only protein present had a molecular weight of 92,000.

These data suggest an explanation for the wide range of molecular weights reported for homogenous preparations of xanthine oxidase. It appears that various isolation procedures yield molecules that have been proteolyzed to differing degrees but still possess full enzymatic activity.

CHARACTERIZATION OF XANTHINE OXIDASE IN THE FAT GLOBULE MEMBRANE

By

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INTRODUCTION

The fat globules that occur in milk are secreted from the mammary gland by a method described as reverse pinocytosis. At the time of secretion, these fat droplets are surrounded by a membrane layer about 90 A thick.

Recently workers employing the electron microscope, comparative chemical analysis and enzymatic studies have presented strong evidence in support of the theory that the milk fat globule membrane arises from the plasma membrane of the mammary gland. Studies of the milk fat globule membrane should give insight into the composition and structural organization of other, more difficult to isolate, membrane systems.

The composition of the lipid moieties of the milk fat globule membrane has been well elucidated while the protein components have long resisted characterization, partially due to their inherent insolubility. The advent of sodium dodecylsulfate - polyacrylamide gel electrophoresis has partially overcome this problem.

The patterns obtained by such treatment are

difficult to interpret, however, and have been shown to reflect the treatment the membrane material has undergone before analysis. Furthermore, such complex patterns reveal little useful information in relation to the original function of the proteins in the intact plasma membrane.

Membrane proteins may serve as structural components, carrier proteins or as enzymes necessary to the metabolic process of the intact cell. The goal of much of the current research in membrane proteins is to identify the role such proteins play in normal membrane function. Most progress in this area has been in the identification of membrane proteins that contain biological activities (e.g., enzymes).

The enzyme present in the greatest quantity in milk fat globule membranes is xanthine oxidase, which comprises about 10% of the total membrane protein. Even though xanthine oxidase has been isolated by a number of workers and comprises such a large portion of the membrane portein it has not been identified on gel patterns of total membrane proteins. This is due, in part, to the wide range of molecular weights assigned to this protein (75,000 - 270,000). This range may be due to the use of proteolytic enzymes that are often employed in the isolation of xanthine oxidase from whole milk.

This study was undertaken firstly to devise a method that does not employ proteolytic treatment to isolate xanthine oxidase directly from fat globule membranes. Characterization of such "native" xanthine oxidase was to be carried out to compare the effects of isolation procedure on the kinetic, compositional and molecular weight parameters of the enzyme. Lastly, it was the purpose of this study to explain the wide variations in molecular weights assigned to xanthine oxidase and to determine which protein(s) in the sodium dodecylsulfate - polyacrylamide gel electrophoretic patterns of milk fat globule membranes were derived from xanthine oxidase.

REVIEW OF THE LITERATURE

Historical Survey of the Fat Globule Membrane

Ascherson (1840) was the first to report the existence of a thin membrane surrounding the fat globules found in milk. He called this boundary the "haptogenic membrane" and believed that it arose from the condensation of albumin at the fat/plasma interface.

Babcock (1885) detected no change in the appearance of fat globules upon the churning of cream. He also failed to detect any membrane fragments in the serum fraction and therefore concluded that no membrane existed. He later (Babcock, 1889) reported the existence of a protein responsible for the clustering of fat globules during the creaming process. This protein, he felt, could cover the surface of the fat globules and thus serve as a membrane. The creaming of milk was likened to the coagulation of blood and the protein responsible was called lactofibrin.

Storch (1897) realized that the possibility existed for skim proteins to adhere to the fat globules during the separation procedure. He, therefore, devised a method

of washing fat globules to remove any absorbed skim proteins. The washed fat globules were then ether extracted to remove neutral lipids and yielded a mucin-like protein. This protein differed from other milk proteins and gave a positive reaction when tested for carbohydrate.

Hattori (1925) used a system of chloroform and water to wash fat globules and obtain the residual protein. The "haptein" thus isolated differed from other milk proteins in physical and chemical characteristics. Noting the extreme insolubility of the protein fraction in a variety of solvent systems he described it as keratin-like.

Titus (1928) and co-workers isolated fat globules without prior washing of the cream to remove skim proteins. Examination of the residue led them to conclude that the membrane-like material surrounding the fat globules was, in fact, casein.

Palmer (1924, 1933, 1936) and co-workers in a series of experiments were the first to demonstrate the association of phospholipid with membrane protein. Characterization of the protein led them to conclude that it was similar to the globulins and contained no carbohydrate moieties. They theorized that the fat globule membrane was composed of a single globulin-like protein associated with various amounts of phospholipid.

Brunner et al. (1953) demonstrated physical and chemical changes in the protein material of fat globules upon

homogenization. The amino acid composition of unhomogenized membrane protein was different from that on any other milk protein. The isolated membrane material exhibited sedimentation characteristics similar to lactoglobulins in the analytical ultracentrifuge. On the basis of this observation, the protein was provisionally classified as globulin-like.

Herald and Brunner (1957) fractionated membrane material into two components by centrifugation at 25,000 x g in 0.02M sodium chloride for one hour. The pelleted material was insoluble in phosphate and veronal buffers and was termed "insoluble." The mucoidal supernatant was designated the "soluble" fraction. These two fractions differed in amino acid composition and this report represented the first demonstration that the membrane complex contained more than one protein entity. On the basis of amino acid composition and solubility characteristics the insoluble fraction was classified as a pseudokeratin.

In a subsequent report (Brunner and Herald, 1957) the "insoluble" fraction was solublized by treatment with a variety of dissociating agents. Moving boundary electrophoresis of this material showed it to contain more than one protein species.

Thompson and Brunner (1959) substantiated the earlier observation of Storch that membrane proteins were glycoproteins. Thompson (1960) also studied the insoluble membrane proteins and on the basis of low angle x-ray

diffraction data described these proteins as fibrous in nature.

Alexander and Lusena (1961) fractionated membrane material into five pellets and a soluble fraction by treating washed cream that had been stored at -20°C with deoxycholate. They found that the lipid to protein and phospholipid to total lipid ratios were much higher in the supernatant fraction than in any of the pelleted fractions. Physical characterization of the membrane proteins was not attempted because of their insolubility in normal dispersing agents.

Harwalker and Brunner (1965) studied the effects of dissociating agents on membrane fractions and concluded that the insoluble material was a heterogenous mixture of lipoproteins. Analytical ultracentrifugal examination of the solublized membranes yielded a large number of boundaries indicating heterogenity. They concluded that hydrophobic bonding was the principal stabilizing factor in the membrane complex with significant contributions from covalent disulfide bonds.

In an experiment similar to that of Alexander and Lusena, Hayashi and Smith (1965) treated washed cream with 1% deoxycholate. After centrifugation, 45% of the membrane protein and 67% of the phospholipid was found in the supernatant. The pelleted material contained a much higher protein to phospholipid ratio. From these data they hypothesized that the milk fat globule membrane

(MFGM) was composed of an insoluble protein region covered by a more easily dissociated lipoprotein layer.

Chien and Richardson (1967) employed a combination of physical stirring and centrifugation to separate membrane proteins into five fractions. An easily removed layer accounted for approximately one half of the original protein. They envisioned the membrane as having an insoluble inner layer of protein covered by a more readily dispersed region of lipoproteins. Their results were compatible with those of Hayashi and Smith.

Swope and Brunner (1970) used differential sedimentation to separate undissociated membrane proteins into a soluble supernatant containing approximately 6% of the total protein and three pellet fractions. They found that as the density of these fractions increased the protein to lipid ratio increased and the carbohydrate to protein ratio decreased. The most dense fraction contained 89% protein and only 11% lipid. From electron microscopic examination of this fraction combined with the changes in chemical composition of the isolated membrane fractions they hypothesized that the original membrane contained a layer of highly associated protein upon which lipoprotein subunits were absorbed.

Mangino and Brunner (1975) separated membrane proteins into different solubility classes by sequential extraction with 0.6M KCl followed by centrifugation. They reported that the least soluble fractions contained

the lowest amounts of phospholipid and carbohydrate. Examination of the protein moieties of these fractions by gel electrophoresis in a SDS containing system revealed that as phospholipid and carbohydrate were removed from them, the proteins underwent an irreversible aggregation phenomenon. They cautioned against attempts to relate compositional data of membrane fractions to the structure of the original membrane material because of the interactions that can occur at each stage of any isolation procedure.

The Structure of Biological Membranes

Davson and Danielli (1935, 1952) proposed the first widely accepted model describing the organization of membrane proteins and lipids. They envisioned an undetermined number of layers of phospholipids oriented such that their polar heads were aligned towards the aqueous phase and their hydrocarbon tails pointed back towards the center of the membrane. Located between the layers of phospholipid was a layer of neutral "lipoidal" material. Membrane proteins were extended along the polar phospholipid aqueous phase.

Robertson (1959, 1961, 1964) modified this concept by restricting the phospholipids to a bilayer and removing the "lipoidal" material from the center of the membrane and placing it between the neighboring phospholipid

molecules. He further extended the concept to include all cellular membranes. The evidence for this model was based upon the uniformity of membrane structure observed with the electron microscope and on measurements of membrane dimensions obtained from such micrographs.

This concept received support from the reports of Green and co-workers (Criddle et al., 1962; Richardson et al., 1963) of the isolation of "structural" membrane protein subunits, isolated from a variety of membrane types. The subunits, isolated from a variety of membranes, were very similar, if not identical, and were reported to have no enzymatic functions. These were strictly "structural" proteins whose only function was to add strength and stability to the phospholipid bilayers and preserve membrane integrity.

Later reports (Halder et al., 1966; Lenaz et al., 1968; Senior and MacLennon, 1970) however, demonstrated gross heterogenity between membrane protein fractions and cast doubts upon the existence of such universal "structural" proteins.

Further arguments against this model were presented by many authors which were based upon thermodynamic principles (Leonard and Singer, 1966; Wallach and Zahler, 1966; Glaser et al., 1970). Their arguments may be summarized as follows:

1. The attachment of membrane proteins to the polar phospholipid heads must be of an ionic

nature. Such attractions should be readily dissociated by exposure to buffers of high ionic strength. The effects of such buffers on membrane protein phospholipid interactions are minimal.

2. The layer of proteins extended along the outer portion of the bilayer prevents the polar phospholipid heads from obtaining contact with the aqueous phase. The polar interactions between the proteins and phospholipids would not be as energetically favorable as would a direct contact of the phospholipid heads with the bulk aqueous phase.
3. The extended conformation of the proteins does not allow for maximal hydrophobic interactions to occur between their apolar amino acid residues. The exposure of these residues to water is energetically unfavorable.

Further criticisms of this model can be made upon predictions that it makes concerning the nature of the protein-lipid interactions involved.

The Davson-Danielli-Robertson model places an extended layer of proteins over the phospholipid bilayer. Such proteins would be in the extended confirmation and would contain little or no α-helical structure. Circular dichroism studies (Ke, 1965; Maddy and Malcom, 1965; Glaser and Singer, 1971) indicate, however, that membrane

proteins contain from 30-40% α-helical structure and little or no β-structure.

The lipid bilayer model would also severely restrict the motion of proteins within the membrane. The movement of proteins within the bilayer has been demonstrated for a wide variety of cells (Frye and Edidin, 1970; Pinto Da Silva, 1972; Rosenblith et al., 1973). Such protein movements are clearly in contradiction to the model as proposed.

The Davson-Danielli-Robertson model also does not allow for proteins to span the membrane structure. The classic study of Jackson et al. (1972) demonstrated that the major glycoprotein of the red blood cell, glycophorin, completely spans the membrane. Their work clearly demonstrates that NH_2-terminal of glycophorin is located outside of the cell while its C-terminal is intracellular.

In answer to these strong objections to the classic lipid bilayer model, Singer and co-workers (e.g., Leonard and Singer, 1966; Singer and Leonard, 1972) proposed a new model for membrane structure: a phospholipid bilayer as in the Robertson modification of the Davson-Danielli model but allowed for the insertion of proteins into and in some cases through the bilayer. The proteins are free to assume whatever conformation is most energetically favorable within the membrane environment (probably globular) and the polar portions of the phospholipids are free to interact completely with the aqueous phase.

Implicit in this model is the fact that carbohydrate moieties would be attached to portions of the protein or lipid in contact with water.

The fluid mosaic model of membrane structure described above seems to be feasible when tested by thermodynamic considerations but does not completely answer all the criteria by which such a model must be judged. The older model with its "structural" proteins explained the added strength a living membrane possesses over a simple phospholipid bilayer. The simple intercalation of hydrophobic membrane proteins does not a priori predict an increased stability.

MacLennan and co-workers (MacLennan, 1970; Osterald and MacLennan, 1974; Stewart and MacLennan, 1974) isolated a Ca-Mg ATPase from sarcoplasmic reticulum membranes that accounts for over 50% of the membrane protein. When this ATPase was resuspended with membrane lipids and glycolipids, membrane-like residues were formed. These residues show surface topography identical to intact sarcoplasmic reticulum membranes. The addition of a second membrane protein, calsequestrin, to such mixtures added internal structure. These two proteins, both having a clearly defined function, make a large contribution to the integrity of the membrane and can be considered as "structural-functional" proteins.

Weltman and Dowben (1973) proposed a link between functional membrane proteins, ATPases, and a known

structural protein, actin. They compared the amino acid compositions of these proteins by the statistical procedure of Marchalonis and Weltman (1971) and concluded that membrane ATPases and actin were so similar in amino acid composition that they may have descended from a common "ancestral" gene.

What emerges is a model incorporating the advantages of both the previous membrane models. Such a membrane would be composed of a phospholipid bilayer with both proteins and neutral lipids extending into or through the hydrophobic membrane core. Structural integrity would be offered by the incorporation of "actin-like" proteins into the bilayer. These proteins could also extend into the aqueous phase and possess enzymatic activities. Transport and receptor phenomenon would be attributed either to proteins that completely span the membrane or to proteins making contact with the aqueous phase on one side of the membrane that are able to interact with proteins making contact on the other side. The latter case would require protein movement within the membrane and is the mechanism proposed by Cuatrecasas (1973) to explain the increase of adenyl cyclase activity in fat cells after exposure to purified cholera toxin.

Origin of the Fat Globule Membrane

The membrane material that surrounds the fat globules in milk is derived from the apical plasma membrane of the secretory cell through a process known as membrane flow (Keenan, 1972; Brunner, 1975).

Lipid molecules are synthesized in the basal region of the cell where they begin to coalesce into droplets (Stein and Stein, 1967). These lipid droplets migrate towards and reach the apical plasma membrane. Upon contact with the plasma membrane the lipid is "pinched" out of the cell being surrounded in the process by a layer of plasma membrane (Bargmann and Welsch, 1969; Linzella and Peaker, 1971 a; Patten and Fowkes, 1967).

The constant removal of plasma membrane caused by fat secretion is replenished by addition of membrane material derived from the Golgi apparatus (Morre et al., 1971).

Proteins synthesized within the mammary gland are produced on the rough endoplasmic reticulum and packaged into the Golgi apparatus (Helminen and Ericsson, 1968; Willings et al., 1960). The Golgi vacuole then fuses with the plasma membrane and evacuates its contents into the lumen (Morre et al., 1971). Thus the milk fat globule membrane is derived directly from the plasma membrane via membrane material of the Golgi apparatus.

Evidence for the plasma membrane as the source

of MFGM was presented by Dowben et al. (1967). They demonstrated the presence in MFGM of many enzymes known to be present in plasma membrane. Also antibody to MFGM material was shown to hemolyze and agglutinate bovine red blood cells further indicating a relatedness between MFGM and plasma membrane.

Keenan et al. (1970) demonstrated that the phospholipid composition of MFGM and plasma membranes were remarkably similar. In a later study (Keenan and Huang, 1972) the protein and lipid composition of MFGM, rough endoplasmic reticulum, Golgi apparatus and plasma membranes were compared. The phospholipids of MFGM were almost identical to those of the plasma membrane. The distribution in the Golgi was intermediate between the rough endoplasmic reticulum and plasma membranes. Comparisons of the molecular weight profiles of the membrane derived proteins yielded similar results as did amino acid analysis of these proteins.

Three of the enzymes reported to be present in MFGM are of special significance or interest.

Xanthine oxidase found in MFGM presents an enigma. This enzyme is normally considered to be one of a group of enzymes required for purine degradation. Its presence in the mammary gland is difficult to explain. In MFGM it is the most prevalent enzyme and estimates of its abundance range from 8 to 10% of the total membrane protein (Swope and Brunner, 1968; Briley and Eisenthal,

1975). The presence of such large amounts of this seemingly superfluous enzyme could be of structural and/or functional significance.

Brodbeck and Ebner (1966) reported the presence of lactose synthetase, i.e., galactosyl transferase, in MFGM preparations. This enzyme is considered to be specific to the Golgi (Keenan et al., 1970) and its occurrence in MFGM can be taken to represent residual Golgi enzyme activity in plasma membrane. No other Golgi specific enzymes have been detected in MFGM and evidence of recent workers (Roth and White, 1972; Patt and Grimes, 1974; Webb and Roth, 1974) indicates that galactosyl transferase may actually be present in all mammalian plasma membranes. If this is the case, the presence of small quantities of galactosyl transferase in MFGM preparations cannot be used as evidence of a Golgi membrane origin of the plasma membrane and hence the MFGM.

Dowben et al. (1967) reported the presence of both Mg-activated and Na-dependent ATPases in MFGM preparations. The presence of Na-ATPase was considered to be of great significance in that it is a marker enzyme for plasma membranes.

Huang and Keenan (1972) confirmed the presence of Mg-ATPase in MFGM but failed to find any Na-dependent ATPase activity.

The fact that the apical plasma membrane of the mammary gland is freely permeable to Na ions (Linzella

and Peaker, 1971 b) and that Kenura (1969) demonstrated that the apical membrane portion of the secretory cell failed to stain for Na-dependent ATPase activity probably rule out the existence of this enzyme in MFGM.

Baumrucker and Keenan (1975) reported the presence of a Mg-dependent ATPase in Golgi membranes that functioned as a calcium pump much like the ATPase found in sarcoplasmic reticulum membranes. Residual activity was reported for MFGM material but the enzyme was no longer ion-dependent for activity. They, too, failed to find evidence for the existence of a Na-dependent ATPase in MFGM.

These data present a rather unique situation in that the apical portion of the plasma membrane in mammary secretory cells does not contain a typical plasma membrane enzyme, Na-dependent ATPase, but rather has a unique non-ion dependent enzyme.

Xanthine Oxidase

Morton (1953) was the first to demonstrate the association of xanthine oxidase with membrane material. He found the activity of the enzymes was greater in cream than in skim milk. The enzymatic activity that was recovered in the skim milk was found to be associated with lipoprotein particles which he called milk "microsomes."

Zittle et al. (1956) reported the loss of xanthine oxidase in the water washes used to remove residual skim proteins from cream. They found the activity of the enzyme was concentrated in the lipid phase upon separation. The xanthine oxidase found in the skim was not associated with whey proteins or casein. Centrifugation of the cream resulted in the pelleted fraction having a higher specific activity than any other fraction.

Herald and Brunner (1956) reported that centrifugation of purified membrane material resulted in the concentration of xanthine oxidase activity in the insoluble fraction.

Alexander and Lusena (1961) treated pelleted membranes obtained from frozen washed cream with 2% deoxycholate. The membranes were then centrifugally fractionated into five pellets and a supernatant fraction. Centrifugation occurred immediately after the addition of the bile salt. They reported this treatment solublized 24.6% of the xanthine oxidase present, while over 60% of the enzyme was recovered in the first two pellet fractions.

Hayashi et al. (1965) in a similar experiment used washed cream as a starting material. After the cream was made to 1.0% with regard to deoxycholate they allowed an incubation period of 1 h before centrifugation. Under these conditions 81.0% of the total xanthine oxidase activity was recovered in the supernatant fraction. Differences in the source of membrane material used and

the 1 h incubation after the addition of the bile salt probably account for the differences in enzyme distribution reported by these workers.

Xanthine oxidase has been isolated in the crystalline form from milk (Avis et al., 1955). The isolation procedure included a 3.5 h digestion with pancreatin. Modifications of this procedure to increase yields have also employed this digestion step (Gilbert and Bergel, 1964; Massey et al., 1968).

A method for isolating xanthine oxidase without exposure to proteolytic enzymes has also been reported (Mackler, 1951). This procedure allows only limited recovery of the enzyme - 2% - but reaction parameters have been reported to be identical for the enzyme purified by either procedure (Hart et al., 1970).

Carey et al. (1961) compared xanthine oxidase prepared by the two methods and found the pancreatin treated preparation to be heterogeneous in its elution profile from hydroxyapetite columns. While proteins were eluted at different concentrations of phosphate ion, each fraction collected had the same specific activity. Enzyme prepared without exposure to pancreatin was found to be homogeneous under the same conditions. Exposure of the homogeneous enzyme to pancreatin or prolonged storage at $4^{o}C$ converted it into a heterogeneous mixture of proteins identical to the enzyme isolated by the pancreatin procedure. They concluded that exposure of the

enzyme to proteolytic enzymes cleaved portions of the enzyme that were not essential for full xanthine oxidase activity but the loss of which altered its elution profile on hydroxyapetite columns. They also suggested that the changes occurring upon storage of the enzyme at $4^{o}C$ might be due to proteolytic enzymes endogenous to the milk system.

Andrews et al. (1964) determined the molecular weight of xanthine oxidase isolated by the proteolytic procedure using both gel filtration and analytical ultracentrifugation. They obtained a value of 278,000 by both procedures and concluded that this was a monomer molecular weight despite the fact that an enzyme with this molecular weight would contain two molecules each of flavin and molybdenum. In a second report Hart et al. (1969) concluded that the molecular weights of the enzyme prepared with and without exposure to pancreatin were essentially similar.

Nelson and Handler (1968) found the weight of the non-pancreatin exposed enzyme to be 304,000 by analytical ultracentrifugation. They reported a 10% reduction in weight for the pancreatin derived enzyme, i.e., 274,000. Exposure of xanthine oxidase to .02M HCl, .1M NaOH or 7M guanidine at pH 3 reduced the molecular weight to 150,000 which was considered to be the monomeric species. Treatment with 4M guanidine and .1M mercaptoethanol yielded a variety of molecular weights ranging from 92,000 to 130,000. They also reported the appearance

in many of the preparations exposed to 7M guanidine of a prominent species having a molecular weight of 100,000. In an attempt to determine a minimum molecular weight, peptide maps were obtained after extensive digestion of the enzyme with trypsin. A theoretical maximum of 310 peptides would be expected if the molecular weight of a monomer was 304,000. Results of the mapping yielded between 70 and 75 ninhydrin positive spots, supporting the existence of four identical subunits. The authors acknowledged the difficulties involved in obtaining complete separation of such a large number of peptides. The results of the peptide mapping experiment were thus considered to be inconclusive.

Nathans and Hade (1975) reported a comparison of the physical properties of xanthine oxidase prepared by using Triton X-100 with and without proteolytic treatment. They determined the molecular weight of the enzyme isolate, reported to be of high specific activity, by employing SDS-disc gel electrophoresis. The Triton X-100 derived preparation yielded five major and three minor zones upon electrophoresis. Molecular weights were reported only for the three zones of highest molecular weight. These three polypeptides accounted for approximately 50% of the total protein as estimated by visual examination of the staining intensity. The weights reported were 155,000, 125,000 and 85,000. Enzyme prepared by the pancreatin procedure yielded none of these high molecular weight

proteins. They concluded that the proteolytic enzyme complement of pancreatin was being co-purified with the xanthine oxidase when this procedure was employed. They also stated that dialysis of these samples in preparation for electrophoresis (length of time not reported) at room temperature allowed for the proteolytic enzymes present to degrade the xanthine oxidase. The Triton X-100 preparation was reported to be free of contamination by proteolytic enzymes.

Nagler and Vartanyan (1973) reported that isolated xanthine oxidase treated with SDS and mercaptoethanol yielded two zones upon gel electrophoresis with molecular weights of 150,000 and 135,000. The 150,000 species comprised most of the preparation. Longer exposure to SDS and mercaptoethanol at 37°C resulted in the appearance of zones having molecular weights of 100,000, 70,000, 42,000, 28,000 and 15,000. They concluded that exposure to heat and the denaturants employed was causing a stepwise breakdown of xanthine oxidase into its native subunit structure. A complex scheme was proposed to account for the appearance of all of the low molecular weight species as being derived from larger polypeptides. It is of interest to note that one or more of the higher molecular weight species reported, i.e., 150,000, 135,000 and 100,000 are present in the preparations of Andrews et al. (1964), Nelson and Handler (1968) and Nathans and Hade (1975).

The data concerning the heterogeneity of xanthine

oxidase prepared by the proteolytic method and the multitude of molecular weights assigned to the enzyme prepared by other procedures present a somewhat confusing picture. Elucidation of the molecular weight of the native monomeric species and definition of the mechanism by which molecules of smaller molecular weights would be a valuable contribution to the study of this membrane protein.

EXPERIMENTAL

Preparative Procedures

Preparation of Membrane Material

Fresh, warm milk was obtained from the Michigan State University Holstein dairy herd and separated within one hour at 37°C. The separated cream was diluted with four volumes of distilled water at 37°C and reseparated. This "washing" procedure was repeated three times to ensure adequate removal of skim milk proteins (Swope, 1968). The washed cream was chilled to 4°C in an ice bath and churned in Erlenmeyer flasks on a rotary shaker at room temperature. After the fat emulsion was broken, the aqueous phase was filtered through four layers of cheese cloth. Unchurned butter granules were removed by centrifugation at 1000 x g for 30 min. The resulting aqueous suspension served as membrane material for all subsequent fractionation procedures.

Total Membrane Proteins

Total membrane proteins were prepared from the original membrane material by the procedure of Herald and Brunner (1957). Membrane material was concentrated

in 50% saturated ammonium sulfate, followed by an alcohol-ethyl ether wash and a series of ether washes to remove residual lipoidal material. The proteinaceous fraction was lyophilized subsequent to chemical analysis.

Isolation of Xanthine Oxidase

The original membrane material was centrifuged to yield a 100 S pellet which was transferred to four volumes of buffer solution containing .12M NaCl, .05M phosphate at pH 7.4 containing 1mM sodium salicylate. The mixture was comminuted for 15 sec in a Waring blender and centrifuged for 40 min at 44,000 x g (max). A 10% solution of sodium deoxycholate (DOC) was added dropwise to the supernatant to yield a final concentration of .1 mg DOC/mg protein. Sodium salicylate was added to bring the total salicylate concentration to 5 mM and the solution was allowed to incubate at room temperature for 10 min. The mixture was then centrifuged for 50 min at 105,000 x g (max). The clear supernatant was carefully removed, avoiding the loose layer on top of the pellet, and 50 mg of calcium phosphate gel was added. The mixture was allowed to stand at room temperature for 10 min and centrifuged for 10 min at 1000 x g. The pelleted gel was resuspended in 100 ml of .1M phosphate buffer at pH 6.8 containing 5mM salicylate and recentrifuged. The enzyme was released from the pelleted gel by mixture with the same buffer containing 5% w/v $(NH_4)_2SO_4$ and the gel removed by

centrifugation. The enzyme containing supernatant was then dialyzed against one of the following solutions:

1. .1 M tris-HCl, pH 8.3 containing 5mM salicylate (enzymatic analysis)
2. .1 M tris-HCl, pH 8.3 without salicylate (spectral analysis)
3. Deionized-distilled water (amino acid analysis)

Preparation of Deflavo Xanthine Oxidase

The flavin moiety of xanthine oxidase was removed by the method of Komai et al. (1967). This method consisted of adding 3 volumes of 3.4 M $CaCl_2$ to 2 volumes of xanthine oxidase solution and allowing the mixture to stand at room temperature for 90 min. The mixture was then dialyzed against several changes of .1 M tris-HCl, pH 8.3 until the dialyzate showed no fluorescence.

Enrichment of Alkaline Phosphatase

Original membrane material was centrifuged to a 100 S pellet which was resuspended by stirring overnight at 4°C in 10 volumes of .6 M KCl. The suspension was centrifuged for 1 h at 44,000 x g (max) and the supernatant was dialyzed overnight against several changes of distilled water. The dialyzed material was recentrifuged at 44,000 x g (max) for 1 h and ice cold acetone was added to a final concentration of 45%. The acetone precipitate was resuspended in .1 M tris-HCl, pH 8.3 in a volume equal to the volume before the acetone precipitation. One half

volume of cold butanol was added with constant stirring and the mixture was centrifuged at 1000 x g for 30 min. The lower aqueous layer was carefully removed with a syringe and cold acetone added to yield a final concentration of 45%. The resulting precipitate was collected by centrifugation at 1000 x g for 10 min and resuspended in a minimal amount of tris-HCl, pH 8.3 containing .1% DOC. The suspension was dialyzed against several changes of the same buffer at 4°C.

Protease Enrichment Fractions

Portions of whole milk, skim milk, washed cream and buttermilk were saved from a preparation of aqueous membrane material. The original membrane material was centrifuged for 2 h at 44,000 x g (max) and the supernatant and pellet fractions were collected. All of these fractions were assayed for proteolytic activity.

Microbiological Procedures

Serial dilutions of membrane fractions were spread on plate count agar and incubated at either 7 or 37°C. Colonies on each plate were counted after 3, 7, and 10 days.

Samples of purified xanthine oxidase were also passed through sterile membrane filters having a mean pore size of .45 m into sterile containers and samples were removed daily under aseptic conditions to assay for proteolytic breakdown. A portion of each sample removed

was examined for microbial growth.

Enzymatic Assays

Xanthine Oxidase

The method of Avis et al. (1955) was used to monitor the conversion of xanthine to uric acid by the increased absorbance of uric acid at 295 nm. The concentration of uric acid formed was determined by using the extinction coefficient difference between xanthine and uric acid, which, according to Kackler (1947), was 9.6×10^3 $cm^{-1}M^{-1}$. Enzymatic activity was reported as moles substrate converted/mg protein/min at 23.5°C and pH 8.3 in .1M tris-HCl. The pH optimum of the enzyme was determined from the initial velocity of the reaction when .1M tris-HCl or .1M tris-acetate buffers at various pH's were substituted for the pH 8.3 buffer.

Energy of activation of the enzyme was determined at temperatures ranging from 15 to 40°C for 5 min. The activities were determined during the first minute of the reaction to minimize the effects of temperature changes during the assay procedure.

To determine values for K_m and V_{max} the concentration of xanthine was varied from 5 to 100 mM and the initial velocity of the reaction at each concentration of substrate was recorded. The data were treated graphically by the procedure of Lineweaver and Burke (1934) and the statistical

procedure of Wilkinson (1961).

The inhibitory effects of ATP on the xanthine oxidase were determined by repeating the K_m and V_{max} analysis in the presence of ATP. Four concentrations of ATP were employed and the data were treated graphically by the Lineweaver and Burke procedure; K_i was also calculated by the method of Whitaker (1972).

Alkaline Phosphatase

The conversion of p-nitrophenol phosphate to p-nitrophenol by alkaline phosphatase was monitored by the absorbance of liberated p-nitrophenol at 420 nm. The reaction mixture contained 3.0 ml of .2M tris-HCl at pH 9.8 and 3.0 ml of 2.5×10^{-3}M p-nitrophenol phosphate. The reaction was initiated by the addition of .1 ml of enzyme solution. All reactants were incubated at 37°C for 3 min prior to mixing. Assays were performed at 37°C. Units of activity were expressed as moles p-nitrophenol released/mg protein/min.

ATPase

The substrate medium contained .1M KCl, .05 M tris-HCl, pH 8.3, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and 5 mM ATP (disodium salt). To 1 ml of reaction mixture .1 ml of enzyme solution was added and the reaction was allowed to procede at 37°C. At appropriate times (usually 5 min) the reaction was quenched by the addition of 1 ml of cold 5% trichloroacetic acid (TCA). Phosphorus liberated by the reaction

was measured in TCA supernatants by the method of Sumner (1944) which employs freshly prepared ferrous sulfate as the reducing agent.

Values of K_m and V_{max} were determined for the reaction as they were for the xanthine oxidase reaction employing varying concentrations of ATP as substrate.

The effect of xanthine on the release of PO_4 from ATP was determined by including various amounts of xanthine in the reaction mixture. Reagent blanks consisting of enzyme alone, an enzyme-free reaction mixture and heat denatured enzyme plus the reaction mixture showed no color development.

The release of phosphorus from ATP resulting from H_2O_2 was assessed by adding various amounts of H_2O_2 to a mixture of the heat-denatured enzyme plus the reaction mixture blank described above. The mixtures and blanks (no H_2O_2) were placed in a boiling water bath for 10 min to decompose residual H_2O_2 before determining the release of phosphorus.

Protease Activity

Proteolytic activity of membrane fractions was determined by using the "universal" substrate Azocol (Knufermann et al., 1973). Twenty-five milligrams of Azocol were added to 3 ml of .1M tris-HCl at pH 8.3 which contained .5% merthiolate as a preservative. One milliliter of the membrane fraction was added and the mixture incubated

at 37°C. Aliquots (1 ml) were removed at intervals ranging from 3 to 24 h and mixed with 2 ml of 10% TCA. These samples were centrifuged at 1000 x g for 10 min and the absorbance of the supernatant at 520 nm was recorded. Units of activity were expressed as .01 absorbance units at 520 nm/mg protein/h.

Trypsin Treatment of Xanthine Oxidase

Trypsin, 2 mg/ml in .1M tris-HCl, pH 8.3 containing 5 mM $CaCl_2$ was added to xanthine oxidase at a ratio of 1:5, respectively. Aliquots were removed at various time intervals (5 min to 5 h) and immediately assayed for xanthine oxidase activity. Other aliquots were mixed with 1% SDS and 1% mercaptoethanol and placed in a boiling water bath for 15 min to stop proteolytic activity. The SDS-treated samples were electrophoresed in SDS-PAG gels.

Chemical Analysis

Amino Acids

Amino acid analysis of membrane proteins were performed on a Beckman/Spinco 120°C amino acid analyzer according to the method of Moore et al. (1957), employing 22 and 72 h acid hydrolysis. Sulfur-containing residues were determined on specimens previously oxidized with performic acid as described by Hirs (1967). Tryptophan was determined on pronase digested proteins by method of Spies and Chambers (1949). Disulfide and free SH

determinations were made on isolated membrane proteins by the DTNB procedure of Ellman (1959) as modified by Beveridge et al. (1974).

Total phospholipid content was estimated from a determination of phospholipid phosphorus according to the method of Ames (1969), utilizing ascorbic acid as the reducing agent. A factor of 25 was used to convert phosphorus to phospholipid.

Total hexose was determined by the phenol-H_2SO_4 method of DuBois et al. (1956) with an equimolar mixture of glucose and galactose as the reference mixture.

Sialic acid was determined by the thiobarbituric acid method of Marier et al. (1963) with N-acetylneuraminic acid as the reference standard.

Hexosamine was estimated by the method of Johansen et al. (1960) with Ehrlich's reagent as a receiver for the steam distilled chromogen. An equimolar mixture of galactosamine and glucosamine served as a reference mixture for this procedure.

Protein was determined in aqueous solutions by measuring absorbance of the biuret formed by the complex of peptide bonds with copper at an alkaline pH (Layne, 1957). For dried samples, a micro-Kjeldahl determination was performed (i.e., %N x 6.25) as described by Swaisgood (1964).

Statistical Comparisons

The SΔQ Index method of Marchalonis and Weltman (1971) was employed to estimate the relatedness of various proteins and is derived by comparing the amino acid composition (Mole %) of any two proteins. The concentration difference for each amino acid residue is squared and the individual values summed, yielding the SΔQ Index, i.e.:

$$S\Delta Q = (X_{ij} - X_{kj})^2$$

where:

i = protein 1

k = protein 2

X_j = content of a given amino acid of type j

Sixteen amino acids were used in these calculations.

H ∅ Ave

The average hydrophobicity of various proteins was determined by the method of Bigelow (1967). The mole % of each amino acid residue was multiplied by the free energy required for its transfer from an organic to an aqueous environment. The sum of these products divided by 100 is equated to the average hydrophobicity (H ∅ Ave) of the protein.

RESULTS

Isolation of Xanthine Oxidase

Table I presents the recovery and purification data for the isolation of xanthine oxidase from whole milk. A total of 75 mg of enzyme was recovered from 5 gal of milk representing a 374-fold purification of the enzyme. Recovery of xanthine oxidase from washed-cream buttermilk amounted to 12.4%. The purity of the preparation is demonstrated in Figure 1 which shows one zone in SDS-PAG. The molecular weight of the enzyme was estimated to be 153,000 by comparing its relative mobility to those for proteins of known molecular weights. Table II presents data related to properties of the purified enzyme. The A_{280}/A_{450} ratio of 5.05 and the specific activity of 3.55 mole/mg/min agree favorably with results of 5.0 to 5.1 for A_{280}/A_{450} and 3.5 - 3.6 mole/mg/min for the specific activity obtained by Hart et al. (1970) and Massey et al. (1969).

Amino Acid Composition of Xanthine Oxidase

The amino acid composition of xanthine oxidase is presented in Table III. The data represent the average

of duplicate 22 h acid hydrolyzates; sulfur-containing residues were determined after performic acid oxidation. The low value of tryptophan (by the method of Spies and Chambers, 1949) is in good agreement with the data of Bray and Malmstrom (1964) and of Nelson and Handler (1968) and seems to be characteristic of the enzyme. Statistical comparisons ($S\Delta Q$) of the amino acid composition of xanthine oxidase with various milk and muscle proteins are presented in Table IV. Values of less than 50 by this procedure are suggestive of homology of primary sequence (Marchalonis and Weltman, 1971). The values of 17 and 15 obtained when xanthine oxidase was compared to sarcoplasmic reticulum-derived ATPase and actin, respectively, are significant. The highly conserved molecule, cytochrome C, gave an average value of 20 when the compositions of the molecule obtained from a variety of sources were compared. This observation together with the knowledge of the primary sequences of these molecules led Marchalonis and Weltman (1971) to conclude that values of less than 20 for any two proteins indicated a high degree of structural homology. By this criterion and according to the considerations of Weltman and Dowben (1973), xanthine oxidase should be considered closely related to actin. Comparisons of a larger number of contractile and membrane-associated proteins are presented in Table V. Examination of these data indicates that a large number of membrane and contractile proteins can be considered "actin-like."

Table III presents the amino acid composition of xanthine oxidase determined in this study and compared to compositions reported by Nelson and Handler (1968) and by Bray and Malmstrom (1964). The calculated values of $S\Delta Q$ are presented at the bottom of the table (1.89 and 2.42) and demonstrate excellent agreement between analyses, since a value of 4 was considered by Weltman and Dowben (1973) as the limit of analytical precision between different laboratories.

The average hydrophobicities of various proteins listed in Table V are presented in Table VI. All values were compared to xanthine oxidase and the percentage differences listed. β-lactoglobulin and β-casein, representing typical milk proteins, were included for comparison. Eight of the ten membrane-associated proteins differed by less than 4% from xanthine oxidase, 5 of which differed by less than 2%. By comparison β-lactoglobulin differed by 8.8% and β-casein by 22%. These data indicate a relatedness between membrane and contractile proteins.

Composition of Total Membrane Proteins

The composition of total MFGM proteins prepared by the method of Herald and Brunner (1957) is given in Table VII. Proteins prepared by this procedure contained 79.4% amino acids, 10.2% residual phospholipid and 6.7% carbohydrate. The total membrane material contained .74 g

free -SH and 1.28 g S-S per 100 g protein, giving a total of 2.02 g -SH/100 g protein compared to the 1.97 g -SH/100 g protein obtained from cysteic analysis of oxidized membrane proteins. SΔQ values derived for MFGM proteins from two different species and for three other types of plasma membranes are compared in Table VIII. The magnitude of these values suggest that these plasma membranes are composed of proteins with very similar amino acid compositions.

Enzymatic Properties of Xanthine Oxidase

When isolated xanthine oxidase preparations were assayed for ATPase activity, positive results were observed. Figure 2 illustrates the release of phosphorus from ATP when ATP and xanthine oxidase were mixed. This observation, coupled with the "actin-like" composition of the protein resulted in its mistaken identity as a membrane derived ATPase during the early phase of this study. Thus, the discovery of high xanthine oxidase activity associated with the isolate required a reevaluation of the ATPase reaction. Figure 3 shows the increase in phosphorus released from ATP by the addition of increasing amounts of xanthine to the ATPase reaction mixture. These data suggested that a product of the xanthine oxidase reaction might be responsible for the hydrolysis of ATP.

The data presented in Figure 4 illustrate that the

direct addition of hydrogen peroxide to the reaction mixture results in the liberation of PO_4 from ATP. Figure 5 represents a double reciprocal plot of the initial velocity of PO_4 release versus ATP concentration. The apparent K_m obtained by treatment of these data by the statistical linear regression procedure of Wilkinson (1961) was 1.31 $\pm$ .09 x 10^{-4}M. Figure 6 represents a double reciprocal plot demonstrating the effect of added ATP on the values of K_m and V_{max} for xanthine oxidase. Analysis of the data by the method of Whitaker (1974) gave a K_i of 1.01 x 10^{-3}M, indicating that ATP was a competitive inhibitor for xanthine oxidase. Figure 7 represents a double reciprocal plot of initial velocity versus xanthine concentration. Treatment of these data by the Wilkinson (1961) procedure yielded a K_m of 1.29 $\pm$ .08 x 10^{-5}M and V_{max} of 3.62 $\pm$ .07 µmole/mg/min.

The activity of xanthine oxidase over a range of pH values is presented in Figure 8. A pH optimum of 8.3 was obtained. Analysis of the effects of temperature upon the reaction are shown in Figure 9 where the natural log of the initial velocity was plotted against the reciprocal of the absolute temperature. The energy of activation of the reaction yielded a value of 14.1 k cal/mole.

Data illustrating the loss of apparent ATPase activity paralleling the formation of deflavo xanthine oxidase for three membrane fractions are presented in Table IX. In each case a fraction exhibiting ATPase activity completely lost its activity when the flavin moiety was

released from xanthine oxidase.

Breakdown of Xanthine Oxidase

Electropherograms of freshly prepared xanthine oxidase (A), the preparation stored at 4°C for 30 days (B), and a membrane fraction, containing 7 major MFGM proteins (C) as described by Anderson *et al.* (1974) are shown in Figure 1. The original 153,000 dalton component (gel A) yielded three components with weights of 90,000, 42,000 and 24,000 (gel B). The original molecule and the three storage-derived peptides correspond to 4 of the 7 major peptides of MFGM preparations. The specimen held at 4°C for 30 days had a total plate count of < 30. The effects of storing a fresh preparation of xanthine oxidase (sterilized by membrane filtration) at room temperature for 24 h is shown in Figure 10. The 153,000 molecular weight species were almost completely degraded and was replaced by a new component with a weight of 138,000 which is one of the 7 major proteins observed in electropherograms of MFGM.

Figure 11 demonstrates that antibody to purified xanthine oxidase gave a precipitin zone characteristic of antigen homology in double diffusion experiments utilizing pure xanthine oxidase and the preparation yielding 3 zones as antigens (Crowle, 1961).

The Effects of Trypsin on Xanthine Oxidase

The specific activity of xanthine oxidase exposed to trypsin at a weight ratio of 5:1 at 37°C for times ranging from 5 to 50 min is illustrated in Figure 12. Within the limits of experimental error, the specific activity of the enzyme remained unchanged during the course of the experiment. Electropherograms obtained for samples removed at 5, 10 and 50 min and placed in a boiling solution of 1% SDS containing 1% mercaptoethanol to inactivate the trypsin are shown in Figure 13. The 153,000 dalton species was cleaved into a number of smaller subunits. The 92,000 subunit seemed resistant to further breakdown. Figure 14 shows an electropherogram of the digestion mixture after 24 h. The only species present was the 92,000 subunit. This sample retained 13% of the specific activity observed prior to proteolysis.

Distribution of Protease Activity

Protease activities of four milk fractions made to 0.5% with merthiolate (w/v) are presented in Table X. The buttermilk fraction, i.e., membrane material, showed a 39-fold enrichment in activity over skim milk. Supernatant and pellet fractions derived from buttermilk also were enriched in protease activity over skim milk.

TABLE I

Isolation of Xanthine Oxidase

Fraction	Volume (ml)	Proteins (mg)	Units[a]	Specific Activity[b]	Fold Purification	Recovery (%)
Whole Milk	18,900	6.62×10^5	6.29×10^3	.0095	1	-----
Washed Cream	1,400	1.12×10^4	2.80×10^3	.25	26.3	-----
Buttermilk	1,180	7.9×10^3	2.13×10^3	.27	28.4	100
1st Pellet	40	1.21×10^3	8.57×10^2	1.05	110.5	40
Extraction Supernatant	65	430	5.41×10^2	1.26	132.6	25.4
DOC Supernatant	50	79	2.79×10^2	3.55	337.8	13.1
Hydroxy Apetite	50	75	2.65×10^2	3.55	337.8	12.4

[a] μmoles xanthine oxidized/min

[b] μmoles xanthine oxidized/mg protein/min

TABLE II

Properties of Purified Xanthine Oxidase

Specific Activity	3.55 μm/mg/min
$E^{.1\%}_{280}$	11.42
$A_{280}:A_{450}$	5.05
Biuret $^{.1\%}_{540}$	0.10
$E^{.1\%}_{450}$	2.26

TABLE III

Amino Acid Composition
of Three Preparations of Xanthine Oxidase

Residue	Amino Acid Content (mole %) Bray and Malmstrom (1964)	Nelson and Handler (1968)	This Study
Lysine	6.8	6.9	6.6
Histidine	2.3	2.3	2.3
Arginine	4.4	4.7	4.9
1/2 Cystine	2.6	2.7	2.7
Aspartic Acid	8.4	8.6	8.9
Threonine	7.1	7.0	6.9
Serine	6.5	6.5	6.3
Glutamic Acid	10.2	10.0	10.5
Proline	5.5	5.5	5.3
Glycine	8.2	8.2	8.2
Alanine	7.5	7.6	7.5
Valine	6.9	6.8	6.7
Methionine	2.0	2.2	2.9
Isoleucine	5.0	4.8	5.2
Leucine	8.7	8.9	8.8
Tryosine	2.6	2.4	2.5
Phenylalanine	5.0	4.9	4.1
Tryptophan	0.4	0.4	0.4

S Δ Q	This study vs
Bray and Malmstrom	2.48
Nelson and Handler	1.81

TABLE IV

S Δ Q Comparisons Among Muscle And Milk-Derived Proteins

Protein (Reference)	MFGM Glyco-protein	β-Lacto-globulin	Calce-questrin	α_s-Casein	β-Casein	Sarco-plasmic ATPase	Actin[a]
Xanthine Oxidase[b]	62	153	251	164	287	17	15
MFGM Glycoprotein[c]	--	149	252	138	322	84	66
β-Lactoglobulin[d]	--	---	153	137	294	113	161
Calcequestrin[e]	--	---	---	188	429	232	227
α_s-Casein[d]	--	---	---	---	182	170	155
β-Casein[d]	--	---	---	---	---	279	319
Sarcoplasmic ATPase[f]	--	---	---	---	---	---	40

[a]Elzinga (1970).

[b]This study.

[c]Swope et al. (1968).

[d]Dayhoff (1972).

[e]MacLennan (1970).

[f]Stewart and MacLennan (1974).

TABLE V

S Δ Q Comparisons Among Membrane-Associated Proteins

Protein (Symbol) Source	A	G	C	MS	AE	SF	MF	T	S	AP	SA
Xanthine Oxidase (XO)[a]	15	56	251	37	28	16	19	11	60	34	17
Actin (A)[b]	--	66	227	49	55	16	26	14	72	39	40
MFGM Glycoprotein (G)[c]	--	--	252	123	93	59	90	52	66	119	84
Calcequestrin (C)[d]	--	--	---	317	247	181	256	159	190	179	232
Mitochondrial Structural (MS)[e]	--	--	---	---	55	63	41	50	122	45	51
Acetylcholinesterase (AE)[f]	--	--	---	---	---	57	65	38	88	62	42
Strep. feacalis ATPase (SF)[g]	--	--	---	---	---	---	14	21	41	38	20
Mitochondrial F1 (MF)[h]	--	--	---	---	---	---	---	28	56	24	18
Tubulin (T)[i]	--	--	---	---	---	---	---	---	53	83	28
Spectrin (S)[j]	--	--	---	---	---	---	---	---	---	37	52
Alkaline Phosphatase (AP)[h]	--	--	---	---	---	---	---	---	---	---	35
Sarcoplasmic ATPase (SA)[l]	--	--	---	---	---	---	---	---	---	---	---

[a]This study.
[b]Elzinga (1970).
[c]Swope et al. (1968).
[d]Osterwald and MacLennan (1973).
[e]Criddle et al. (1962).
[f]Leuzinger and Baker (1967).
[g]Schnebli et al. (1970).
[h]Knowles and Penefsky (1972).
[i]Mohri (1968).
[j]Robertson (1964).
[k]Simpson et al. (1968).
[l]MacLennan (1970).

Table VI

Comparison of H ø Ave of
Membrane-Derived Xanthine Oxidase With
Selected Muscle, Membrane and Milk-Derived Proteins

Protein (reference)	H ø Ave (cal/us)	Difference from Xanthine Oxidase (Δ)	Δ %
Xanthine Oxidase[a]	1074	---	----
Tubulin[b]	1073	1	0.1
Spectrin[c]	1069	5	0.5
Mitochondrial F1[d]	1056	18	1.6
Strep. feacalis ATPase[e]	1050	24	2.2
Acetylcholin-esterase[f]	1109	35	3.5
Mitochondrial Structural[g]	1083	9	0.8
Actin[h]	1058	16	1.5
MFGM Glycoprotein[i]	991	83	7.7
Calcequestrin[j]	1020	54	5.0
Sarcoplasmic ATPase[k]	1116	42	3.9
β-Lactoglobulin[l]	1168	94	8.8
β-Casein[l]	1310	236	22.0

[a]This study.
[b]Mohri (1968).
[c]Robertson (1964).
[d]Knowles and Penefsky (1972).
[e]Schnebli et al. (1970).
[f]Leuzinger and Baker (1967).
[g]Criddle et al. (1962).
[h]Elzinga (1970).
[i]Swope et al. (1968).
[j]Osterwald and MacLennan (1973).
[k]MacLennan (1970).
[l]Dayhoff (1972).

Table VII

Composition of Delipidized MFGM

Residue	Mole %	g residue/100g Proteins[a]	g residue/100g Sample
Lysine	5.56	6.44	5.11
Histidine	2.31	2.87	2.28
Arginine	4.63	6.54	5.19
Aspartic Acid	8.51	8.86	7.03
Threonine	6.09	5.57	4.42
Serine	6.49	5.11	4.06
Glutanic Acid	9.60	11.12	8.83
Proline	5.96	5.24	4.16
Glycine	8.00	4.13	3.28
Alanine	7.23	4.65	3.69
1/2 Cystine[b]	2.13	1.97[d]	1.56
Valine	7.69	6.83	5.42
Methionine[b]	2.45	2.91	2.31
Isoleucine	5.11	5.23	4.15
Leucine	9.49	9.44	7.49
Tyrosine	2.83	4.17	3.31
Phenylalanine	4.61	6.14	4.87
Tryptophan[c]	1.61	2.81	2.23
Total Amino Acids	100	100	79.4
Residual Phospholipid			10.2
Hexose			3.0
Hexosamine			2.6
Sialic Acid			1.1
TOTAL			96.3

[a]Amino acid content based on duplicate 22 and 72 h digestion with values corrected for destruction during hydrolysis to the equation of Hirs et al. (1954).

[b]Determined by the method of Hirs (1967).

[c]Determined by the method of Spies and Chambers (1949).

[d]0.74 g -SH, 1.28g S-S.

Table VIII

S Δ Q Comparisons Among Total Membrane Proteins

Membrane (reference)	Human Erythrocyte	Ehrlich Ascite Plasma Membrane	Murrah Buffalo FGM	Tharparkar cow FGM	Holstein Cow[a] FGM
Cardiac Plasma Membrane[b]	26	7	13	11	9
Human Erythrocyte[c]	--	23	29	22	35
Ehrlich Ascite Plasma[d]	--	--	10	12	14
Murrah Buffalo FGM[e]	--	--	--	8	9
Tharparkar Cow FGM[e]	--	--	--	--	10

[a]This study.

[b]Saccomani et al. (1974).

[c]Rosenberg and Guidotti (1968).

[d]Wallach and Zahler (1966).

[e]Banerjee et al. (1974).

Table IX

Loss of ATPase Activity Paralleling the Removal of Flavor from Xanthine Oxidase in Crude Membrane Preparations

	Intact		Deflavo	
Sample	Xanthine Oxidase Activity	ATPase Activity	Xanthine Oxidase Activity	ATPase Activity
1	1.25	0.096	0	0
2	1.32	0.102	0	0
3	1.53	.122	0	0

[a] μmoles xanthine oxidized/mg protein/min.

[b] μmoles Po_4 liberated/mg protein/min.

Table X

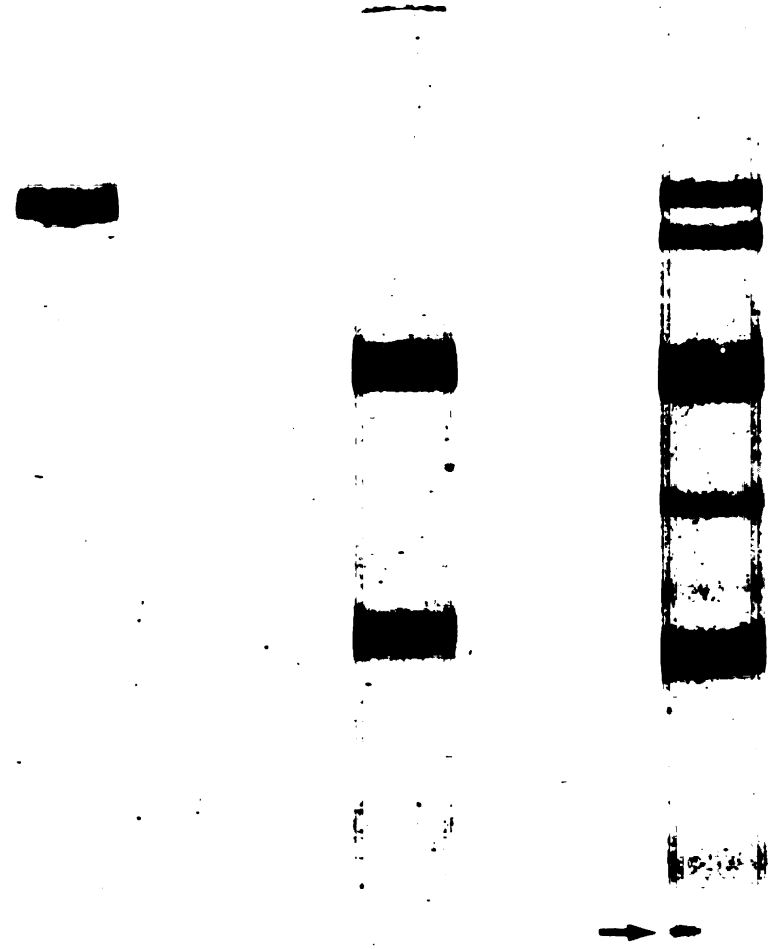
Protease Distribution Between Milk Fractions

Fraction	Units[a]	Proteins (mg/ml)	Specific Activity[b]
Skim milk	2.14	35.1	0.06
Washed-cream buttermilk	15.8	6.7	2.36
100-S supernatant	5.0	5.2	0.96
100-S pellet	9.8	15.1	0.65

[a] 0.01 absorbance units/h.

[b] 0.01 absorbance units/mg proteins/h.

Figure 1. Polyacrylamide SDS gel electrophoretograms of: (A) purified xanthine oxidase; (B) xanthine oxidase stored at 4°C for 30 days; and (C) total MFGM proteins as described by Anderson *et al.* (1974).



A B C

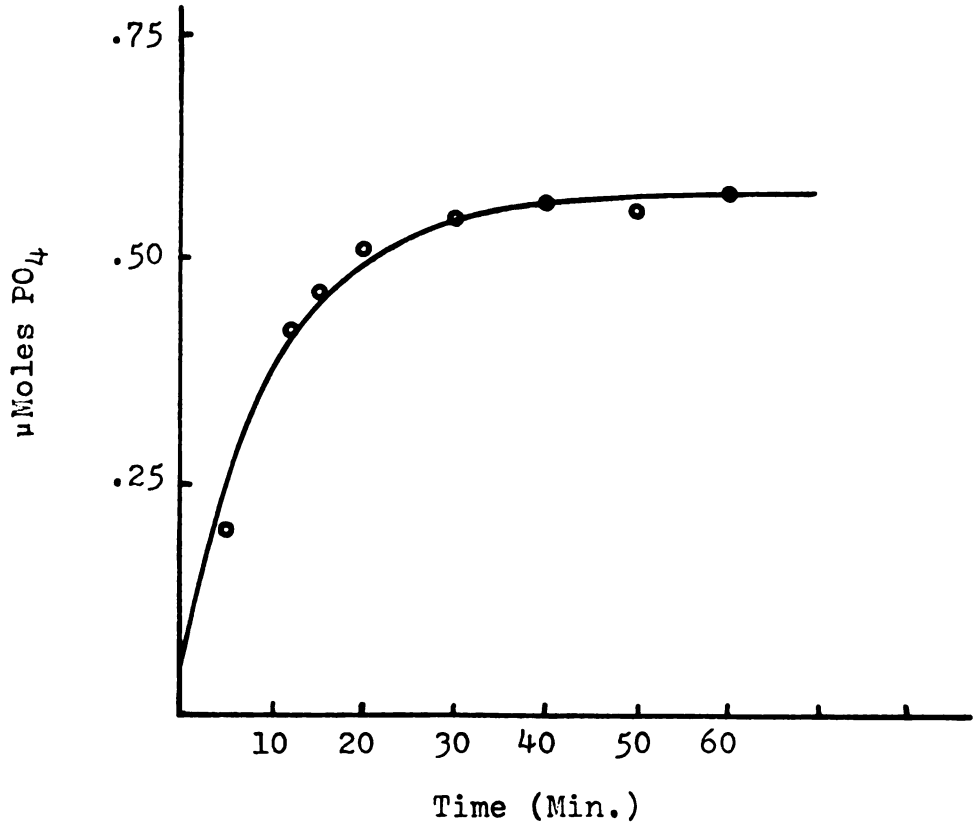


Figure 2. Release of phosphate from ATP by xanthine oxidase. Reaction mixtures contained .1 M Tris-HCl, pH 8.2, 5 mM ATP and .15 mg protein.

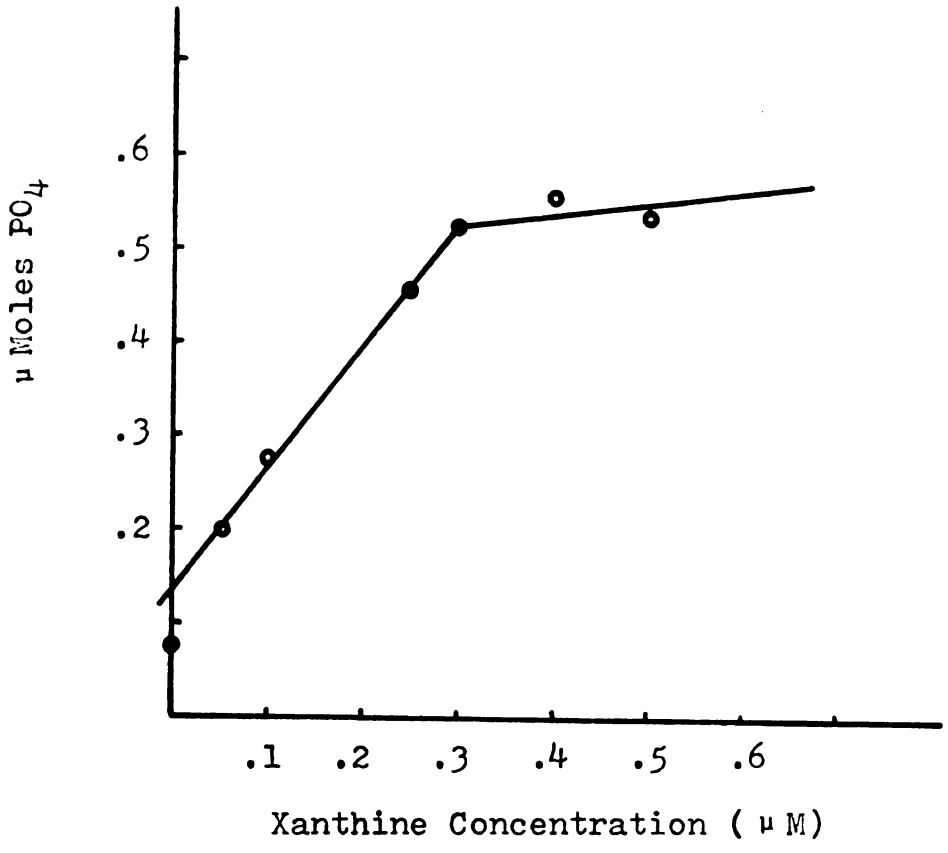


Figure 3. Stimulation of apparent ATPase activity of xanthine oxidase by addition of xanthine. Reaction mixtures contained 5 mM ATP, .1 M Tris-HCl, pH 8.3 and .1 mg protein.

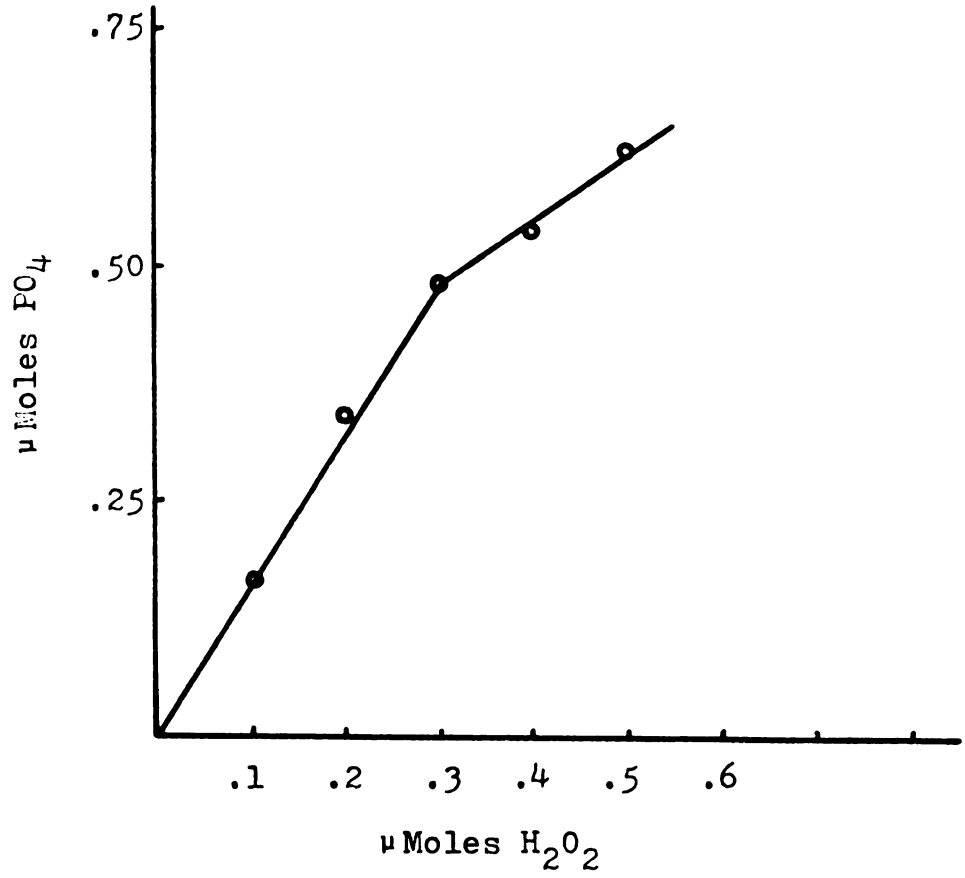


Figure 4. Release of phosphate from ATP by addition of H2O2. Reaction mixture contained .1 M Tris-HCl, pH 8.2, 5 mM ATP and 1 mg heat denatured xanthine oxidase

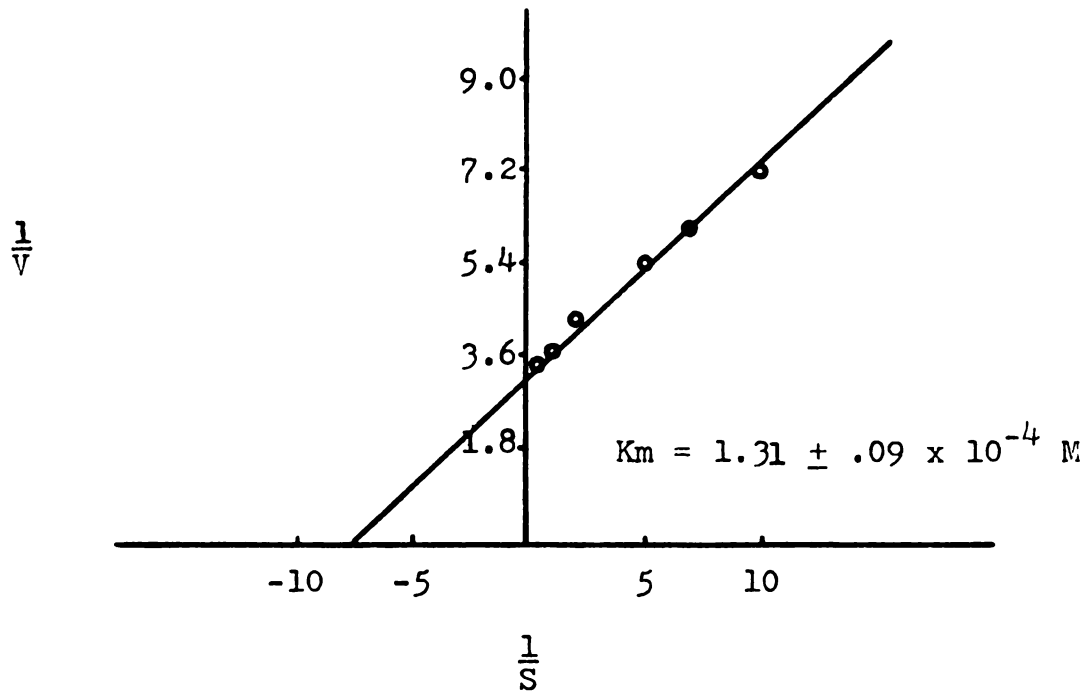


Figure 5. Reciprocal plot of the rate of phosphate release from ATP by xanthine oxidase. Reaction mixture contained 0.1 M Tris-HCl, pH 8.2, .1 - 1 mM ATP and 1 mg protein. $\frac{1}{S}$ is reciprocal units of ATP concentrated and $\frac{1}{V}$ is reciprocal units of velocity in micromoles phosphate/min.

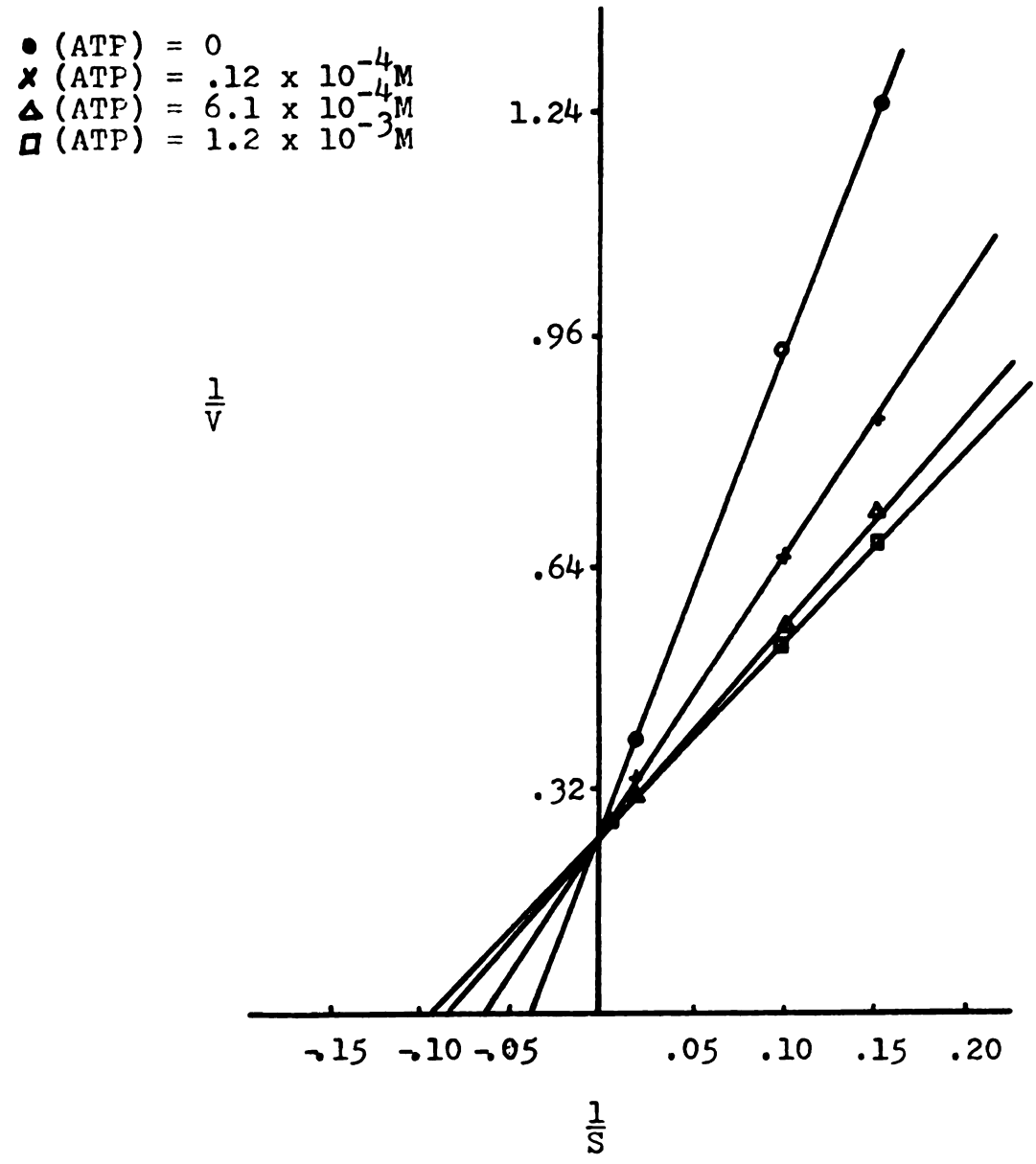


Figure 6. Reciprocal plot of the effect of ATP on the oxidation of xanthine. Reaction mixtures contained .1 M Tris-HCl, pH 8.2, 5 - 100 micromolar xanthine and 1 mg protein. $\frac{1}{S}$ is reciprocal units of xanthine concentration and $\frac{1}{V}$ is reciprocal units of velocity in micromoles/min. The concentration of ATP was as indicated.

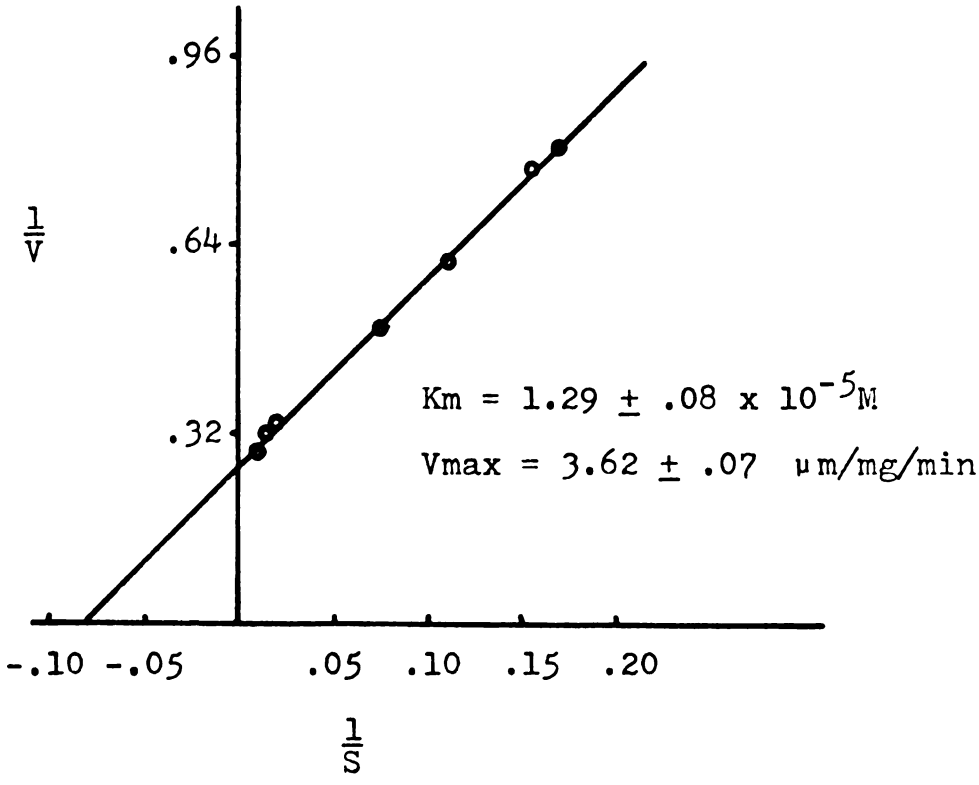


Figure 7. Reciprocal plot of xanthine oxidation. Reaction mixture contained .1 M Tris-HCl, pH 8.2, 5 - 100 micromolar xanthine and 1 mg protein. $\frac{1}{S}$ is reciprocal units of xanthine concentration S and $\frac{1}{V}$ is reciprocal units of velocity in micromoles/min.

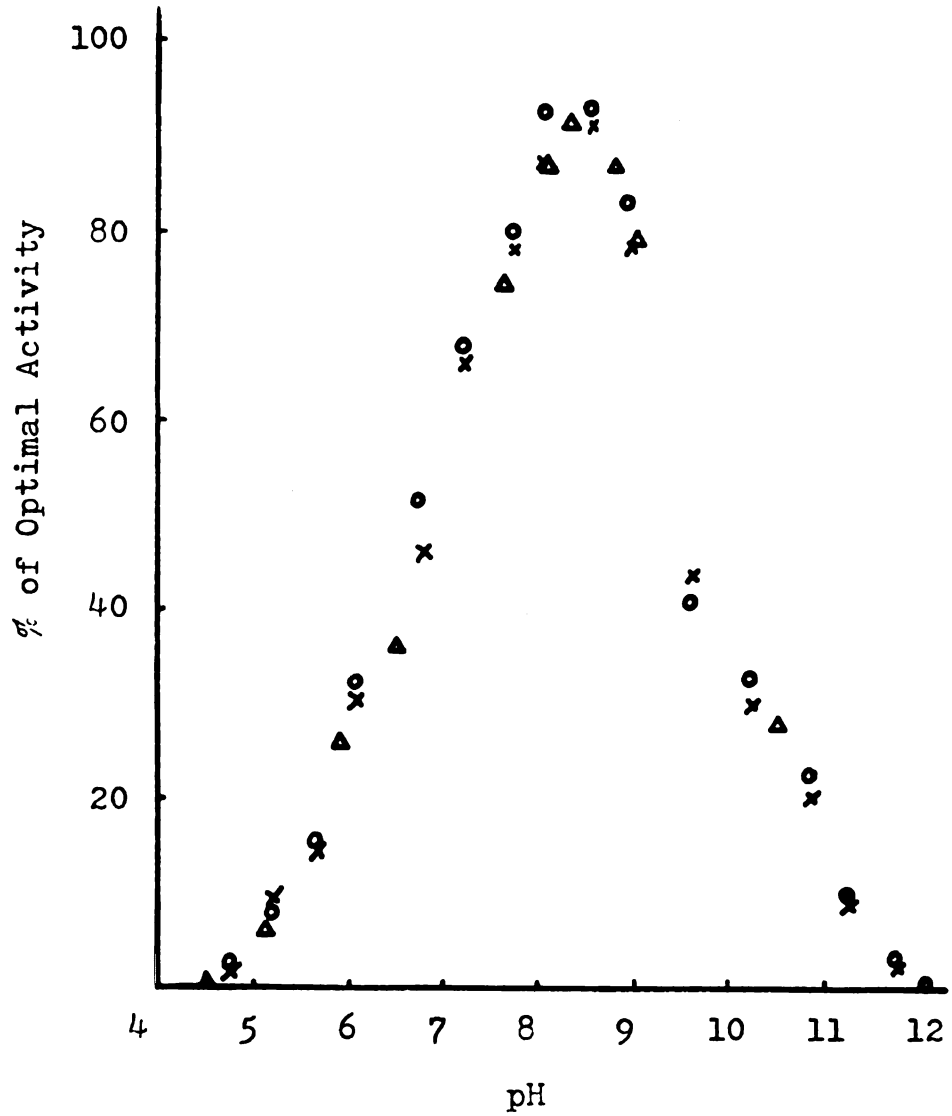


Figure 8. pH profile of xanthine oxidase.

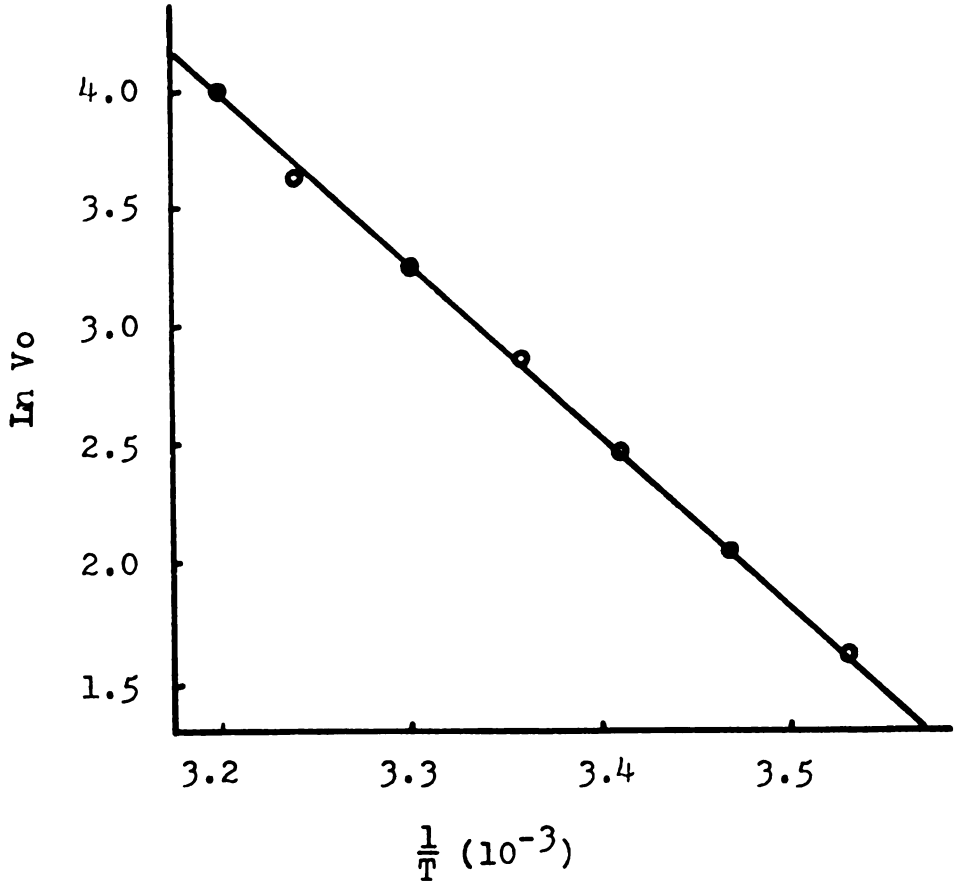
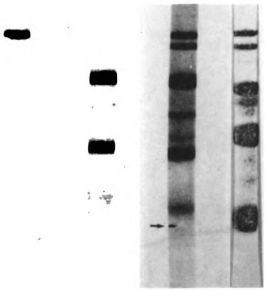


Figure 9. Effect of temperature on the initial velocity of the xanthine oxidase reaction.

Figure 10. Polyacrylamide-SDS gel electrophoretograms of: (A) purified xanthine oxidase; (B) xanthine oxidase stored at 4°C for 30 days; (C) total MFGM protein as described by Anderson *et al.* (1974); (D) "purified" xanthine oxidase of Nathans and Hade (1975); and (E) xanthine oxidase membrane filtered and stored at RT for 24 hr.

Figure 11. Double diffusion analysis of whey: (A) purified xanthine oxidase and (B) xanthine oxidase stored at 4°C for 30 days served as antigens and antibody to purified xanthine oxidase was placed in the center wall.


A
B
C
D
E


A
B

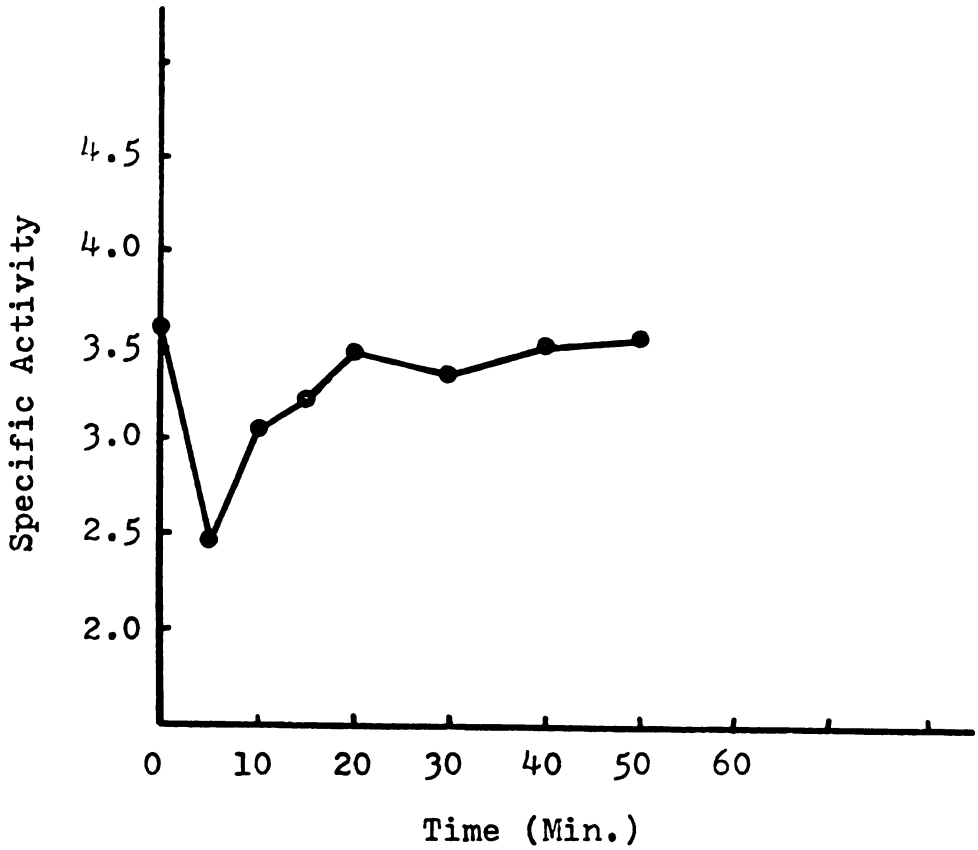
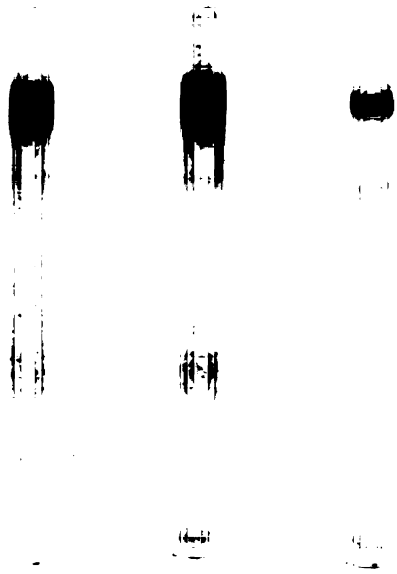


Figure 12. Effect of trypsin on the specific activity of xanthine oxidase. Trypsin and xanthine oxidase (1:5) were incubated at 37°C and samples were removed at the indicated times for xanthine oxidase activity measurements.

Figure 13. Polyacrylamide-SDS gel electrophoretograms of xanthine oxidase and trypsin (5:1) after incubation at 37°C for the indicated times.

Figure 14. Polyacrylamide-SDS gel electrophoretogram of xanthine oxidase-trypsin reaction mixture after 24 h.


5
10
50
Time (min)

DISCUSSION

Purification of Xanthine Oxidase

The yield of xanthine oxidase prepared from washed-cream buttermilk was 12.4%. The hydroxy-apetite adsorption procedure was not required in every preparation. The necessity for including this step in the isolation procedure seemed to be related to the care taken in decanting prior supernatant fractions. The specific activity of the preparation before and after the hydroxy-apetite adsorption was similar. However, in some preparations the DOC supernatant was not electrophoretically homogeneous. The fact that the adsorption step resulted in homogenity without an increase in specific activity indicates that some of the lower molecular weight "contaminants" removed by this process possessed enzymatic activity.

Gilbert and Bergel (1964) reported a procedure that gave high yields of xanthine oxidase (estimated to be 70 - 80%) of variable purity (85 - 95%). Massey et al. (1969) modified the procedure to obtain 44.7% yields of the enzyme that was consistently 90% pure. Hart et al. (1970) achieved 24.4% yields of xanthine oxidase that was considered to be 100% pure. The preparation in this study

was also 100% pure as estimated from a spectral analysis, as well as by electrophoretic analysis.

The three procedures reported above involve a pancreatin digestion step and require a minimum of 2 days to final preparation. The procedure utilized in this study avoids the use of proteolytic enzymes and the time required for enzyme purification has been reduced to 7 h for small scale preparations (e.g., 25 mg) and 14 h for larger scale preparations (e.g., 75 - 100 mg). The effects of pancreatin on the properties of the purified enzyme have been discussed by Carey et al. (1961), who demonstrated that the enzyme purified with the use of proteolytic enzymes was heterogeneous when chromatographed over hydroxy apetite, suggesting that its molecular weight characteristics may have been altered.

The largest loss of xanthine oxidase activity in the current preparative procedure occurred during the first centrifugation. Also, this was the quantity and time limiting step in the isolation procedure. However, the centrifugation step did decrease the volume of material from 1180 ml to 80 ml and all subsequent steps were facilitated by this decreased volume. Also with careful decanting of supernatants in the subsequent operations, the need for hydroxy apetite chromatography was eliminated.

Increased yields and total amounts of material might result from an alternative method for concentrating the

xanthine oxidase-containing membrane material. One method that appeared promising on a small scale preparation was the use of cold acetone to precipitate the xanthine oxidase from buttermilk. This could be accomplished on a larger scale and would require shorter time to accomplish. With this procedure, however, the batch adsorption of the enzyme must be replaced by an elution of the enzyme from a column of this material. Further work would be required to determine if increased yields could actually be achieved.

Criteria of Purity of Xanthine Oxidase

The specific activity of xanthine oxidase prepared for this study was 3.55 μmole/mg/min, comparing favorably with the highest reported value of 3.6 (Hart et al., 1970). The A_{280}/A_{450} ratio of the enzyme was 5.05 which also compared favorably with the lowest literature value of 5.0 (Avis et al., 1955). One milligram of dried xanthine oxidase added to 1 ml of buffer gave a biuret reading of 0.100 at 450 nm. This value was slightly higher than that obtained for crystalline bovine serum albumin under the same conditions, i.e., 0.091.

The enzyme yielded one zone on SDS-PAG when assayed immediately after purification but yielded more zones after prolonged storage. The molecular weight of the enzyme was estimated to be 153,000. Literature values

for the molecular weight of xanthine oxidase range from 150,000 to 370,000. Andrews et al. (1964) reported a value of 278,000 for the enzyme by both gel filtration and ultracentrifugation. They reported that this value could not be lowered by the addition of reducing reagents and they considered this to be a monomer molecular weight. Nelson and Handler (1968) found the native molecule to have a weight of 304,000 by ultracentrifugal studies. They reported, however, that in the presence of guanidine HCl at pH 3 the value was reduced to 150,000. Addition of mercaptoethanol yielded weights that ranged from 92,000 to 130,000. Massey et al. (1969) calculated a native molecular weight of 370,000 and a monomer molecular weight of 181,000 on the basis of the ratio of protein to flavin molecules. These values have to be considered high because the method of calculation assumes a 100% pure xanthine oxidase preparation and analysis of their specific activity and spectral data showed that their preparation had a purity of 90%. Carey et al. (1961) and Nathans and Hade (1975) have suggested that the use of proteolytic enzymes in the preparation of xanthine oxidase may affect its physical properties. That this contributes to the confusion over the molecular weight of xanthine oxidase will be considered below.

Amino Acid Composition of Xanthine Oxidase

Table III compares the amino acid composition determined in this study with those reported by Bray and Malmstrom (1964) and Nelson and Handler (1968). The SΔQ values of 2.42 and 1.89 respectively, indicate that the analysis are in excellent agreement. Common to all is the extremely low value for tryptophan (.4%). When xanthine oxidase (this study) was compared to various milk and muscle proteins by the SΔQ method, interesting results were obtained. The enzyme gave values of 15 and 17 when compared to actin and sarcoplasmic reticulum ATPase, respectively. According to Marchalonis and Weltman (1971), values this low indicate homology of primary sequence. Weltman and Dowben (1973) stated that values of less than 50 were suggestive of homology of sequence and found that a number of diversified contractile and ATP utilizing membrane proteins gave values in this range. They argued that such relatedness of structure among diversified proteins suggested evolutionary divergence from a common gene. The fact that all the related proteins hydrolyzed ATP added credence to their argument.

The argument for divergence was weakened somewhat when xanthine oxidase was included with these proteins because it does not hydrolyze ATP directly. It could be argued, however, that the enzyme can be considered to be related because of its role in purine metabolism.

The ability to recognize the purine ring may thus be responsible for some of the conservation of amino acid sequence these numbers employ.

To further examine the relatedness of membrane and contractile proteins, SΔQ values for a larger number of proteins are presented in Table V. Twelve different proteins were compared and xanthine oxidase was shown to be related to actin, mitochondrial structural protein, acetylcholinesterase, Strep. faecalis ATPase, mitochondrial coupling factor F1, sperm tubulin, sarcoplasmic reticulum ATPase and human placental alkaline phosphatase. While many of these proteins can in some way be related to purine metabolism, three cannot be. Mitochondrial structural protein, acetylcholinesterase and alkaline phosphatase are in no way connected with purine metabolism and the descent from a common ancestral gene of all of these proteins is suspect. The commonality within these proteins is that they are either membrane bound or form insoluble structures in water. A more logical explanation for their close relatedness may be an evolutionary convergence towards similar amino acid composition due to the environment these proteins are associated with. The membrane-associated protein that did not exhibit even the slightest degree of relatedness with any of the other proteins was the sarcoplasmic reticulum calcium binding protein, calciquestrin. This protein contains extremely large amounts of aspartic

and glutamic acid and is extremely low in basic amino acids. This composition is probably reflective of its role in calcium binding.

Further illustrations of similarities of membrane proteins are seen when their average hydrophobicities were calculated by the method of Bigelow (1967). The milk proteins β-lactoglobulin and β-casein differed from xanthine oxidase by 9.8 and 22% respectively. Calciquestrin which differed greatly in SΔQ and has a very specialized role in membrane metabolism reflected its association with other membrane proteins by this method and differed from xanthine oxidase by 5%. In general, the average hydrophobicity procedure lacks the descrimination of the SΔQ method but indicated a relatedness among membrane associated proteins.

To examine this concept in further detail, the amino acid composition of a variety of plasma membranes were compared. Complete analysis was not available for MFGM proteins so compositional analysis was performed on delipidized MFGM prepared by the method of Herald and Brunner (1957). Membranes prepared by this procedure contained 12.75% nitrogen in agreement with the data of Herald and Brunner (1957). Amino acids accounted for 79.4% by weight of this membrane preparation. Residual, i.e., unextracted phospholipid, accounted for 10.2% of the total and carbohydrate was responsible for 6.7% of the material. The carbohydrate was distributed between

total hexose 3.0%, sialic acid 1.1% and hexosamine 2.6% in general agreement with the data of Swope and Brunner (1970). The 2.02 g residues of 1/2 Cys/100 g protein were composed of 0.74 g -SH and 1.28 g S-S/100 g protein. For comparison with other plasma membranes the amino acids were expressed as mole % of the total amino acids present.

The SΔQ values showed that all plasma membranes examined had almost identical amino acid compositions. Exclusive of red blood cell membranes the values ranged from 7 to 14 with a mean of 10.3. With erythrocyte membranes included, the values ranged from 7 to 35 with a mean of 15.9. Either set of numbers indicates that the amino acid composition of these 6 types of plasma membrane are extremely similar. To apply an SΔQ comparison between mixtures of proteins and conclude homology of sequence or relatedness from the results would be open to severe criticism, but the low values do illustrate how similar in total amino acid composition plasma membrane proteins are. It can be assumed that this closeness of amino acid composition, in spite of a diversity of lipid to protein and carbohydrate to protein ratios between various plasma membranes (Robinson, 1975), is reflective of their common structural and functional characteristics.

Enzymatic Properties of Xanthine Oxidase

ATPase Activity

The isolated protein from MFGM was initially thought to be the Mg-ATPase reported in such preparations by Huang and Keenan (1972) and Baumrucker and Keenan (1975). When the enzyme was added to a solution of ATP, free phosphate was released as is shown in Figure 2. The discovery that the isolate was actually xanthine oxidase called for a more thorough review of the literature on the existence of Mg-ATPase in MFGM and for a possible mechanism to explain this phenomenon.

By-products of the aerobic reaction of xanthine oxidase with substrate are hydrogen peroxide and the super oxide anion. Both of these are highly reactive and the last two phosphate bonds in ATP are of limited stability. The enzyme is also rather non-specific and will oxidize a large number of purines, pyrimidines, aldehydes and analogs of these compounds. Adenine has been shown to be oxidized at both the 2 and 8 position by xanthine oxidase (Krenitsky *et al.*, 1972).

It was hypothesized that if ATP could be oxidized either the hydrogen peroxide or the super oxide anion produced would be responsible for the release of free phosphate from ATP. This being the case it would be expected that anything increasing the rate of their production would increase the apparent ATPase activity

of the enzyme. Thus, addition of xanthine to the ATPase reaction mixture caused an increase in phosphate release which plateaued at the point where the enzyme was operating at maximal velocity and further additions yielded no further increase in activity. An 8.6-fold increase in phosphate release was accomplished at maximal stimulation. These data argue against the possibility that the xanthine oxidase preparation was contaminated with ATPase because the addition of a non substrate for ATPase should not have accelerated the reaction. The fact that a substrate of xanthine oxidase accelerated the reaction and that a plateau was reached at a concentration of xanthine that insured the enzyme was approaching V_{max} argue strongly for the case that a by-product of the xanthine oxidase reaction was responsible for the apparent ATPase reaction. Furthermore, the addition of hydrogen peroxide to the ATPase reaction mixture containing heat-denatured xanthine oxidase also caused a release of phosphate from ATP.

Dixon and Lemberg (1934) found that a crude preparation of milk xanthine oxidase oxidized inosine, inosinic acid, adenosine and adenylic acid. They concluded, however, that in all cases the substrates were first converted to xanthine or hypoxanthine and then oxidized. They further stated that this must be due to the presence of other enzymes in the crude xanthine oxidase preparation. In the case of adenylic acid, for example, three additional enzymes would be required. This conclusion was supported

by Kackler (1947) who stated "No known enzyme can directly oxidize a nucleoside or nucleotide."

Krenitsky et al. (1972) have recently demonstrated however, that purified xanthine oxidase was fully capable of oxidizing adenine, purine ribonucleoside, inosine and xanthosine without requiring other enzymes to first deaminate or remove the ribose group from these molecules. The contradiction is probably due to the increased sensitivity of the procedure employed.

Further evidence that purified xanthine oxidase is capable of reacting with ATP is demonstrated in Figure 6. ATP was shown to be an inhibitor of the xanthine oxidase reaction and treatment of the data obtained by the method of Lineweaver and Burke (1934) graphically demonstrates that the inhibition was of a competitive type. Treatment of the data by the method of Wilkinson (1961) also demonstrates that K_m was reduced in the presence of ATP while V_{max} remained unchanged. A value of 1.01×10^{-3}M was obtained for K_i. These data, demonstrating competitive inhibition of xanthine oxidase by ATP, suggest that the molecule interferes with the enzyme by blocking the active site. If ATP can be bound it should be oxidized at the 2 and 8 positions as is adenine. Such oxidation would then produce hydrogen peroxide and super oxide anion which could explain the release of phosphorus from ATP.

If xanthine oxidase does mimic ATPase the problem of assaying for a "true" ATPase in the presence of xanthine

oxidase arises. The most extensive studies of MFGM ATPase were conducted by Huang and Keenan (1972) and Baumrucker and Keenan (1975). The first study dealt only with the enzyme in MFGM while the second study reported its activity in Golgi apparatus and MFGM. Baumrucker and Keenan hypothesized that the presence of the enzyme in Golgi apparatus served an analogous function to a similar enzyme found in sarcoplasmic reticulum membranes. The former would pump calcium ions into the Golgi to aid in the micellerization of casein while the latter pumps calcium into the sarcoplasmic reticulum to allow for muscle relaxation. Activity found in MFGM was very low and was thought to represent residual enzyme from the Golgi that was transferred to the plasmalemma during membrane flow.

Comparison of the properties of the enzyme from the two sources revealed some important differences. In the Golgi the ATPase was Mg^{++} dependent while in MFGM it showed no Mg^{++} dependency. The pH optimum changed from 7.5 for the enzyme in the Golgi to 8.5 for MFGM ATPase. The energy of activation of the Golgi ATPase was 6.03 k cal/mole while that of the MFGM enzyme was 10.2 k cal/mole. These rather drastic differences are difficult to explain by changing the source of the enzyme from the Golgi to MFGM, but are consistent with the complete loss of ATPase activity in the conversion of Golgi to plasmalemma and the appearance of a pseudo-activity caused by the presence of xanthine oxidase.

To see if this latter possibility was reasonable the kinetic parameters of xanthine oxidase were determined and compared to those reported for the MFGM ATPase.

The pH optimum for xanthine oxidase occurred between pH 7.8 - 8.5. The center, pH 8.3, was reported as the optimum. This compares favorably with the pH 8.5 reported for MFGM ATPase but is further from the 7.5 reported for the Golgi enzyme. The energy of activation of xanthine oxidase was found to be 14.0 k cal/mole compared to the 10.2 k cal/mole for MFGM ATPase. The value for the MFGM enzyme occurs directly between the values of the other two enzymes so these data are inconclusive.

Huang and Keenan (1972) reported the MFGM ATPase had a K_m of 5.68 x 10^{-5}M and a V_{max} of 21.5 μmoles/mg/min. These data were determined on total MFGM proteins. Purified xanthine oxidase when assayed for ATPase activity with varying amounts of ATP yielded a K_m of 1.31 ± .09 x 10^{-4}M and a V_{max} of 0.32 ± .03 μmoles/mg/min. The value for K_m is in reasonable agreement with that determined by Huang and Keenan but the value for V_{max} was too small by a factor of 67. Examination of the data presented by Huang and Keenan make it difficult to rationalize their value for V_{max} with their results.

The crude data, including a double reciprocal plot, when analyzed revealed a V_{max} of from 0.02 to 0.04 μmoles/mg/min. It can only be assumed that the authors or printers made a 1,000 fold error in their conversion,

e.g., m became mm, of the raw data to a value for V_{max}.

The average of their V_{max} from the raw data (0.03 μm/mg/min) then agrees well with the value determined in this study. They analyzed whole MFGM which is from 8 - 10% xanthine oxidase (Swope and Brunner, 1968; Briley and Eisenthal, 1975). Complete purification of xanthine oxidase should then yield a value of from 10 to 12.5 times higher or 0.3 to 0.37 μmoles/mg/min in excellent agreement with the 0.32 ± .03 μmoles/mg/min determined in this study.

To summarize these comparisons:

1. The pH optimum for Golgi ATPase was 7.5, for MFGM ATPase 8.5, for xanthine oxidase 8.3
2. Golgi ATPase was Mg^{++} dependent, MFGM ATPase was not, xanthine oxidase has no dependency on Mg^{++}
3. K_m for MFGM ATPase was 5.68×10^{-5}M, for xanthine oxidase utilizing ATP as a substrate $1.31 \pm .09 \times 10^{-4}$M
4. V_{max} for MFGM ATPase was reported to be 21.5 μmole/mg/min, analysis of the raw data reveals a value of 0.03 μmole/mg/min - 10 to 12.5 increase in this value (assuming pure xanthine oxidase) yields a V_{max} of from 0.30 to 0.37 μmole/mg/min, the V_{max} determined for pure xanthine oxidase in this study was 0.32 ± .03 μmole/mg/min.

These comparisons indicate that the ATPase activity seen in MFGM preparations could actually be an artifact due to the presence of large amounts of xanthine oxidase.

Plantz and Patton (1973) studied MFGM derived from skim milk and found it to be devoid of ATPase activity. They found this difficult to reconcile with the existence of the enzyme in the cream derived membrane preparations. Membrane material is isolated from skim milk by high speed centrifugation. The membrane is located as a "slide" layer above the pelleted casein. The material so isolated contains typical plasma membrane markers, e.g., 5' nucleotidase but no ATPase. If the ATPase activity were due to a true ATPase this would present a dilemma. If, however, the ATPase activity was really due to xanthine oxidase activity the answer to this problem can be found in other literature.

Zittle et al. (1956) and Kitchnen et al. (1970) studied the distribution of enzymes upon the centrifugation of skim milk. These workers found no xanthine oxidase activity associated with casein. In the study reported here, antibody to membrane xanthine oxidase gave a precipitin line (double diffusion in agar) with skim milk indicating its presence there but failed to react with a casein preparation, indicating the absence of xanthine oxidase in casein. It may be more than coincidental that the membrane material Plantz and Patton isolated and found to be devoid of ATPase activity was

also devoid of any xanthine oxidase activity.

Xanthine oxidase can be rendered inactive by removal of the flavin moiety from the molecule. This was accomplished by exposing the enzyme to 2M $CaCl_2$ (Komai et al., 1967). The mixture was then exhaustively dialyzed to remove free flavin and $CaCl_2$. The resulting deflavo enzyme was completely devoid of xanthine oxidase activity. When this procedure was utilized to produce deflavo xanthine oxidase on three preparations of membrane proteins, the disappearance of xanthine oxidase activity was accompanied, in each case, by a complete loss of an apparent ATPase activity.

The fractions analyzed were not pure xanthine oxidase but rather a mixture of membrane proteins. The denaturation of xanthine oxidase (by removal of flavin) would not a priori inactivate non-flavin enzymes. For example, the $CaCl_2$-treated fractions still maintained alkaline phosphatase activity. While it cannot be stated unequivocally that the conditions used to inactivate xanthine oxidase did not also inactivate endogenous ATPase, it is of interest that membrane fractions devoid of xanthine oxidase activity were also without ATPase activity. The evidence for the apparent ATPase in MFGM, which indeed represents a ramification of membrane xanthine oxidase, may be summarized as follows:

1. The reaction of xanthine oxidase with substrate

yields hydrogen peroxide and super oxide anion.

2. Hydrogen peroxide has been demonstrated to release phosphate from ATP.
3. Xanthine oxidase is a rather non-specific enzyme. Two of its substrates are adenine and the nucleoside, inosine.
4. ATP is a competitive inhibitor of the xanthine oxidase reaction.
5. Golgi and sarcoplasmic reticulum ATPase require Mg^{++} for activity while MFGM ATPase requires no ions for activity.
6. The reported pH optimum for MFGM ATPase (8.5) is consistent with the pH optimum determined for xanthine oxidase (8.3) but varies by one full unit from Golgi ATPase (7.5).
7. The reported K_m for MFGM ATPase (5.68×10^{-5}M) is in reasonable agreement with the K_m determined for xanthine oxidase utilizing ATP as a substrate ($1.3 \pm .09 \times 10^{-4}$M).
8. The reported V_{max} for MFGM ATPase (0.03 µmole/mg/min) in unfractionated membrane material is in excellent agreement with the V_{max} determined for purified xanthine oxidase ($.32 \pm .03$ µmole/mg/min) when the fact that xanthine oxidase comprises from 8 to 10% of total MFGM is considered.
9. Literature reports indicate that fractions of

MFGM that lack ATPase activity also lack xanthine oxidase activity.

10. In this study, the loss of xanthine oxidase activity was accompanied by a loss of ATPase activity.

Consideration of these data make a strong case for the concept of the ATPase activity detected in MFGM being due to a reaction of ATP with the xanthine oxidase present. It cannot, however, be stated that there is no ATPase activity in MFGM preparation without further investigation of this interesting phenomenon.

Molecular Weight of Xanthine Oxidase

The molecular weight of xanthine oxidase determined by SDS-PAG was 153,000. Electrophoresis of the molecule after 30 days storage at $4^{o}C$ yielded three zones with molecular weights of 90,000, 42,000 and 24,000. The original 153,000 species was completely absent. As can be seen in Figure 1, the original molecule and the three "breakdown" products correspond to 4 of the 7 major membrane proteins. The method of breakdown of the original molecule is subject to speculation. It is hard to conceive of a cold dissociation in a molecule that resisted boiling in SDS and mercaptoethanol. On the other hand, proteolysis of proteins generally results in a smeared pattern of SDS-PAG and fails

to yield discreet zones. If proteolysis was the cause of the observed breakdown it must have been very limited or specific. The total bacterial count of the material after 35 days at 4°C was <30/ml indicating a low amount of microbial contamination.

In an attempt to elucidate the mechanism of molecular breakdown, antibody to purified xanthine oxidase was used in a double diffusion experiment against fresh xanthine oxidase (one zone) and aged xanthine oxidase (three zones). The antibody formed completely fused precipitin arcs with both antigens, indicating antigenic identity. Failure to observe spurs on either end of the precipitin arc suggests that either:

1. Only one of the three derived polypeptide chains was antigenic, or
2. The three zone antigen migrated as a single molecule due to physical forces that held the three peptides together.

Closer examination of the precipitin zones show them to be nearly linear indicating that the antibody and antigen have approximately equal molecular weights (Crowle, 1961). The determined molecular weight of xanthine oxidase, 153,000, and the known molecular weight of IgG - 150,000 are in agreement with this observation. It appears, then, that the sample of xanthine oxidase held for 30 days at 4°C contains three polypeptides that are dissociated by SDS and mercaptoethanol but that

migrate in single diffusion through agar as a single molecule with a weight of ~ 150,000.

To further investigate the possible role of microorganisms in the observed breakdown of xanthine oxidase a sample was prepared on a small scale yielding 25 mg of xanthine oxidase in 7 h from the time of milking. Except for the separation of the milk (37°C) and the churning of the washed cream (~ 12°C) all subsequent procedures were carried out at 4°C. The enzyme solution (TPC < 30/ml) was then sterilized by passage through membrane filters. The count after filtration was 0. After 24 h at room temperature, a sample was electrophoresed by SDS-PAG, assayed for enzymatic activity and another total count made. The enzyme was fully active, i.e., 3.5 μmole/mg/min, had a plate count of 0 and yielded the patterns shown in Figure 10 after electrophoresis. The 153,000 molecular weight species was almost completely converted to a species with a molecular weight of 138,000. It is of interest to note that the 138,000 polypeptide is also one of the 7 major zones seen in electrophoresis of total MFGM. These data indicate that the breakdown occurring was independent of microbial contamination. It is possible that before membrane filtration the organisms present had produced enough extracellular protease to cleave the enzyme but the low count before filtration and the rapid isolation procedure at low temperatures would argue against this

possibility. If breakdown were due to microbial contamination other procedures for isolation of the enzyme with their 37°C incubation step with pancreatin and longer isolation times would also be expected to exhibit similar breakdown properties.

The fact that the majority of isolation procedures employ a pancreatin digestion step coupled with the observed propensity of the enzyme to break down upon storage made an examination of the effects of trypsin on the enzyme highly desirable. When trypsin was added to freshly prepared xanthine oxidase at a weight ratio of 1:5 and incubated at 37°C for 50 min, no appreciable change in the specific activity of the enzyme occurred with time. Gel electropherograms of samples removed 5, 10 and 50 min after the addition of trypsin indicate, however, that limited breakdown of the enzyme had occurred. After 50 min, about half of the original enzyme was still present as the 153,000 species and about one half as breakdown products. Of these products, the 90,000 dalton species seemed to be fairly resistant to further breakdown by trypsin. The cleavage of xanthine oxidase by trypsin, as evidenced by the electropherograms, was not accompanied by a decrease in the specific activity of the sample indicating that lower molecular weight species had full enzymatic activity. An aliquot of the reaction mixture was removed after 24 h of digestion and yielded one zone

after electrophoresis with a molecular weight of 90,000. This sample had 13% of the specific activity of the original enzyme assuming constant amounts of protein were present. This is clearly a lower limit of the specific activity of this sample because much of the protein had been completely proteolyzed. This observation indicates that the species with a molecular weight of 90,000 had at least partial enzymatic activity.

Closer examination of the specific activity data and molecular weights obtained from the trypsin treatment and the storage experiments indicate that full enzymatic activity is present in molecules of 153,000, 138,000 and 92,000. Some of the controversy over the molecular weight of xanthine oxidase can be resolved if it is assumed that the enzyme as isolated by various workers has undergone various degrees of proteolysis. Andrews et al. (1964) estimated a molecular weight 274,000 by ultracentrifugal procedures. They employed thiols in attempts to reduce the molecular weight of the enzyme but did not use guanidine HCl or SDS. This value is in excellent agreement with the value of 138,000 obtained after very limited proteolysis. A dimer would yield a weight of 276,000, well within experimental error. Nelson and Handler (1968) obtained a value for enzyme prepared without a pancreatin digestion step of 304,000. Again, this value agrees well with the native value of 153,000 (dimer = 306,000).

Treatment of their enzyme preparation with 7 M guanidine HCl at pH 3 yielded a molecular weight of 150,000, further indicating that the value of 153,000 obtained in this study is the monomeric species. When their preparation was exposed to 4 M guanidine HCl and 0.1 M mercaptoethanol, they reported molecular weight values of from 92,000 to 130,000 -- again in excellent agreement with the values observed in this study.

Nathans and Hade (1975) isolated xanthine oxidase by employing Triton X-100 instead of pancreatin. They presented an electropherogram of their "purified" enzyme, showing 5 major and 3 minor zones. The preparation was reported to be over 90% pure. Molecular weights of the three largest peptides were given--155,000, 125,000 and 85,000, again in good agreement with the weights determined for the original molecule and its active subunits determined in this study. These authors also prepared xanthine oxidase by the pancreatin digestion procedure and found it to show evidence of extensive proteolysis. They concluded that the proteolytic enzymes in pancreatin were being co-purified with xanthine oxidase when this procedure was used. They stated that their preparation was free of such contamination. That proteolysis was occurring in all of these preparations seems apparent from the data. That proteolysis occurs in enzyme isolation prepared without the use of pancreatin and that molecular weights of the subunits obtained

from both types of preparations are similar suggests that a source of protease other than pancreatin was common to all preparations.

Source of the Proteolytic Activity Associated with Xanthine Oxidase

Xanthine oxidase has been shown to break down after isolation independent of microbial contamination. The only other possible source of the proteolytic enzyme would have to be in the milk itself. Harper et al. (1960) have demonstrated the existence in milk of a protease that was not of microbial origin. The protease was precipitated with casein from skim milk. Dulley (1972) demonstrated that milk protease did not result from leakage through the circulatory system but rather originated in the mammary gland. Fractionation of mammary tissue showed that the protease was bound to particulate material. Presumably the only particulate material derived from the mammary gland that enters milk in significant quantities are the portions of the plasmalemma that surround lipid droplets upon their secretion (Keenan et al., 1970). The concept of membrane associated proteases is not a new one and proteolytic activity has been demonstrated to be associated with the plasma membranes of a variety of mammalian cells (Morrison and Neurath, 1953; Schnebli and Burger, 1972;

Tokes and Chambers, 1975).

Table X represents the distribution of protease between milk fractions containing 0.5% merthiolate to inhibit microbial growth. Specific proteolytic activity was increased 39-fold in washed-cream buttermilk over skim milk, indicating that the protease was concentrated in the membrane fraction. Centrifugation of the buttermilk into supernatant and pellet fractions produced an almost even distribution of protease activity between the supernatant and pellet fractions. Thus, it appears that the protease reported by previous workers to exist in milk is of membrane origin. The concentration of proteolytic activity in the casein fraction of skim milk can be explained by the fact that the membrane material of skim milk sediments with the casein.

The 39-fold enrichment of protease activity in the membrane fraction of milk and its appearance in both supernatant and pellet fractions derived from the membrane fraction strongly suggests that the breakdown of xanthine oxidase observed in this study was due to contamination of the preparation with a membrane associated protease. The amount present was too low to be detected electrophoretically but its effects on xanthine oxidase could be monitored by SDS-PAG electrophoresis and the breakdown of Azocol by the protease could be monitored spectrophotometrically.

This phenomenon may also explain why the gel pattern presented by Nathans and Hade (1975) yielded 8 zones. In preparation for electrophoresis, they dialyzed their enzyme preparation against several changes of buffer at room temperature before denaturation with SDS and mercaptoethanol. During this process proteolysis may have occurred. In this study when freshly prepared xanthine oxidase was denatured by boiling in SDS and mercaptoethanol no breakdown of the enzyme occurred, indicating that exposure to boiling temperatures or SDS or both resulted in inactivation of the protease.

Xanthine Oxidase And MFGM: Speculations

Koblyka and Carraway (1972) and Anderson *et al.* (1974) independently devised a numbering nomenclature for the major proteins observed in MFGM preparations. Both groups number the membrane proteins I through VI and both groups have an additional protein component visible on most of their electrophoretograms (frequently this component stains more intensely than do some of the "major" proteins). Figure 10 presents gels that represent: (a) purified xanthine oxidase; (b) xanthine oxidase which was stored at $4^{o}C$ for 30 days; (c) the major proteins described in the papers previously

cited; (d) the xanthine oxidase preparation published by Nathans and Hade (1975) which was assumed to be a pure preparation; and (e) xanthine oxidase which was sterilized by membrane filtration and stored at room temperature for 24 h. Five of the major stained zones of total MFGM can be matched with patterns for xanthine oxidase or its breakdown products. Also, the pattern of purified xanthine oxidase presented by Nathans and Hade resembles the pattern for the major MFGM proteins. The electropherograms are not directly comparable because they were not electrophoresed identically. Nevertheless, the patterns (distribution of and intensity of zones) are remarkably similar. The five stained zones observed, when compared with total MFGM proteins, show molecular weights corresponding to 5 of the 7 major proteins in MFGM preparations. To indicate identity of proteins solely on the basis of molecular weight data would be open to severe criticism. Furthermore, it is of interest to note the striking similarities in the electrophoretic patterns of whole MFGM proteins and the patterns generated by the breakdown of xanthine oxidase. If identity of the observed components can be assumed it would appear that xanthine oxidase and its derived products represent the majority of MFGM proteins.

Estimates of the amount of total membrane protein composed of xanthine oxidase have been based on either

specific activity measurements or on the amount of molybdenum and flavin found in milk (Swope and Brunner, 1968; Briley and Eisenthal, 1975). Hart et al. (1967) demonstrated the existence of two forms of xanthine oxidase in milk which are enzymatically inactive--one of which completely lacks molybdenum. Prior estimates, therefore, must be regarded as lower limits for the amount of xanthine oxidase present. Also, the observation that one molecule of xanthine oxidase can yield three polypeptides and still maintain enzymatic activity must be considered. Possibly, the amount of inactive xanthine oxidase present in milk may be quite high.

Wooding (1971) demonstrated the presence of a limiting boundary surrounding intracellular fat droplets. Briley and Eisenthal (1975), employing the criteria suggested by Vanderkooi (1972), classified xanthine oxidase as an integral MFGM protein. They speculated that xanthine oxidase may represent the internal limiting boundary of the unsecreted fat globules and thus may be obtained from a source other than the plasmalemma of the secretory cell. While there is no evidence to support this suggestion, neither is there evidence demonstrating that xanthine oxidase is actually associated with the plasma membrane of secretory cells. Huang and Keenan (1972) isolated plasma and Golgi membranes from bovine mammary tissue but did not

report on the presence or absence of xanthine oxidase activity in these preparations.

The source of the large amounts of xanthine oxidase associated with MFGM is a matter for speculation. If the enzyme does function as an intracellular coating for non-secreted fat globules, its point of origin within the secretory cell has yet to be determined. On the other hand, if xanthine oxidase is actually associated with the plasmalemma, the rationale explaining the presence of such large quantities of this enzyme is lacking. A determination as to whether xanthine oxidase arises as a pre-membrane coating of fat globules or as a membrane associated enzyme would greatly aid further investigations into the source of MFGM material.

The evidence for a plasmalemma origin of MFGM is strong. The similarities of the phospholipid distribution within these two membranes and the presence of marker enzymes typical of plasma membranes support the concept of membrane flow (Keenan et al., 1970). The large amounts of xanthine oxidase present in MFGM, the question relative to its source, and the electrophoretic patterns obtained from partially degraded xanthine oxidase and whole MFGM suggests that the MFGM may be comprised of components from two sources--one derived intracellularly (xanthine oxidase) and the other directly from the plasmalemma. At present there

is only electron microscopic evidence to support this possibility and the interpretation of such micrographs is not unambiguous. Further work is in the progress utilizing antibodies to purified xanthine oxidase in an attempt to clarify the origin of the enzyme and the nature of its association with MFGM.

CONCLUSIONS

1. Xanthine oxidase can be isolated directly from MFGM without exposure to proteolytic enzymes by utilizing the bile salt, DOC.
2. Xanthine oxidase isolated in such a manner is identical in activity towards xanthine and in spectral characteristics to xanthine oxidase isolated by the use of proteolytic enzymes.
3. Xanthine oxidase can mimic ATPase, i.e., release phosphate from ATP.
4. H_2O_2 or the super oxide anion, both by-products of oxidation of substrate by xanthine oxidase, are implicated as causing the release of phosphate from ATP.
5. It is possible that the ATPase activity reported to be associated with MFGM is actually a ramification of the presence of large amounts of xanthine oxidase in this material.
6. Freshly isolated xanthine oxidase yields one zone after electrophoresis in SDS containing polyacrylamide gels. Molecular weight equals 153,000.

7. After storage of xanthine oxidase at 4°C for 30 days gel electrophoresis yields 3 zones.
8. Xanthine oxidase that has been membrane sterilized and stored at room temperature for 24 h yields two zones after electrophoresis.
9. Treatment of xanthine oxidase with trypsin (5:1) at 37°C for 60 min causes no decrease in the specific activity of the xanthine oxidase while cleaving portions of the molecule as determined by gel electrophoretic analysis.
10. Polypeptides having molecular weights of 153,000, 138,000 and 92,000 have full xanthine oxidase activity.
11. Protease found in milk was shown to be associated with the membrane fraction.
12. The breakdown of xanthine oxidase into subunits upon storage has been hypothesized to be the result of this membrane associated protease.
13. Statistical comparisons of xanthine oxidase with muscle proteins caused xanthine oxidase to be classified as "actin-like."
14. Statistical comparisons of a large number of membrane-associated proteins with actin caused them to also be classified as "actin-like."
15. The diversity among these membrane-associated proteins argues for evolutionary convergence towards similar amino acid composition rather

than divergence from a common ancestral gene.

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