XANTHINE OXIDASE IN THE LUNG

by

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ABSTRACT

The generation of oxygen free radicals by the cytosolic enzyme, xanthine oxidase (XO), has been implicated in post-ischemic or reperfusion damage in several organs. XO catalyzes the conversion of hypoxanthine to urate with the concomitant production of superoxide anion free radical ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2. Oxygen free radical-mediated injury has also been demonstrated in inflammatory lung disease. The possible involvement of XO in oxidative injury in the lung has not yet been studied. Therefore, this research project was designed to determine whether XO is present in the lung and to investigate its characteristics in porcine, bovine, rat and human lung and other tissues.

Immunochemical analysis of xanthine oxidase in the tissues employed on polyclonal antibody raised to bovine milk XO. Proteins were separated by SDS-polyacrylamide gel electrophoresis of tissue homogenates. Proteins were transfered from the gels to nitrocellulose filters by Western blotting. After incubating the filters with a antisera containing the antibody to the purified bovine XO. XO on the filter was detected by its reaction with an enzyme-conjugated second antibody. XO was immunologically detectable in bovine lung and milk. Rat lung, kidney and liver all showed XO reactivity. XO was detectable in porcine liver but not detectable in porcine lung or kidney. Thus, the antibody to bovine XO was cross-reactive with porcine and rat XO. XO protein was not immunologically detectable in

human lung possibly because the antibody was not cross reactive with the bovine antibody.

In vivo, xanthine oxidase exists predominantly as a dehydrogenase rather than an oxidase. In this form as xanthine dehydrogenase (XDH) the enzyme does not produce either $O_2^{-\cdot}$ or H_2O_2. The activity of both XDH and XO was measured in several tissues using a fluorometric assay which uses an artifical substrate, pterin which is catalytically converted to the fluorescent product isoxanthopterin (IXP). XO activity in porcine liver was of 1.1 x 10^{-3} µg IXP/mg protein/min although XO activity was not detectable in porcine lung and kidney, in rat lung of 1.7 x 10^{-2} µg IXP/mg protein/min, rat kidney of 1.5 x 10^{-2} µg IXP/mg protein/min, and rat liver of 2.2 x 10^{-2} µg IXP/mg protein/min. Seven human lung biopsy samples were obtained after lung resection and initially tested for viability by determination of NADH oxidase activity and then assayed for XO-XDH. Three of these samples showed NADH oxidase activity indicating tissue viability, but only one of these three showed measurable XO activity of 5.35 x 10^{-6} µg IXP/mg protein/min.

Irreversible conversion of XDH to XO is thought to be the result of limited proteolysis by a Ca^{2+}/calmodulin activated protease, whereas reversible conversion of the enzyme occurs by oxidation of critical thiol groups. Studies on the rate and nature of the conversion of XDH to XO were conducted in rat lung using the

fluorescence assay to detect catalytic activities of both enzyme forms. Incubation of lung homogenates with trypsin for 60 min caused irreverisble conversion of 90% of the XDH to XO. In contrast, incubation of homogenates at 15°C for 10 hours caused conversion of 100% of the XDH to XO. This conversion was reversible to the extent of 80% by reduction of thiol groups with dithiothreitol (DTT). The effects of free Ca^{2+} on the conversion of XDH to XO was examined by using EDTA, a chelator of Ca^{2+} and other divalent cations; and EGTA, a more specific chelator of Ca^{2+}. The presence of these chelating agents during homogenization of either normoxic or ischemic rat lung tissue did not inhibit reversible enzyme conversion. Increased XO activity was reversible by DTT. In the normoxic rat lung, homogenates prepared with EDTA and EGTA showed a similar conversion of 95% of XDH to XO which was reversible to 70% with DTT. In the ischemic rat lung, samples prepared with EDTA and EGTA showed a conversion of 80% and 95% XDH to XO which was similar to control samples. The extent of reversibility to XDH was 75% with DTT incubation. In addition, perfusion of rat lungs with EDTA and DTT via a pulmonary artery cannula prior to 60 min of ischemia and homogenization did not affect the extent of XDH to XO conversion.

These results indicate that irreversible Ca^{2+}-mediated proteolytic conversion of XDH to XO does not occur to a great extent in the rat lung during either normoxia or ischemia. However,

reversible conversion of XDH to XO does occur, suggesting that reversible thiol dependent conversion may play a role in the lung under both physiological and pathophysiological states.

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ABBREVIATIONS

ARDS	Adult respiratory distress syndrome
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Divalent calcium cation
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
$E_{M(295)}$	Molar extinction co-efficient at 295 nm in gm-moles/l with a 1cm light path
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked-immunosorbant assay
FAD	Flavin adenine dinucleotide
H_2O_2	Hydrogen peroxide
HBSS	Hanks balanced salt solution
HMW	High molecular weight standards
HRP	Horseradish peroxidase
IXP	Isoxanthopterin
KDa	Kilodaltons
KPi	Potassiuim phosphate buffer
M199	Gibco Medium 199
MW	Molecular weight

NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide in the reduced form
$O_2^-\cdot$	Superoxide anion free radical
OH	Hydroxyl free radical
PBS	Phosphate buffered saline
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulfonylfluoride
SE	Standard error of the mean
SDS-PAGE	Sodium dodecyl/sulphate (denaturing) polyacrylamide gel electrophoresis
XO	Xanthine oxidase
XDH	Xanthine dehydrogenase
XDH-XO	Xanthine oxidase-xanthine dehydrogenase total enzyme activity

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DEDICATION

I would like to dedicate this to all those who were instrumental in my leaving Toronto to "go West Young Woman" and pursue this degree in B.C., especially Dr. Anne Autor who invited me, my parents and family, Dr. Marlene Rabinovitch, Dr. Hersch Rosenberg, Helen Ives, Dr. Paul Adonis Hamel, Dr. D.G. Butler, and Michelle Mullen.

INTRODUCTION:

Oxygen free radicals are known to be involved in the etiology of disease states induced by the toxic effects of radiation, xenobiotics, pesticides and chemotherapeutic agents. It is now becoming better recognized that endogenously generated oxygen free radicals also are important in responses to injury such as inflammation and post-ischemic tissue damage. The enzyme system xanthine dehydrogenase: xanthine oxidase, which in the oxidase form is capable of generating oxygen free radicals in the oxidation of hypoxanthine and xanthine to urate, is considered to play an important role in free radical induced reperfusion injury, as well as in several other disease states (Table 1). The pathogenesis of several forms of pulmonary injury have been linked to oxygen free radical formation. This thesis investigates the presence and characteristics of xanthine dehydrogenase: xanthine oxidase in the lung with particular interest in whether oxygen free radicals produced by xanthine oxidase could be important in inflammatory lung disease. In order to best introduce the topic of xanthine oxidase in the pulmonary microvasculature it will be necessary to provide a brief introduction to free radical biology in general and explain why this area is of particular concern in pulmonary disease.

Oxygen Free Radicals: Overview

In 1878 Paul Bert showed that the element oxygen, long considered to be the very sustenance of aerobic life, could be damaging to the brain, lungs and other organs of animals. This discovery introduced a very paradoxical concept that was not well accepted. It was almost another

TABLE I

DISEASES FOR WHICH XANTHINE OXIDASE MEDIATION HAS BEEN SUGGESTED

ACUTE PANCREATITIS[1.]

STREPTOZYTOCIN INDUCED DIABETES MELLITUS[1]

ISCHEMIC BOWEL DISEASE[2]

GASTRIC ULCERS[2]

NECROTIZING ENTERITIS[2]

CARCINOGENESIS[3]

PRODUCTION OF CLASTOGENIC FACTORS[3]

RHEUMATOID ARTHRITIS[4]

GOUT[4]

XANTHINURIA[4]

STROKE[4]

DISSEMINATED INTRAVASCULAR COAGULOPATHY[4]

ADULT RESPIRATORY DISTRESS SYNDROME[5]

TRANSIENT RENAL ISCHEMIA LEADING TO ACUTE RENAL FAILURE[6]

ACUTE TUBULAR NECROSIS POST-KIDNEY TRANSPLANTATION[6]

MYCARDIAL INFARCTION WITH REPERFUSION[7]

As per.
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century before Rebecca Gerschman first suggested that the mechanism of oxygen poisoning may have a common mechanism with radiation damage, based on the observation that irradiation of oxygenated solutions caused the formation of highly reactive oxygen species known as oxygen free radicals (1).

A free radical is an atom or molecule which incorporates an unpaired electron into its outermost orbit (2). These species have a very high chemical reactivity due to their ability to abstract an electron from other molecules. Because of its electronic structure, ground state oxygen exists as a diradical, O_2, unlike most elements which exist in the singlet state. In the absence of a catalyst, because of Hund's Rule oxygen is most easily reduced by four sequential univalent electron additions rather than a single concerted four electron reduction. Sequential univalent reductions create short lived reactive intermediates, and establish the potential for the incomplete reduction of oxygen, thus producing oxygen free radicals (See Figure 1)(3).

In aerobic organisms, free radicals are an essential component to many biochemical processes. The catalytic action of enzymes is often dependent upon the generation of free radicals. For example, the production of ATP by the mitochondrial electron transport chain requires organic free radical intermediates in the Q cycle and at NADH dehydrogenase (4). However, a paradox exists due to the highly reactive nature of free radicals which makes them toxic unless kept in check.

REACTIONS OF REACTIVE OXYGEN SPECIES

Reactions	Description
$O_2 + 1e^- \longrightarrow O_2^-\cdot$	One electron reduction of oxygen
$O_2^-\cdot + H^+ \longrightarrow HO_2$	The pKa of this reaction is 4.8
$O_2^-\cdot + O_2^-\cdot + 2H^+ \longrightarrow H_2O_2 + O_2$	Dismutation of superoxide to form hydrogen peroxide and oxygen
$O_2^-\cdot + Fe^{3+}X \longrightarrow O_2 + Fe^{2+}X$	Reduction of iron-containing compounds such as ferritin
$H_2O_2 + Fe^{2+}X \longrightarrow HO\cdot + OH^- + Fe^{3+}X$	Generation of hyroxyl free radicals from hydrogen peroxide in the presence of reduced iron; the Fenton Reaction

Net Reaction: $O_2^-\cdot + H_2O_2 \xrightarrow[\text{catalyst}]{\text{iron}} O_2 + HO\cdot + OH^-$

Net reaction for the generation of hydroxl free radicals from superoxide and hydrogen peroxide

Figure 1. Reduction Reactions of Oxygen

The cell is exposed to several different extracellular and intracellular oxygen free radicals (Figure 1). The one electron reduction of O_2 produces superoxide (designated $O_2^{-\cdot}$). However, at physiologic pH, two molecules of $O_2^{-\cdot}$ rapidly dismutate to produce hydrogen peroxide (H_2O_2) and O_2 thus limiting the ability of $O_2^{-\cdot}$ to react with substrates whose rates of reaction are competitive with the dismutation reaction. These include compounds such as ferricytochrome c, quinones, ascorbic acid, heme proteins and thiols (5) (6). H_2O_2 itself is not highly reactive with most organic compounds (8). However, H_2O_2 in the presence of reduced transition metals such as ferrous iron the extremely powerful oxidant hydroxyl free radical OH$^{\cdot}$(6) is generated in a reaction referred to as the Fenton reaction (according to Figure 1, Reaction 5). The hydroxyl radical oxidizes organic compounds with little selectivity, at reaction rates in the range of 1 x 10^9 to 1 x 10^{10} $M^{-1}s^{-1}$ (9).

Hydroxyl free radical mediated oxidation of organic molecules proceeds by abstraction of an election from the target molecule by OH$^{\cdot}$. This step is termed initiation. An organic free radical is produced from this oxidation which can itself abstract further electrons causing the generation of more free radical moieties in a succession. This second step is termed propagation. The initiation of one organic free radical starts a cascade propagating further free radicals in a chain reaction, thus causing the ultimate oxidative cleavage of organic molecules. This process continues until the free radicals are quenched either by interaction with another free radical or by a molecule which does not in turn produce a free radical.

In vivo, reactive oxygen species are produced in a variety of enzyme systems. These include the cytochrome P450 system, NADPH oxidase of neutrophils, prostaglandin synthetase and xanthine oxidase which catabolizes purine nucleotides. In addition to these normal physiologic processes, oxygen radicals are also generated from exposure to radiation, hyperoxic states, pesticides, chemotherapeutic agents, tobacco smoke and aromatic hydrocarbons (10).

In order to control potentially cytotoxic free radical reactions, the cell contains numerous protective antioxidant mechanisms. As Fridovich has commented "if it were not for a group of antioxidant defenses the cell would not be able to survive in its oxidizing environment" (11). These antioxidant mechanisms employ both enzymes which catalytically remove reactive species and low molecular weight compounds referred to as scavengers, which react with free radicals without generating further free radicals. The most important of the antioxidant enzymes are superoxide dismutase, which catalyzes the dismutation of superoxide; catalase and glutathione peroxidase, which both catalyze the reduction of hydrogen peroxide to H_2O. Low molecular weight scavengers such as ascorbate, α-tocopherol, β-carotene and glutathione act by being oxidzied but do not become free radicals themselves.

Even with this complex array of protective mechanisms, in a

CELLULAR FREE RADICAL TARGETS

Target	Consequence
Low Molecular Weight Molecules	
Unsaturated & thiol-containing amino acids	Protein denaturation & crosslinking, enzyme inhibition Organelle and cell permeability changes
Nucleic acid bases	Cell cycle changes, mutations
Carbohydrates	Cell surface receptor changes
Unsaturated lipids	Cholesterol & fatty acid oxidation Lipid cross-linking Organelle & cell permeability changes
Cofactors	Deceased nicotinamide & flavin-containing cofactor availability & activity, ascorbate oxidation, porphyrin oxidation
Neurotransmitters	Decreased neurotransmitter availability (e.g. serotonin, epinephrine
Antioxidants	Decreased availability, includes α-tocopherol & β-carotene
Macromolecules	
Protein	Peptide chain scission
DNA	Strand scission
Hyaluronic acid	Change in synovial fluid viscosity

Figure 2. Effects of oxygen free radicals on cellular components. Reproduced from Freeman and Crapo 1982, (10).

strongly oxidizing environment all of these antioxidant defenses may prove
to be inadequate. Essentially all cellular constituents are susceptible to
injury from free radicals (12) (Figure 2). Once produced, free radicals
will interact with protein, DNA, membrane phospholipid, lipoprotein and
glycoprotein in self propagating reactions which may alter the native
structure of both the intracellular components and the extracellular matrix.

Because reactive oxygen species will readily react with unsaturated
and sulphur-containing molecules, the amino acid residues tryptophan,
tyrosine, phenylalanine, histidine, methionine and cysteine are especially
sensitive to oxidation (13).

Specific residues required for enzymatic function may be modified
enough to interfere with activity (both augmenting or arresting function).
More permanent damage may be due to protein crosslinking and fragmentation
may have structural and ultrastructural consequences (14) (15). It is known
that oxidation of critical groups makes proteins more susceptible to
proteolytic breakdown, thus the suggestion has been made that oxidative
modification may be the first step in normal protein turnover (16) (17).
This implies that free radicals produced during the inflammatory response
could have multiple functions, such as isolation of the area of damage,
killing bacteria, activation of the immune system via superoxide dependent
chemotractants and initiating breakdown of damaged structural components in
order to facilitate repair.

Highly reactive free radicals are also capable of inducing DNA strand
breakage as demonstrated in vitro with a xanthine oxidase + xanthine
superoxide generating system (18). If unrepaired this may cause point

mutations (19) and chromosomal aberrations (20). Free radicals generated
enzymatically, and in several disease states have also been shown to have a
clastogenic effect, such that further damage to DNA is perpetuated (21).

The plasma membrane and organelles are especially susceptible to
oxidation of the polysaturated fatty acids. (For a recent review see
Sevanian and Hochstein, 1985 (22). Oxidative cleavage of polyunsaturated
fatty acids results in the accumulation of short chain fatty acid
derivatives. Such a process becomes autocatalytic if unchecked due to the
production of lipid peroxides, lipid alcohols and aldehyde by-products,
which will cause further oxidation. Membrane viscosity, permeability,
membrane transport proteins, secretory functions, cell surface interactions
and integrated cellular metabolic processes will all be affected (12). In
addition, such by-products as malondialdehyde, which is diffusable across
cell membranes, may augment protein cross linking and mutate DNA (23).

Effects of Reactive Oxygen Species on the Pulmonary Microvasculature

Because the microvascular endothelium acts as a selective diffusion
barrier between the circulation and interstitium, loss of endothelial
integrity through the loss of critical endothelial cell functions has
serious consequences for organ function. The effects of oxygen free radical
damage on the pulmonary endothelium include increased capillary permeability
to macromolecules and edema formation (17)(24). This is particularly
detrimental in the lung where gas exchange for the entire organism takes
place.

Studies with endothelial cells in culture have shown that reactive
oxygen species are particularly detrimental to the endothelial membrane

integrity. Shasby et al. showed an increase in albumin transfer across a diffusion barrier of endothelial cells grown on micropore filters after treatment with oxygen free radical generating systems (25). This was shown to be associated with cytoskeletal changes attributable to Ca^{2+} levels and to be reversible. Decrease in thymidine incorporation into DNA is a sensitive index of cellular dysfunction. Hyperoxia or H_2O_2 treatment has been shown to reduce thymidine kinase activity with implied effects on cellular protein production (26). While the exact mechanism of lethal oxidant injury to endothelial cells remains to be clarified, it is unequivocably established that reactive oxygen species have lethal consequences for the endothelial cell (27) (28). At physiologic pH, $O_2^-\cdot$ acts preferentially as a reducing agent, whereas $HO^\cdot$ is an extremely powerful oxidizing agent. The $OH^\cdot$ species is however very short lived and does not cross cell membranes but rather will react with membrane macromolecules before it reaches the cytosol. However, in the presence of transition metals such as iron, $O_2^-\cdot$ which is produced and then dismutated into H_2O_2 will generate $OH^\cdot$ (Fig. 1 Reaction 6). Iron has been shown to be essential for superoxide free radical induced injury (29). Thus, while $OH^\cdot$ is the most strongly oxidizing species, the means of producing $HO^\cdot$ via the H_2O_2 dependent pathway is important (30) in lethal oxidant injury to endothelial cells and injury resultant from stimulated neutrophils (31). This is presumably because H_2O_2 is uncharged and freely diffusable across the cell membrane, unlike superoxide and hydroxyl radicals which are not. The pathogenesis of several forms of pulmonary damage have been linked to oxygen free radical formation.

Hyperoxia has been long established as a cause of pulmonary damage (38). The pulmonary pathology resultant from hyperoxia appears to originate from enhanced autoxidation of cellular components which are normally found in the reduced state, as well as exhaustion of cellular antioxidant defense mechanisms. Interestingly, oxidative damage to endothelial cells has been shown to augment their susceptibility to further neutrophil-mediated damage by increasing adhesive interaction between neutrophils and endothelial cells (31) (32) (33).

In vivo, the extensive destruction of the pulmonary microvasculature associated with hyperoxic insult is likewise combined with an infiltrate of inflammatory cells in the lung (34), thus perpetuating free radical damage due to superoxide generated by NADPH-oxidase of neutrophils which produces $O_2^{-\cdot}$ in the following reaction:

$$NADPH + 2O_2 + \xrightarrow{\text{NADPH-oxidase}} NADP^+ + 2O_2^{-\cdot} + H^+$$
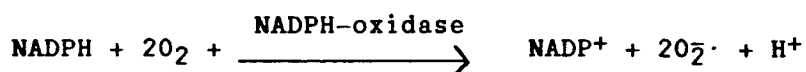
The effects of oxygen free radicals are particularly detrimental in the lung (35) which may react by microvascular leakage of proteins and disruption of alveolar epithelial integrity (36). The earliest stages of microscopically visible damage occur in the pulmonary capillary bed (37). The pulmonary toxins paraquat (40), and nitrofurantoin (41) act by the generation of superoxide through redox cycling, while bleomycin interacts via binding reduced iron to produce superoxide (39).

Oxygen radicals generated by activated polymorphonuclear leukocytes (PMN's) are of particular significance in the pathogenesis of acute and chronic pulmonary injury (42). It has been estimated that up to 50% mortality occurs in cases of acute non-cardiogenic pulmonary edema,

otherwise known as adult respiratory distress syndrome (ARDS) (43). The neutrophil has been strongly implicated in the pathogenesis of ARDS (44) (45) (46) (47). The consquences of edema due to increased microvascular permeability (48) (49) in the lung are especially detrimental because the filling of interstitial spaces with fluid interferes with the normal process of gas exchange upon which the entire organism is dependent.

Oxygen Free Radical Damage Associated with Ischemia-Reperfusion Injury in Other Tissues

Oxygen derived free radicals are now thought to play an important role in another form of damage, described as post-ischemia or reperfusion injury. It has been well documented in other organ systems (kidney, gut, heart, skin, liver, pancreas and brain) that reperfusion of a previously ischemic tissue produces, paradoxically, more damage than that caused by ischemia alone (50) (51) (52). The initial site of reperfusion injury appears to be the microvascular endothelium (53). This results in increased transcapillary filtration, interstitial edema and influx of inflammatory cells which can lead to the loss of the specialized functions characteristic of the tissue, such as acute tubular necrosis in the kidney (54) or loss of contractility in the myocardium (55). Oxygen free radicals are thought to be introduced by the reperfusion of the tissue with oxygenated blood. This mechanism has been confirmed by several studies which show that use of superoxide dismutase and catalase or dimethylsulphoxide (DMSO) as free radical scavengers will ameliorate the detrimental effects of reperfusion (56) (57). The enzyme xanthine oxidase (ECO 1232 oxidoreductase)

produces $O_2^{-\cdot}$ and H_2O_2 during the oxidation of its substrates xanthine and hypoxanthine. Because of the effects of the above described inhibitors xanthine oxidase was suggested as the molecular source of the oxygen radicals produced in reperfusion injury (58). This proposal has now been confirmed by many studies showing that allopurinol (54) (55) (59), a specific inhibitor of xanthine oxidase which acts as a tightly binding competitive substrate, prevents the development of the reperfusion injury syndrome.In an analogous manner perfusion of specific organs with a mixture of xanthine oxidase plus xanthine (33) (60) creates injury similar to reperfusion injury. Perfusion of the lung with a solution containing xanthine oxidase plus xanthine is likewise shown to increase vascular permeability apparently via compromised endothelial cell function (61) (62) resulting in similar pathologic findings to acute pulmonary edema. Since it has been shown that oxygen derived free radical damage to pulmonary endothelium increases the cells susceptibility to adhesion of neutrophils and thus magnifiying damage due to inflammatory reactions, this leads to the interesting speculation that xanthine oxidase may play a role in the development of ARDS (63). Since an episode of transient ischemia may be an initiating factor in the activation of xanthine oxidase mediated pathology in other tissues, (see the following section on biochemistry of XO)(93) (Figure 4), under what circumstances could the lung be considered "ischemic"?

ARDS was first recognized as a discrete syndrome in 1967 by Ashbaugh when it was observed to occur subsequent to traumatic, hemorrhagic and septic shock and given the name "shock lung" (64), although infections,

Decreased perfusion
Decreased O_2

ATP $\longrightarrow$ AMP $\longrightarrow$ adenosine

inosine

hypoxanthine

Xanthine dehydrogenase

1. reversible thiol oxidation

2. Ca^{2+} mediated proteolysis

Hypoxanthine $+2O_2 + H_2O$ xanthine oxidase $\longrightarrow$ xanthine $+ 2O_2^-\cdot$
$2H^+$

Reperfusion: increase in O_2

xanthine
oxidase

uric acid $+ O_2^-\cdot$

Result: $O_2^-\cdot + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

and $\quad 2O_2^-\cdot + 2H^+ \longrightarrow O_2 + H_2O_2$

$\quad\quad H_2O_2 + Fe^{2+}(X) \longrightarrow HO\cdot + OH^- + Fe^{3+}(X)$
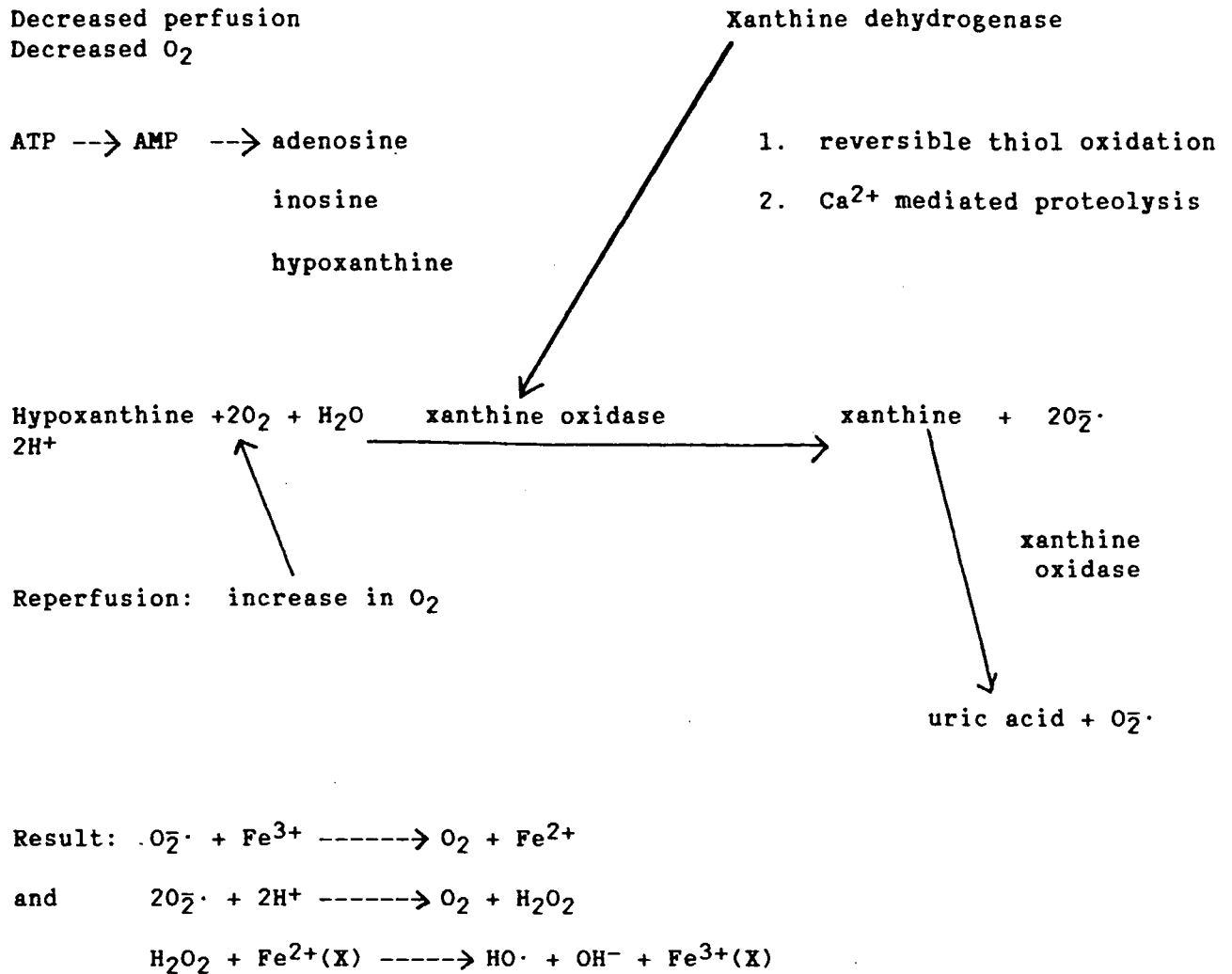
Figure 4. Reactions of Xanthine Oxidase. Proposed sequence for superoxide
production in reperfused post-ischemic tissue and subsequent
generation of the powerful oxidizing agent OH·

pancreatitis, inhalants and ingestants and near drowning were also implicated. All of these factors compromise pulmonary gas exchange. Interestingly, microthrombi and microemboli due to fat, gas, air and ammiotic fluid are also occasionally followed by the development of ARDS. These could lead to areas of focal ischemia in the lung. Cardiopulmonary bypass has also been shown to result in secondary pulmonary injury which resembles ARDS at the ultrastructural level.Surgical bypass is another instance of when the lung would be deprived of its blood supply for a period of time (65). Unilateral pulmonary edema is also known to be a complication of re-expansion of a pneumothorax (66). In some models lung injury leukocytes are necessary for the development of increased pulmonary vascular permeability (67) and superoxide dismutase in combination with heparin can prevent its development (68).

In the dog lung experimental model it has been noted that decreased pulmonary blood flow will increase leukocyte uptake in the lung (64). Clinically, an early transient leukopenia has been associated with ARDS (70). There is some speculation that free radical induced damage to lipid bilayers can create chemoattractants which sequester and activate neutrophils (Figure 3)(71). While all of these studies seem to implicate hyperfusion in activating neutrophils for ARDS the association with reperfusion injury has not been acceptably established.

Xanthine oxidase has been found in the lung of several species both by activity assay (72) and immunological means (73). The enzyme has been demonstrated to be localized in microvascular but not large vessel endothelium (73). Several recent studies have been designed to examine whether inhibition

of xanthine oxidase with allopurinol could prevent the development of pulmonary edema due to a variety of insults such as air-embolism, re-expansion of pneumothorax and hypoxia (72) (73) (74). Conflicting results of these studies have not yet been resolved. Nevertheless, the activity of xanthine oxidase in the lung must be further investigated in order to elucidate whether this enzyme may play a similar role in inflammatory lung disease to that which has been demonstrated in ischemia-reperfusion injury (63).

Biochemical Aspects of Xanthine Oxidase

Xanthine oxidase (XO) is one of the most well characterized and complex of the flavoproteins. It was first isolated and studied extensively in bovine milk, where it is found in great abundance In 1902 Schardinger first observed that methylene blue was reduced by formaldehyde in the presence of fresh milk. This enzyme was designated Schardinger's enzyme until it was later denoted xanthine oxidase. Xanthine oxidase contains FAD, iron and molybdenum in the molar ratios of 1:4:1 as well as inorganic sulphide in two subunits of approximately 150,000 molecular weight. Xanthine oxidase is known to have a relatively broad substrate specificity, high activity, easy purification and stability as well as low specifity for the electron acceptor. In addition to hypoxanthine and xanthine, xanthine oxidase will also oxidize many other purines, pyrimidines, pterins and aldehydes. Xanthine oxidase transfers the substrate electrons to acceptors such as molecular oxygen, cytochrome c, ferricyanide and artificial dyes such as methylene blue (77). The biological function of XO appears to be as the rate-limiting enzyme in nucleic acid degradation through which all purines are channelled for terminal oxidation.
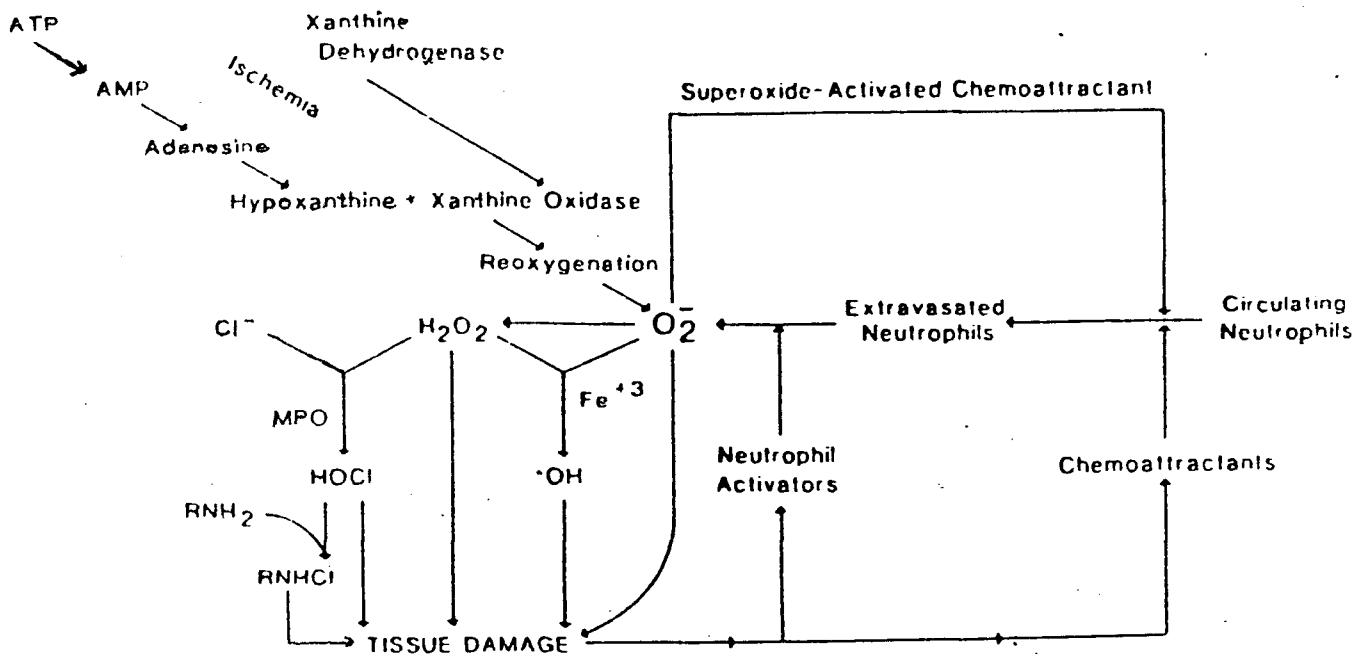
Figure 3   Activation of neutrophils by oxygen free radical derived
            chematractants. Tissue damage due to the generation of
            hydroxyl free radicals and hypohalides in though to cause
            lipid peroxidation, generating short chain fatty acid
            derivatives which will activate neutrophils leading to
            further oxygen free-radical damage. Taken from Grisham,
            Hernandez and Granger 1986 (60).

In vitro hypoxanthine and xanthine are oxidized to urate with molecular oxygen as an electron acceptor by the oxidase form. A superoxide free radical by-product is thus generated (Figure 4). This reaction proceeds by first order enzyme kinetics and is product inhibited by urate (78) (79). Xanthine oxidase has long been utilized in conjunction with hypoxanthine or xanthine as one of the best enzymatic sources of oxygen free radicals available.

Allopurinol, a drug used in the treatment of gout which acts by competitive inhibition of the enzyme is a known specific inhibitor (80) of XO useful for mechanistic studies (81) as well as allowing for regulation of the enzyme in vivo (75) (59) (82). For extensive general review of the mechanism and structure of xanthine oxidase see Bray et al. (83).

The original established source of xanthine oxidase was bovine milk. Early on it was recognized that xanthine oxidase was found in most species, and more tissues than milk (84). In the tissue xanthine oxidase is found in highest concentration in liver and small intestine, with variable levels in other tissues (72). Xanthine oxidase has been demonstrated in liver, heart, lung and kidney (85), but is virtually undetectable in serum (85) (89). Brain and cornea have the lowest activity. Xanthine oxidase is found in many species and the amino acid sequence of xanthine oxidase is remarkably well conserved, even in Drosophila melanogaster (85). It is found in a variety of human tissues most notably liver (86) and jejunum, (87), but is absent from cultured human fibroblasts and Hela cells (88). However, recently the enzyme was localized exclusively to microvascular endothelial cells using sensitive immunofluoresent techniques, (73) (87) and these have yet to be assayed in culture. The enzyme is absent from large

vessel endothelium (73).

When the enzyme was first isolated from liver it was found to exist in two forms, a dehydrogenase (XDH) with capability of utilizing NAD^+ as an electron acceptor which does not produce a superoxide radical by-product and the oxidase which cannot transfer electrons to NAD^+. The significance of these different activities had previously been ignored because early methods of isolation did not prevent the proteolytic degradation of the enzyme to its oxidase form. The milk enzyme has now been shown to have some dehydrogenase component (91) (92) when isolated by non-proteolytic methods. Normally, the enzyme is thought to exist almost exclusively in the dehydrogenase form in vivo.

Factors such as anaerobiosis, storage at $-20^{o}C$ of whole tissue homogenate and solvent treatment are known to cause conversion of the enzyme from the dehydrogenase to oxidase (93). Interestingly, some of the conversion to oxidase is reversible, indicating that there are at least two different structural alterations of xanthine dehydrogenase possible which still retain enzymatic activity (93). Further work showed that reduction of thiol containing groups of xanthine dehydrogenase by dithiothreitol could prevent conversion of the enzyme to the oxidase form caused by anaerobiosis, storage at $-20^{o}C$ and solvents. Conversion caused by proteolysis such as trypsin treatment or incubation with whole tissue homogenates at $37^{o}C$ was irreversible (94). These data thus support the proposal that there are two mechanisms of conversion, an irreversible process due to proteolytic cleavage of a polypeptide essential for binding with NAD^+ and a second process due to modification of thiols resulting in a reversible conformational change.

In the intestine of mammals xanthine dehydrogenase predominantly converts irreversibly and very rapidly to the oxidase form showing complete conversion in 16s (95), giving rise to the speculation that the enzyme may be acted upon by a specific protease in high concentration in the intestinal mucosa (96) which is low or absent in other tissues. Other tissues such as heart with 60 min conversion time may be susceptible only to non-specific proteolysis (94).

Electrophoretic studies in intestine and liver have shown that irreversible conversion of the enzyme coincides with changes in molecular weight from 150,000 Daltons to 130,000 Daltons (90) in agreement with the previous observation that liver and intestine enzyme show different electrophoretic mobility (98). When tissues are homogenized, the dehydrogenase converts to oxidase as a result of limited proteolysis which was prevented by addition of EDTA, a chelator of Ca^{2+} and other divalent cations in the rat liver model (99). Use of protease inhibitors such as soybean trypsin inhibitor prevent this proteolytic conversion (100). Current suggestions are that ischemia activates a Ca^{2+}-calmodulin mediated protease (101) which acts on the enzyme which then cannot be converted back to dehydrogenase. Inhibition of calmodulin with trifluorperazine also prevents irreversible conversion of the enzyme to the oxidase form in the rat ileum, as does treatment with soybean trypsin inhibitor (100).

It is also of note that ischemia or hypoxia cause a rise in hypoxanthine, the substrate for xanthine oxidase/dehydrogenase as a breakdown product of ATP in the lung and in the plasma (102)(103). The events occurring during an incidence of ischemia could encompass both

conversion of the enzyme to its oxygen dependent form and concommitant increase in substrate. With the reintroduction of molecular oxygen upon reperfusion of the tissue with oxygenated blood, the necessary electron acceptor is supplied for the reaction to proceed to the production of urate with superoxide radical by-products being generated. Xanthine oxidase has also been shown to be capable of mobilizing iron from ferritin by both superoxide dependent and independent mechanisms, thus potentiating the effect of free radical damage by generating the more highly reactive hydroxyl radicals (71), (Figure 4). This effect has been demonstrated to be blocked by 70% by superoxide dismutase, while uric acid removal does not affect the mobilization when a system of bovine milk xanthine oxidase and horse spleen ferritin is employed (104).

During a period of ischemia ATP levels are diminished, as documented by NMR spectroscopy studies using an ischemic rat kidney model that from the onset of ischemia until reperfusion was initated after 45 min the α, β, and γ ATP peaks virtually disappeared while the inorganic phosphate peak rose by four fold (105). Loss of cellular ATP promotes an influx of calcium when energy dependent channels which maintain the lower intracellular concentration of this cation are inactivated.

Since xanthine oxidase is known to be an active enzymatic source of oxygen free radicals and this enzyme has been demonstrated in the lung (73) it is necessary to investigate the conversion properties of xanthine dehydrogenase/oxidase in the lung in order to determine whether this enzyme may play a part in the etiology of pulmonary edema attributed to reactive oxygen species (49).

↓ Perfusion/O_2 xanthine dehydrogenase

Then:

ATP $\rightarrow$ AMP $\rightarrow$ adenosine Ca^{++} dependent
                                                proteolysis activated
                                                by ischemia
              inosine

              hypoxanthine

                                        xanthine

hypoxanthine + $2O_2$ + H_2O $\xrightarrow{\text{oxidase}}$ xanthine + $2O_2^-\cdot$ + $2H^+$
                                                        xanthine
                                                        oxidase

                                                uric acid + $O_2^-\cdot$

Reperfusion   ↑ $2O_2$

    Then:
        O_2 + Fe^{+2} $\xrightarrow{\hspace{2cm}}$ $O_2^-\cdot$ + Fe^{+3}

    and: $2O_2^-\cdot$ + $2H^+$ $\xrightarrow{\hspace{1cm}}$ O_2 + H_2O_2

        H_2O_2 + $Fe^{+2}(X)$ $\xrightarrow{\hspace{1cm}}$ $HO\cdot$ + OH^- + $Fe^{+3}(X)$ (14)
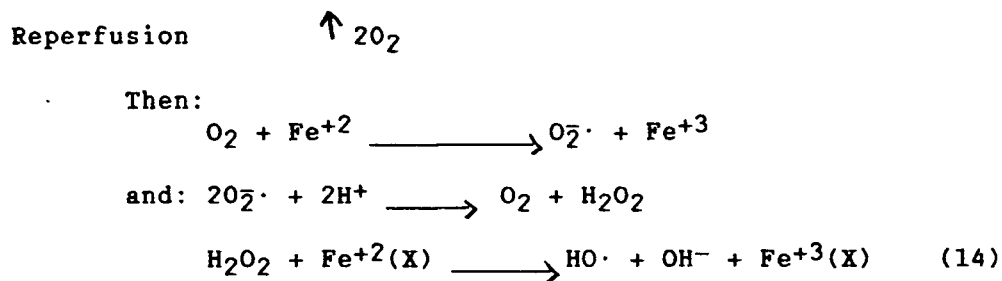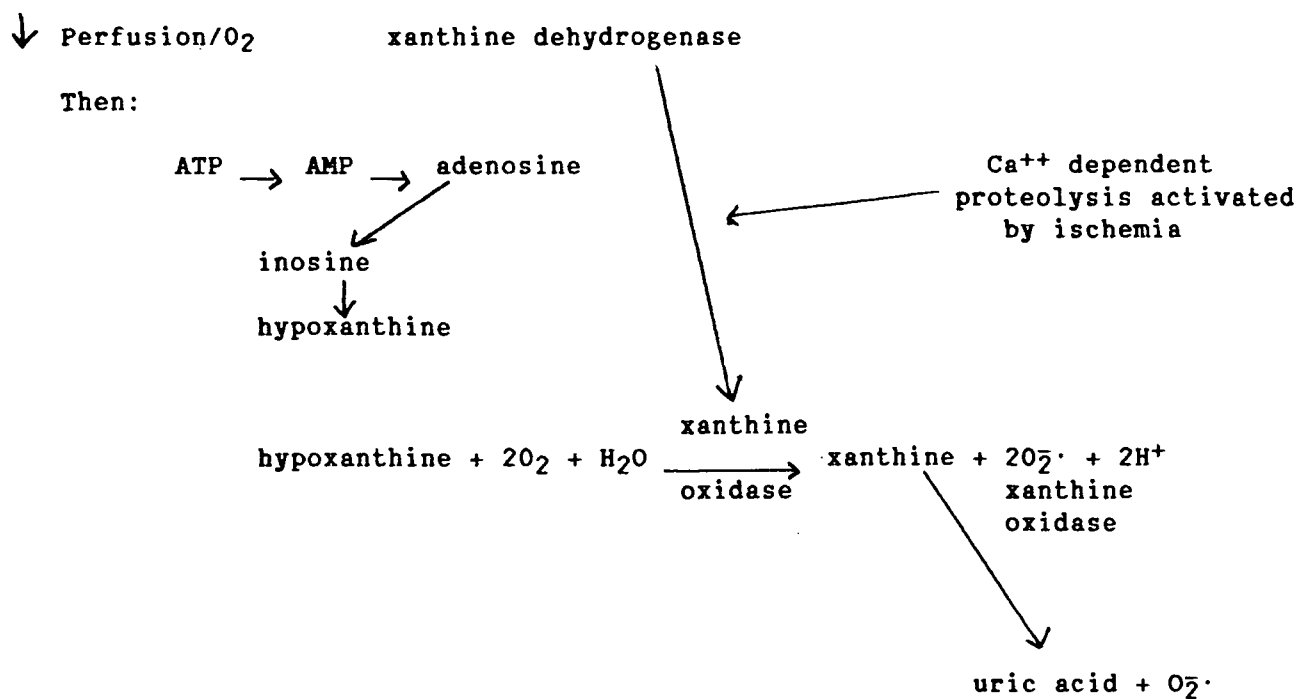
Figure 4. Reactions of XO in Ischemia and Reperfusion

PURPOSE OF THESE STUDIES

1. To determine the presence of xanthine dehydrogenase/oxidase in human, pig and rat lung and to measure its level of activity.

2. To compare enzyme content and activity in various organs within the same species using immunoreactivity, gel electrophoresis and specific enzyme assays.

3. To assess the relative content of the enzyme in the dehydrogenase or the oxidase form under standard conditions of enzyme purification.

4. To study factors which might affect conversion from the dehydrogenase to the oxidase form in the lung and to determine under what conditions the conversion can be inhibited or reversed.

METHODS

Methods of Detection of Xanthine Dehydrogenase - Oxidase Activity

Several different assays for XDH-XO have been developed and each has advantages and disadvantages which contribute to its usefulness. The earliest assay was developed in 1914 and employed the normal in vivo function of the enzyme in oxidizing hypoxanthine or xanthine to urate (106). The formation of urate in the presence of enzyme and substrate was determined by measuring the increase in absorbance at 295 nm using a molar extinction coefficient for urate of 9.6×10^{-3} cm^{-1}. The assay carried out with oxygen as an electron acceptor measures oxidase activity. Addition of NAD^{+} as the second electron acceptor measures total XDH plus XO activity. The technique was modified by Morell (106), who found removal of endogenous substrate increased activity. There are two major drawbacks to this method, the first being that it is not very sensitive and the second being that most organisms lower than man contain uricase which degrades urate to allantoin and thus production of urate cannot be accurately measured without inhibition of uricase activity.

Several more sensitive methods for the measurement of xanthine oxidase activity have been developed. These include the use of [14C]-labelled xanthine (107), use of high pressure liquid chromatography (108), a manometric assay (109) and an assay utilizing sulphite-enhanced oxygen uptake (110). A fluorometric assay developed by Lowry (111) is currently the preferred method because it is extremely sensitive due to the

highly fluorescent nature of its product and is also more convenient, since it does not require isotopes. This assay was modified by Glassman (112) who found that removal of endogenous small molecular weight substrates and inhibitors increased the sensitivity enough to make possible the determination of enzyme activity in a single Drosophila melanogaster. A modification of this assay was the first to make possible the determination of the very low levels of xanthine oxidase activity in brain (113). In an extensive comparison of several assays, Haining and Legan (114) found the modified fluorescence method (113) to be the optimal technique.

Initial studies were carried out with the urate assay until it was determined to be unsatisfactory for our purposes, since porcine tissue contains uricase. The fluorescence method as modified (113) was then used and proved to be more sensitive and less prone to interference from contaminating components.

A synthetic pterin is employed as the substrate and is enzymatically oxidized to a highly fluorescent product, isoxanthopterin (IXP). Both molecular oxygen and methylene blue act as electron accepters in this reaction allowing for determination of oxidase to dehydrogenase ratios. With oxygen as the electron acceptor oxidase activity alone is measured. When methylene blue is added as the electron acceptor both oxidase and dehydrogenase activity are measured.

Determination of XDH-XO Activity by the Determination of the Rate of Urate Production (115)

1.   The tissue was excised and trimmed. Pig liver and kidney obtained fresh from a local slaughterhouse had capsule membranes removed prior

to mincing. Pig lung had the pleural membrane removed and large airways dissected out. Whole rat kidney and livers were minced. Major bronchi of rat lungs were dissected out before mincing. The tissue was minced in four volumes of ice cold 50 mM potassium phosphate buffer (KPi), pH7.4 containing 0.1mM EDTA and 1mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. Homogenization was accomplished with a Sorvall Omnimixer at 75% speed in from 3 to 8 fifteen second bursts on ice, depending on collagen content of the tissue. Cells were broken after 10 passes of the homogenized tissue in an electrically driven Potter-Eljevheim mortar equipped with a Teflon pestle.

2.   The homogenate was fractionated by ultracentrifugation at 100,000g for 60 minutes at 4°C. Supernatant was removed by pipet.

3.   A 2 ml aliquot of supernatant fraction was loaded onto a Sephadex G-25 column (2 x 25 cm) equilibrated with 50 mM KPi + 1mM EDTA. The rate of flow of the buffer was 0.8 ml/min. A Pharmacia FRAC 300 fraction collector equippped with a protein detection unit was used to collect five fractions of 450 drops (15.6 ml) each. This fractionation removed endogenous small molecular weight substrates and inhibitors from the crude homogenate containing enzyme activity. Activity was associated with the first of two protein peaks.

4.   XDH-XO activity was determined by measuring the production of urate. The following procedure was used. The absorbance change was measured at 295 nm using a Beckman 35 Spectrophotometer with a 1ml quartz cuvette at 25°C. Recorder sensitivity range was set at 0 to 0.5

absorbance units, and the chart speed was 1 inch/min. The reaction mixture contained sample + Na-pyrophosphate (0.1M) pH 8.3 in a total volume of 990 μl + 10 μl of 10 mM xanthine in Na-pyrophosphate. The initial absorbance change measured oxidase activity. Addition of 15 mM NAD$^+$ as a second electron acceptor measures total dehydrogenase + oxidase activity. 20 μMoles allopurinol was added to inhibit the reaction and verify that the urate production was catalyzed by XDH-XO.

Calculation: $\dfrac{\Delta \text{ Absorbance 295 nm/min}}{(9.6 \times 10^{-3} \text{M}^{-1}\text{cm}^{-1})(\text{sample size in ml})} = \text{mU/ml}$

where $9.6 \times 10^{-3} \text{M}^{-1}\text{cm}^{-1}$ is the molar extinction coefficient of urate. One unit (U) is defined as a change of absorbance at 295 nm of 1. Protein concentration of samples was determined by the method of Lowry (121). Enzyme activities were expressed as mU/mg protein/min.

Determination of XDH-XO Activity by Production of IXP (113)

Preparation of tissue proceeded in the same manner as for urate assay steps 1-3.

4. An Aminco SPF-125 Spectrofluorimeter was used to record the changes in fluorescence at an excitation wave length of 345 nm and an emission wave length of 390 nm. The recorder was operated at a sensitivity of 10 mV full scale with the chart rate set at 1 cm/min.

5. Tissue samples were pre-warmed at 37°C in water bath.

6.   The reaction mixture contained:

450 µl of sample

450 µl of KPi (50mM) at 37°C

 25 µl of 900 uM pterin was added to start the reaction.

Oxidase activity was first determined and then 5 µl of a 1mM stock

of methylene blue added as a second electron acceptor to determine

the total XDH + XO activity.

7.   The fluorescent emission at 390 nm was compared to a standard curve

prepared with a known sample of IXP dissolved in 50 mM KPi pH 7.4 at

a concentration range of 0.01 to 1 µg/ml. One unit of relative

intensity was found to correspond consistently to 2.3×10^{-1} µg

IXP/ml.

8.   Initial rates of enzyme activity were determined as µg IXP/ml/min

produced. Protein concentrations of samples were determined by the

method of Lowry (122). Enzyme activities were expressed as ug IXP

produced/minute/mg protein.

9.   The production of IXP could be inhibited by addition of 20 µMoles

allopurinol, thus confirming that the increase in emission at 390 nm

was due to the catalytic action of XDH + XO.

10.   The proportion of XO activity in the tissue samples was expressed as

a ratio of total enzyme activity: $\dfrac{\text{XDH + XO}}{\text{XO}}$ in order to give a linear

rate of conversion.

Experiments Using Purified Xanthine Oxidase

Purification of Xanthine Oxidase A highly purified preparation of xanthine oxidase was required for a base of comparison for the tissue and samples and to use as an antigen for the preparation of a polyclonal antibody. The purification method chosen was based on the non-proteolytic method of Waud, et al. (116) Commercially available sources of XO are known to be contaminated with variable amounts of proteases (117) and thus could not be used for these purposes. The procedure was carried out as follows:

1. 16 liters of raw fresh milk was obtained from the UBC Dairy Barn and centrifuged at 3000 rpm for 10 minutes. This resulted in the separation of 425 ml of cream.

2. Equal volumes of 200 mM K_2HPO_4, 4 mM cysteine-HCl, 2 mM sodium salicylate pH 9.0 containing 0.01% EDTA were added at $80^{\circ}C$.

3. This mixture was cooled to $7^{\circ}C$ and 135 ml of butanol at $4^{\circ}C$ was added with continuous stirring. 10N NaOH was added to maintain the pH at 9.0.

4. 155.25 g of ultrapure ammonium sulphate was slowly added with stirring and the pH of the mixture adjusted to 9.0 with 10N NaOH. Stirring continued for 20 minutes followed by centrifugation at 13,700g and $4^{\circ}C$.

5. The supernatant buttermilk fraction (700 ml) was filtered through glass wool to remove fat globules. Solid ammonium sulphate (135g) was added to the filtrate. This was stirred for 30 min. and the precipitate collected by centrifugation at 13,700 g for 20 minutes.

6. The pellet was suspended in a minimal amount of 50 mM K_2HPO_4 containing 1 mM salicylate and 0.005% EDTA. The suspension was clarified by centrifugation and applied to a Sephadex G-25 ion exchange column that was equilibrated with the same buffer in order to remove residual butanol. The protein eluted from the G-25 column after a volume of 330 ml.

7. The above protein component was applied to a DEAE - Sephadex A-50 column equilibrated with 5mM potassium phosphate pH 7.8 containing 0.005% EDTA and 1mM salicylate. It should be noted here that by accident the cold room temperature dropped to -10° during the sample elution and the column was partially frozen. Despite this problem the enzyme remained active but eluted from the column differently than expected. Because XO contains a flavin prosthetic group, the enzyme could be detected visually by the elution of a bright yellow flavin-containing protein fraction which eluted from the column with the equilibration buffer rather than after gradient elution. The linear gradient between 5mM and 100mM potassium phosphate buffer was not applied therefore. Active fractions were pooled and concentrated using an Amicon PM-30 membrane.

8. 5 ml of sample was chromatographed on a Sephadex G-200 column equilibrated with 50 mM K phosphate pH 7.8 + 0.005% EDTA + 1mM salicylate. Xanthine oxidase activity was contained in four fractions eluted between 32 ml and 56 ml from column. Enzyme activity was determined using both the urate assay and the IXP assay. Molecular weight determinations were carried out using the

Pharmacia PhastSystem SDS-PAGE. This technique was also used to check purity of protein. Pooled fractions were stored as precipitates in 50% ammonium sulphate.

Preparation of Polyclonal Antibody to Purified Bovine Xanthine Oxidase

In order to identify specifically XO in tissue samples an antibody to XO was used against proteins separated by SDS-PAGE gel electrophoresis and transferred by blotting to nitrocellulose sheets. XDH could also be identified since polyclonal antibody to XO has also been shown to cross react with XDH (87). One ml of the partially purified homogenate fraction containing the highest activity of XO was electrophoresed on SDS-dissociating gel with mercaptoethanol 1.5% per volume using the method of Laemmli (118). The gel was lightly stained with Coomassie blue to locate the protein bands. The band containing protein of a molecular weight of 135,000, where XO is known to run, was cut out and lyophilized overnight. Protein was electroeluted from the gel through an agarose gel (1%) and into an attached length of dialysis tubing (20 cm in length and a molecular weight exclusion of 10,000) using a Buchler apparatus. Electroelution was conducted for two days at 4°C, 20 mA, in SDS electrophoresis buffer which was changed every 24 hours. When all the blue stained protein had migrated into the dialysis tubing, the tubing containing protein was removed, knotted and dialyzed for two days against 1 l of distilled H$_2$O. Distilled water was changed every 24 hours.

Purified antigen was emulsified in Freunds complete adjuvant (1 mg protein/ml). One ml of emulsion was injected intramuscularly into a rabbit

at several sites around the body. A blood sample of 15 ml was taken from the rabbit prior to injection to prepare a sample of non-immune serum to be used as a control. Rabbit was caged individually in the animal care facility of St. Paul's Hospital and given water and standard laboratory chow ad libatum. The animal received booster injections of purified antigen in saline every two weeks (0.5 mg in one ml)(119). The antibody titer was measured at these times using ELISA technique (120). After eight weeks a maximal antibody response had been reached and the rabbit was exsanguinated by cannulation of the central ear artery. Blood collected (120 ml) was allowed to clot at 37°C, centrifuged at 1000g for 20 minutes and the serum collected. Aliquots were frozen at -20°C for later use.

Gel Electrophoresis of Tissue Homogenates and Western Blotting with Polyclonal Antibody to Purifed XO

In order to look for immunoreactive XO in various species and organs separation of tissue homogenates was accomplished using SDS denaturing polyacrylamide gel electropheresis (SDS-PAGE) in conjunction with immunoreplication using Western blotting and polyclonal antibody raised against purified XO.

Gel electrophoresis was run using the modified system of Laemmli (118), (121). The following method for sample preparation was used:

1. Fresh tissue obtained from. slaugterhouse, human lung biopies or sacrifice of rats was dissected and finely minced in four volumes of 50mM KPi buffer pH7.4 containing 1mM PMSF and 0.1 mM EDTA at 4°C. Minced tissue was rinsed and filtered through cheesecloth to remove

blood. The retentate was then homogenized in a Sorval Omnimixer (75%
maximum speed 3 to 8 times for 10 seconds each depending on
connective tissue content) on ice and then cells were disrupted using
a Potter-Eljveheim for 10 passes on ice.

2.   Protein was precipitated by the addition of ice cold 33%
trichloroacetic acid at a volume to volume ratio of 1:5 TCA:
homogenate. The pellet was then suspended in electrophoresis sample
buffer of 20 mM Tris pH 6.8, 2.5% SDS and 10% glycerol with the pH
adjusted to between 6 and 8. These samples were heated at $100^{o}C$
for 5 minutes to denature proteins. Samples were clarified by
centrifugation. Protein determinations were made by method of Lowry
(122) and sample volumes adjusted to give a final protein
concentration of 1Ug/µl. A 2:1 mixture of 1.5% beta
mercaptoethanol with bromphenol blue. was added. Prior to
electrophoresis, samples were heated to $100^{o}C$ x 5 min and
centrifuged to clarify in an Eppendorf table top centrifuge. A
molecular weight standard mixture (Sigma, range 60,000 to 220,000
molecular weight) were used for molecular weight determination and
prepared as described above. 100 ug samples were applied in the
slots of the 5% stacking gel of 6% PAGE-SDS slab gels run in
duplicate with electrophoresis buffer containing 0.050M Tris, 0.384 M
glycine with 0.1% SDS. Gels were subjected to a current of 70m amp
for 3 hours. After electrophoresis, one gel is stained with
Coomassie Blue to show protein bands while the other is processed for
immunoreplication.

Immunoreplication Method

Gel was soaked in electrotransfer buffer of 10 g Tris, 48g glycine, 600 ml methanol in 2400 ml H_2O. Gel was then layered with Biorad Immobilon transfer membrane between sheets of Whatman filter paper and supports. The cassette was subjected to a current of 1 amp for 90 min in electrotransfer buffer. When subjected to a strong current, negatively charged proteins will be removed from the gel and transfered to the filter as they migrate towards the cathode.

Immobilon filter is then processed according to Biorad Immuno-Blot Assay techniques. Non-specific protein binding sites are blocked with 3% gelatin. Blot is then incubated overnight at room temperature with 100 ml of a 1:500 dilution of polyclonal rabbit antisera against purified XO (anti-XO). After washing, the filter is then incubated with a second antibody, goat anti-rabbit IgG, covalently linked to horseradish peroxidase (Biorad). 4-chloro-1 napthol substrate (HRP substrate, Biorad) was added which leaves a coloured product at site of antibody binding. Blots were then photographed using black and white print to maintain a permanent record. Photographs were compared for presence of antibody reaction and varying intensity of reaction between tissues.

Comparison of Sensitivity of Urate Assay to Isoxanthopterin Assay

Purified XO was used to test the limits of detection of the urate assay (115) versus the IXP assay (113). Serial dilutions of purified XO (diluted 1:1000) were suspended in the appropriate buffer (Na pyrophosphate pH 8.3 for urate assay or 50 mM KPi pH 7.a4 for IXP assay). 25μ moles pterin was added to start reaction.

Dilutions were as follows:

1.   1:1000 dilution of XO stock = 1 µl in 1 ml buffer

                                 = 24.5 µg XO protein.

2.   1:1 dilution of above   = 0.5 µl in 1ml buffer

                                 = 12.25 µg XO protein

3.   1:1 dilution of above   = 0.25 µl in 1ml buffer

                                 = 6.12 µg XO protein.

4.   1:1 dilution of above   = 0.125 µl in 1 ml buffer

                                 =3.06 µg XO protein.

Both the spectophotometer and spectrofluorimeter were used at the highest sensitivity range of absorbance.

Product Inhibition of IXP Assay

Urate is known to be an inhibitor of XO activity (78) (79). It was important to determine therefore, whether IXP would be similarly competitive with the substrate pterin for XO. Purified XO activity was measured by IXP assay with no additions as a control and in the presence of 3nm IXP. Reaction cuvette contained 10 µl of 1:1000 dilution of purified XO in 900 µl of 50 mM KPi. Twenty-five µ moles pterin was added to start the reaction. The rate of reaction in the control cuvette was compared to rate of reaction in cuvette containing the same amounts of enzyme and pterin with the addition of 3 nm IXP.

Experiments in Porcine Tissue

Assay for XO-XDH Activity in Pig Tissue

Pig heart, lung, kidney and liver were obtained fresh from a local abattoir and transported to the laboratory in ice cold KPi buffer where they were prepared as described in methods (113). Each assay contained 450 μl tissue extract, 450 μl KPi buffer and 25 μ moles pterin in a total volume of 900 μl. The assay was conducted at 37°C. After measuring the aerobic rate of reaction, 5 μ moles methylene blue was added to determine the dehydrogenase activity. In order to confirm the specificity of the reaction for XDH-XO

20 uM allopurninol was added to inhibit the activity attributable to XDH-XO.

Inhibition of Purified XO Activity by Pig Extracts

These tests were carried out in order to ascertain whether the lack of activity found in some semi-purified organ extracts could be due to interference from impurities so that rates of reaction measured did not accurately reflect the actual activity. In order to determine if pig lung extracts could inhibit purified XO activity in vitro, XO was assayed in either KPi buffer alone or buffer plus aliquots of homogenized tissue preparations. Rates of reaction were compared between the following:

1. 10 μl of 1:1000 dilution of purified XO KPi + 900 μl of 50 mM KPi + 0.1 mM EDTA 25 uM pterin.

2. 10 μl of 1:1000 dilution of purified XO in 50 mM KPi + 0.1 mM EDTA + 900 μl of pig lung extract prepared as described in methods. 25 μ moles pterin.

Heat Stability of Active Factors in Pig Tissue

In order to determine if factors in pig tissue which affected the catalytic activity of purified XO were labile to heat, an aliquot of homogenate from pig tissue was incubated in boiling water for 20 minutes. The rates of reaction mixtures were compared as follows:

1.   10 µl of 1:1000 dilution purified XO + 900 µl of 50 mM KPi + 0.1 mM EDTA 25 uM of pterin.

2.   10 µl of 1:1000 dilution purified XO + 900 µl of pig lung extract denatured by boiling for 20 minutes, cooled and centrifuged to remove coagulated proteins + 25 µ moles pterin.

Effect of Pig Tissue Extract on Relative Fluorescent Intensity of Isoxanthopterin

In order to determine whether a component or compounds contained in pig tissue extract could either quench or degrade the product measured in the fluorometric assay (IXP) a comparative determination was conducted. The relative intensity of 3nM of IXP was measured in 900 µl of 50 mM KPi buffer. This was compared to the relative intensity of 3nM of IXP in the presence of 900 µl of pig lung homogenate. The reaction was recorded at 37°C for 10 minutes.

Assay for XO/XDH Activity in Cultured Pig Pulmonary Artery Endothelial Cells

In order to ascertain whether large vessel endothelium in culture contained XO/XDH activity, porcine pulmonary artery endothelial cells were cultured under conditions previously established in this laboratory (123).

Fresh porcine heart-lung sets were obtained from a local abbatoir, and transported at 20-22°C to the laboratory. The pulmonary artery was excised, rinsed briefly in phosphate buffered saline (PBS) and placed in Gibco Hanks balanced salt solution (HBSS) (Gibco) supplemented with penicillin and streptomycin in a Laminar Flow Hood using sterile instruments. Arteries were placed on a gauze covered Petri plate and slit lengthwise. Endothelium was removed from the luminal surface by gentle scraping with a Number 22 surgical blade and sheets of cells placed in sterile HBSS. Cells were centrifuged and the pellet resuspended in Medium 199 (Gibco) supplemented with 20% pig serum and seeded in T75 flasks which had been previously coated with 1% gelatin on the culture surface. Cells were grown in a humidified incubator at 37°C in an atmosphere of 95% air: 5% CO_2. After 24 h most of the cells which remained unattatched to the culture surface were washed free using HBSS. Most of the unattached cells were contaminating fibroblasts and smooth muscle cells. Cells were grown to confluence. One artery provided the cells to seed two T-75 flasks. The average time required to reach confluence was 10 days. At confluence cells were removed with a rubber policeman and reseeded at a ratio of 1:2. In addition to selection by detachment cells at confluence were identified by these characteristics of endothelial cells: 1) their characteristic cobblestone monolayer growth pattern and 2) by the detection of Factor VIII by immunofluorescence (124). Cells for these experiments were used at Passage 3.

For assay, seven T-80 flasks were used with, the cell number being estimated at between 1.1 x 10^7 to 1.8 x 10^7 cells per flask. After

rinsing with HBSS monolayers were removed by scraping with a rubber policeman and the cells suspended in 5 ml of ice cold 50 mM KPi containing 1 mM EDTA. Clumps of cells were broken up using a syringe. This cell suspension was then homogenized by 20 passes with a Teflon pestle in a glass mortar and sonicated for 2s with a Sonifier Cell Disrupter. This preparation was fractionated by ultracentrifugation at 100,000g for 60 min. at 4°C. A

2 ml aliquot of the supernatant was run on a Sephadex G-25 column equilibrated with 50 mM KPi containing 0.1mM EDTA. The equilibration buffer was applied to the column and 15.6 ml fractions were collected. Fraction 2 which contained the first protein peak was assayed for XO-XDH activity by the IXP assay method. 900 μl of Fraction 2 were assayed with 25 μ moles pterin + 5uM methylene blue added to determine XDH activity at 37°C. Fraction 2 was also concentrated three fold using a Spectra-Con Disposable Sample Concentrater (MW limit 8000) and assayed as above.

Isolation of Porcine Pulmonary Microvascular Cells

Because it has been reported on the basis of immunocytochemical localization that XO XDH is found in microvascular but not large vessel endothelial cells (73) we attempted to culture pulmonary microvascular endothelial cells in order to assay for enzyme activity in vitro. Several methods of obtaining capillary endothelium for culture have been developed (125) (126) (127). However, these are unsatisfactory due to potential contamination from large vessel endothelium. The development of a new method of microvascular isolation was attempted based on the observation by

light microscopic examination that to a depth of less than 1 cm from the pleural surface of the pig lung only vessels less than 50 μm diameter are found (128). The methods of microvessel isolation used was based on techniques developed for the isolation of rabbit pulmonary Type II alveolar cells (129) and guinea pig sinusoidal endothelial cells from the liver (130) by using enzymatic dissociation of tissue in combination with centrifugal elutriation (131). This technique depends on the finding that different cell types often have different sizes and densities. Separation by centrifugal elutriation is based on the different rates of sedimentation of particles in a centrifugal field balanced by a counterflow so that particles are maintained in suspension. Fractionation of a mixed cell population is dependent on cell size and can be accomplished by stepwise increases in flow rate (129) A Beckman Elutriator was used. Adult porcine peripheral lung tissue was prepared in the following manner:

Porcine lung tissue was obtained fresh from a local slaughterhouse. Peripheral lung tissue (less than 1 cm from pleural surface) was dissected free of pleura. Fifteen to twenty g of tissue was rinsed in cold PBS without Ca^{2+} or Mg^{2+} (100 ml). Tissue was minced finely in cold PBS containing Ca^{2+} and Mg^{2+} (0.1 g/l each) along with 0.25% BSA, 1 mg/ml collagenase and 1000 μg DNAse (50ml). This mixture was incubated at $37^{o}C$ for 30 minutes. Large chunks of tissue were then filtered out with sterile gauze and the filtrate retained. The filtrate was examined under a phase contrast micrscope to gauge the degree of digestion. When chains of single cells were seen (capillary fragments) the dissociation was judged to be completed. Fifty ml of the above BSA:PBS: collagenase mixture was added

and incubated for approximately another 30 minutes until clusters of 4-5 cells were seen. The cell suspension was then equally divided into six 50 ml screw cap centrifuge tubes. PBS-BSA was added up to a total volume of 50 ml in each tube. Tubes were centrifuged at 300 g for 10 minutes and the supernatant discarded. The cell pellet was washed twice with PBS-BSA and centrifuged again as above. The supernatant was discarded and all pellets were pooled and resuspended in 10 ml of PBS-BSA to be loaded into the elutriator. The elutriation buffer contained: HBSS supplemented with 0.05% DNase by weight and 0.5% BSA at 15°C. During loading the elutriator rotor was spun at 2500 rpm and the flow pump was set at 5 ml/min. The following fractions were collected:

	Flow	Volume
(1)	~13.5 ml/min	150 ml
(2)	~19.5 ml/min	250 ml
(3)	~23.5 ml/min	200 ml
(4)	purge elutriator at zero rpm, ~ 50 ml/min 100 ml	

Fraction 2 was found to contain the endothelial cells. This fraction was centrifuged at 300 g for 10 minutes. The pellet was suspended in 5 ml of culture medium containing 50 ml of endothelial conditioned Medium 199, 50 ml of regular Medium 199, 0.002 g Endothelial Derived Growth Factor (Meloy), 1 ml .1% heparin, 1 ml Fungizone (Squibb) and 20 ml porcine serum (Gibco). Cells in 4 ml of the above medium were plated onto gelatinized T-25 flasks. These seeded flasks were incubated at 37°C for 60 minutes. Unattached cells were then removed by washing with HBSS and cells resuspended in 5 ml of the above medium.

Cells from Fraction 2 were noted to grow with the appearance of newly seeded large vessel endothelial cells in culture. However, cultures did not grow well enough to reach confluence or passage. In addition, cultures became contaminated possibly due to pulmonary infections in pigs before slaughter. An attempt was made to accelerate the growth rate of the cell colony by using newborn (5 day old) pig tissue which has been shown to have greater growth potential than adult tissue. In addition, we used homologous newborn pig sera to supplement culture media since fetal bovine serum is widely used as a supplement because it contains growth factors not found in adult sera. Since this was not commercially available the sera was prepared from the same animal which lung tissue was collected from. Piglet weighed 2 kg and was anaethetized with 1.5 cc sodium pentobarbital.

The pig serum growth supplement was prepared as follows:

Blood was collected into a sterile syringe via the inferior vena cava, allowed to clot thoroughly at 4°C and centrifuged. Serum was collected and heat inactivated at 56°C for 30 minutes then cold sterilized using a 22 micron filter. Serum was then aliquoted and frozen for use as required to supplement artifical cell culture media by 20%. By preparing tissue and serum in the research and cell culture facility on site, it was hoped to eliminate the problem of contamination during killing of the animal and removed of tissue by using sterile conditions and by preparing tissue and serum in the St. Paul's Hospital surgical research and cell culture facilities.

EXPERIMENTS IN RAT TISSUE

Characterization of IXP Assay in Tissue

Rat kidney was used to test the IXP assay system in tissue since this is a well studied source of XDH-XO (54). Reaction mixture was composed of:

450 µl rat kidney extract (119)

450 µl 50 mM KPi + 0.1 mM EDTA

25 µ moles pterin for oxidase activity

5 µ moles methylene blue added to measure dehydrogenase
    activity

20 µ moles allopurinol was added to stop reaction.

Determination of XDH-XO Level in Normal Rat Lung by IXP Assay

Normal rat lung was quickly excised, homogenized and processed according to Markley (113). Tissue extracts were assayed by IXP method to determine the baseline level of XDH-XO activity. Reaction mixture contained:

1. 450 µl rat lung extract

    450 µl 50 mM KPi + 0.1 mM EDTA

    25 µ moles pterin

2.   5 µ moles methylene blue was added to complete the reaction
        and determine XDH activity

3. 20 µ moles allopurinol was added to inhibit XDH-XO.

Investigations into Conversion of XDH to XO

In order to investigate factors affecting conversion of XDH to XO the rat lung was chosen since the enzyme was found to be easily detectable in this readily available tissue. Based on assays published in 1972 using rat liver (93) (94), it was decided to examine the role that proteolysis and Ca^{++} might play in affecting enzyme conversion. This was correlated with two ischemic tissue models. Reversibility of enzyme conversion attributable to redox states of thiol groups was studied with the use of dithiothreitol (DTT) to cause reduction of thiol groups in the enzyme and affect reversal back to XDH activity.

Effect of Trypsinization on Conversion of XDH to XO

Rat lung extracts prepared in 50 mM KPi pH7.4 in the absence of EDTA or protease inhibitors were incubated with trypsin purified from beef pancreas (BDH). 100 µg trypsin and 1ml of extract were incubated for 60 min at 37°C with gentle agitation.

Samples were compared to the same extracts without trypsinzation. The possibility of reversibility of enzyme conversion by a reducing agent was examined by incubating trypsin-treated extracts with 1mM DTT for 60 min. at 37°C prior to assay.

Reaction mixtures contained:

1. 250 µl rat lung homogenate and 650 ul KPi 50 mM were incubated at 37°C for 60 min. 25 µl of 15 µM pterin was added to initiate the oxidase reaction. 5 µl of 1 mM methylene blue was added to measure dehydrogenase plus oxidase acitivity.

2. 250 µl rat lung homogenate with 650 µl KPi 50 mM was incubated with 100 µl of a trypsin solution from a 10 mg/ml stock at 37^oC for 60 min. 25 µ moles of pterin was added to determine the oxidase activity and then 5 µl of methylene blue was added to measure dehydrogenase plus oxidase activity.

3. 250 µl of rat lung homogenate with 650 µl of KPi 50 mM was incubated with 100 µl of the above trypsin stock for 60 min at 37^oC. This sample was then incubated with 1 mM DTT for an additional 60 min at 37^oC. To this 25 µl pterin was added to measure the oxidase activity and then 5 µl of methylene blue was added to determine total XDH + XO activity.

Effect of Endogenous Proteases on Conversion of XDH to XO

In order to examine the effect of endogenous proteases on the conversion of XDH to XO samples were incubated at 15^oC overnight. This temperature was chosen because ambient room temperature (93) has been shown to inactivate XDH-XO, while 4^oC inhibits protease activity. Samples were compared to extracts from the same animal which had not been incubated at 15^oC overnight as control. The extracts were incubated with 1mM DTT in order to assess if enzyme conversion was reversible.

Reaction mixture contained:

1. 450 ul rat lung extract prepared in 50 µm KPi, with no added EDTA incubated at 15^oC for 16 hr.

    450 µl KPi pH 7.4 50 mM 37^oC

    25 µl pterin

    5 µl methylene blue added

2. 450 μul same rat lung extract not incubated at 15$^{\mathrm{o}}$C + 450 μl

    KPi pH 7.4 50 mM 37$^{\mathrm{o}}$C

    25 μl pterin

    5 μ methylene blue added

3. 450 μl same rat lung extract x 15$^{\mathrm{o}}$C overnight) + 450 μl KPi

    50 mM pH 7.4 incubated with 1mM DTT X60 min at 37$^{\mathrm{o}}$C

    25 μl pterin

    5 μl methylene blue added

Effect of Calcium on Conversion of XDH to XO

In order to assess whether calcium might play a role in enzyme
conversion in the rat lung, as suggested by McCord (52) two known calcium
chelating agents which have different affinities were used. Samples with no
calcium chelater present were used as a control. The XDH XO fluorescence
assay method of Markley (113) specifies the addition of EDTA as a chelator
of divalent cations to all homogenization and sample buffers. It was of
interest to ascertain whether the same amount of EGTA, a more specific
chelator of calcium would have a more protective effect on conserving enzyme
in the XDH form. Tissue extracts prepared in the absence of EDTA or EGTA
served as a control. Reversibility of enzyme conversion by the reduction of
sulphydryl groups was tested by the addition of 1mM DTT to tissue extracts
prepared with EDTA, EGTA and in the absence of calcium chelators followed by
incubation at 37$^{\mathrm{o}}$C x 60 min.

Reaction mixtures contained:

1.   450 µl rat lung extract prepared in 50 mM KPi

      450 µl KPi pH 7.4

       25 µl pterin

        5 µl methylene blue added

2.   450 µl above extract, 450 µl KPi pH 7.4, incubated with

      1 mM DTT at 37°C x 60 min.

       25 µl pterin

        5µl methylene blue added

3.   450 µl rat lung extract prepared in 50 mM KPi

      with 1mM EDTA added to all homogenizing, chromatography

      and sample buffers

      450 µl KPi pH 7.4 50 mM + 1mM EDTA

       25 µl pterin

        5 µl methylene blue

4.   450 µ above extract with 450 µl KPi pH 7.4, 50 mM + 1mM EDTA

      incubated with 1 mM DTT at 37°C for 60 min

       25 µl pterin

        5 µl methylene blue added

5.   450 µl rat lung extract prepared in 50 mM KPi with 1mM EGTA

      added to all homogenizing, chromatography and sample buffers

      450 µ KPi pH 7.4 50 mM + 1mM EGTA

       25 µl pterin

        5 µl methylene blue added

6.   450 µl above extract with 450 µl KPi ph 7.4 50mM + 1mM EGTA

incubated with 1 mM DTT at 37°C for 60 min

25 µl pterin

5 µl methylene blue added

Effect of Ischemia on Conversion of XDH to XO

We sought to determine whether ischemia might have an effect on conversion of XDH to XO by the use of two models of ischemia. (A) The first involved incubation of excised lung tissue in PBS at 37°C in a humidified incubator under normoxic conditions for 60 min post-sacrifice, according to the method of Granger (132). (B) The second method involved perfusion of the rat lung via a pulmonary artery cannula with an oxygen depleted buffer mixture of 50 mM KPi pH 7.4 at 37°C through which N_2 had been sparged for 20 min and clamping of the pulmonary artery for 60 min. (C) These were compared to rat lung extracts prepared from lungs which were rapidly cannulated and perfused with cold 50 mM KPi pH 7.4 containing 1 mM EDTA and 1 mM DTT and immediately homogenized in the same buffer containing 1 mM PMSF as a protease inhibitor. Samples were then compared to those incubated with 1 mM DTT at 37°C x 60 minutes to examine the reversibility of XDH to XO conversion.

Sample mixtures contained:

1.   450 µl of rat lung extract prepared by Method (A) with 450 µl of KPi buffer

25 µ moles pterin

5 µ moles methylene blue

2. 450 μl of rat lung extract (A) with 450 μl of KPi buffer

    incubated with 1 mM DTT at 37°C for 60 min

    5 μ moles of methylene blue

3. 450 μl of rat lung extract prepared by method (B) with 450 μl of

    50 mM KPi pH 7.4

    25 μ moles pterin

    5 μ moles methylene blue added

4. 450 μl of rat lung extract (B) with 450 μl of 50 mM KPi, pH 7.4

    incubated with 1 mM DTT at 37°C 60 min

    25 μ moles pterin

    5 μ moles methylene blue added

5. 450 μl of rat lung extract (C)(EDTA + DTT+)

    450 μl of 50 mM KPi pH 7.4 + 1 mM EDTA

    25 μ moles pterin

    5 μ moles methylene blue added

Experiments in Human Lung Tissue

It was of great interest to determine whether active XDH XO was detectable in human lung tissue, since the enzyme as yet has only been localized using immunohistological means (72) (73). Human lung samples of approximately 6-10 g were obtained from peripheral lung sections of patients undergoing pneumectomy or lobectomy for bronchogenic carcinoma. Samples were immediately frozen at -70°C upon arrival to be processed at a later date. Thawed samples were minced and homogenized in cold buffer containing 50 mM KPi pH 7.4 and 1 mM EDTA with 1 mM PMSF, and processed according to the method of Markley (113).

Since activity was much lower than rat lung, the spectrofluorimeter was used on the most sensitive setting.

Reaction mixtures contained:

900 µl of human lung extract

25 µ moles of pterin

5 µ moles of methylene blue

Since many biopsy samples were obtained from tissue which was already necrotic, viability of tissue was determined by assessing the capacity of the tissue to oxidize NADH. NADH (0.1 mM) was added to human lung tissue homogenates and the oxidation of NADH to NAD was measured spectrophotometrically by recording the rate of change of absorbance at 340 nm.

Statistical Analysis

SE was calculated for all mean values of enzyme activity. Comparisons between experimental groups and controls were based on students t-test of xanthine oxidase activities for the studies of enzyme conversion.

RESULTS

EXPERIMENTS WITH PURIFIED XO

Isolation of Milk Xanthine Oxidase

After fractionation of milk protein as described in methods pooled fractions with xanthine oxidase activity obtained had a total protein of 86.8 mg (122). Activity of pooled fractions was 4.78 U/mg protein. One unit is defined as giving a ΔA of one per minute at 295 nm during the conversion of xanthine to uric acid (115).

Ratio of A280 to A450 (protein to flavin ratio) of the purified XO was determined to be 5.75 which is higher than the value of 4.8 reported by the authors of the method (116). These results suggest a higher level of specific activity, due to more flavin-containing active subunits.

SDS-PAGE revealed a single band of 135KDa (Figure 5), the known size of the xanthine oxidase subunit. Protein concentration of ammonium sulphate precipitates dialyzed on Millipore membranes was determined to be 25.4 mg protein /ml. Activity of precipitates was 54 U/ml/min as determined by urate assay; or 69.11 µg IXP produced /min/mg protein by fluorometric assay (113). In accordance with the as per method of Beckman (91), milk xanthine oxidase was found to have up to 10% dehydrogenase activity when samples were incubated with 1 mM dithiothreitol (DTT) at 37°C for 60 min prior to assay.

Characterization of Polyclonal Antisera to Xanthine Oxidase

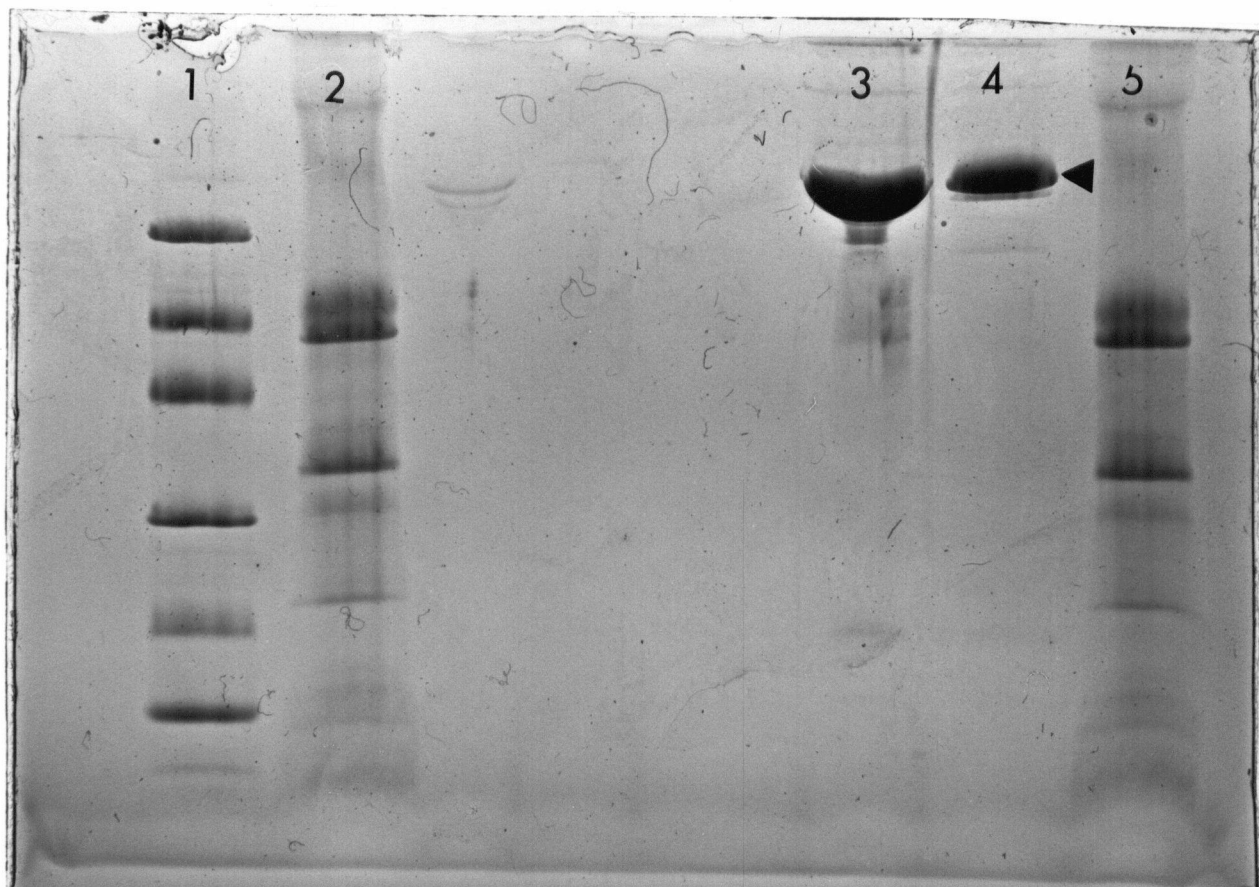When a rabbit was immunized with purified milk XO which had been

Figure 5. Pharmacia Phast System SDS-PAGE analysis of purified bovine milk
XO on a 8-25% gradient gel stained with coomassie blue. Lane 1
LMW markers (from top to bottom) phosphorylase b, 94,000, albumin
67,000, ovalbumin 43,000, carbonic anhydrase 30,000 trypsin
inhibitor 20,000, lactalbumin 14,000. Lanes 2 and 5 contain high
molecular weight markers (Top to bottom) ferritin, 220,000,
albumin 67,000, catalase 60,000, lactate hydrogenase 36,000.
Lane 3:2 μg of purified bovine milk XO. Lane 4: 1μg of
purified bovine milk XO (arrow).

SDS-dissociated, the antibody titer of immune serum increased 5000 fold during the 8 weeks of immunization, as determined by the ELISA technique. The titer against native XO increased 2000 fold increased, in keeping with the fact that the antibody was raised against SDS-dissociated antigen, and thus more determinants were uncovered. At exsanguination, 120 ml of whole blood was collected from which 60 ml of immune serum was obtained.

In tests using immune serum as a first antibody in Western Blotting, anti-XO antiserum was strongly reactive with purified bovine milk XO and XDH separated on SDS-PAGE gels reflecting molecular weights of 130,000 and 150,000 Da respectively (Figure 6). The antibody also reacted with smaller proteolytic fragments of XO. In tests using tissue homogenates and purified bovine serum albumin (BSA) the antibody was strongly reactive against the albumin of several species which ran at 67,000 Da (Figure 6).

SDS-PAGE of Tissue Homogenates with Western Blotting

SDS-PAGE of tissue homogenates containing 100 µg of protein per sample revealed that antiserum to bovine XO cross reacted with bovine lung tissue and bovine milk (powdered, Safeway Brand), showing reactivity at 150,000 and 130,000 Da, as well as at characteristic proteolytic fragment of XO at 90,000 Da (87) (Figure 7) which is a major fragment of the enxyme.

When rat tissue homogenates containing 100 µg protein of rat lung, kidney, liver and heart were examined by SDS-PAGE, XO reactive bands were seen in all tissue samples at 150,000 and 130,000 Da as well as at 90,000 Da (Figure 8).
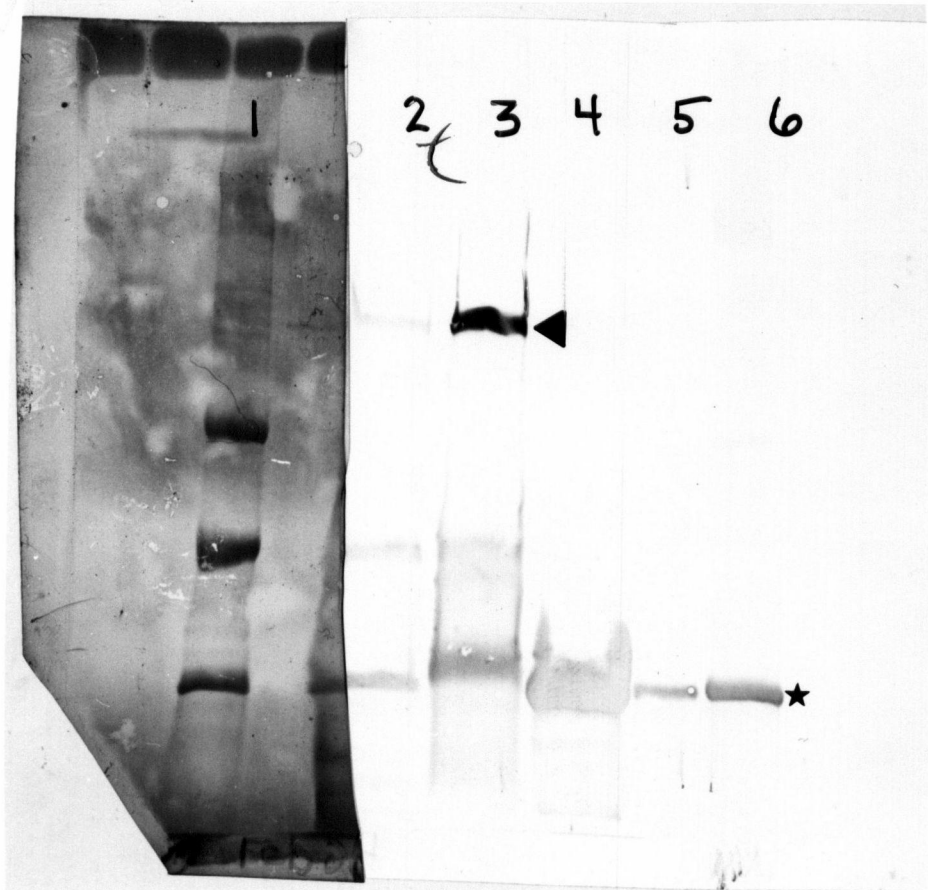
Figure 6. Western blot of purified bovine milk XO. Electrophoresis of proteins was carried out on 6% SDS-PAGE. Protein was electrotransfered onto Immobilon Membrane (Millipore). XO was visualized by incubation of the Western blot with a :500 dilution of polyclonal rabbit anit-bovine XO serum followed by incubation with the second antibody, goat-anti-rabbit IgG, covalently linked to horseradish peroxidase. Addition of substrate produces a purple coloured product bound to the membrane at site of antigen location. Lane 1 high molecular weight stained with amido black. (Top to bottom) myosin 205,000, β-galactosidase 116,000, phosphorylase B 97,000, BSA 66,000, ovalbumin 45,000. Lane 2, 3, XO (arrow). Lane 4, 5, 6 albumin showing reactivity of antibody to albumin (star).
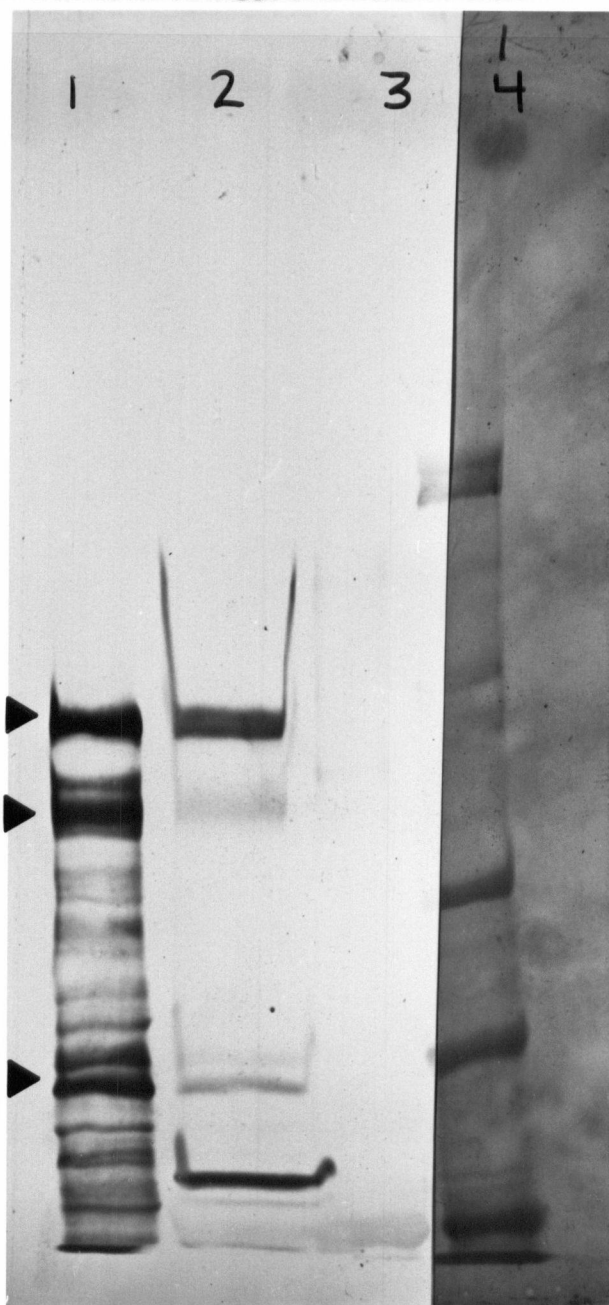
Figure 7.   Western blot of bovine tissues.   100 µg of redissolved
protein precipitates were electrophoresed, blotted and
visualized as previously described (Figure 6).   Lane 1 -
purified XO showing 150 KDa and 135 KDa subunits (arrows)
as well as 90 KDa fragment.   Lane 2 - bovine lung.   Lane
3, BSA.   Lane 4 high molecular weight markers stained with
amido black.   (Top to bottom) myosin 205,000,
β-galactosidase 116,000, phosphorylase B 97,400, BSA
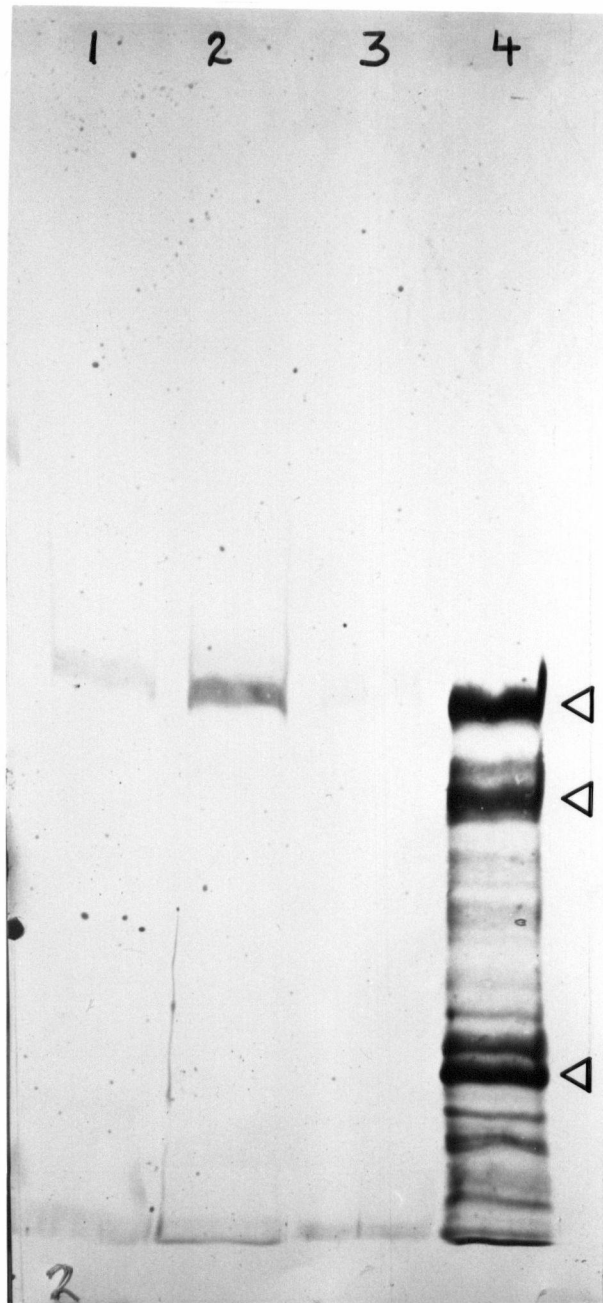66,000, ovalbumin, 45,000.

Figure 8.   Western blot of rat tissues.   100 μg of redissolved
protein precipitates were electrophoresed, blotted and
visualized as previously described (Figure 6).   Lane 1
shows the results from rat lung homogenate, lane 2 rat
liver homogenate, lane 3 rat kidney homogenate.   Lane 4
shows purified bovine milk XO with the 150 KDa and 135 KDa
subunits as well as 90 KDa fragment.

SDS-PAGE of 100 μg protein of porcine whole lung, peripheral lung and kidney did not show protein bands which reacted with anti-bovine XO antisera. Porcine liver reacted positvely for XO (Figure 9). Analysis of peripheral human lung tissue homogenates showed no detectable xanthine oxidase (Figure 10).

Comparison of Sensitivity of Urate Assay to Isoxanthopterin Assay

Graded dilutions of XO were assayed until no activity could be measured. The limits of detection of the urate assay were found to be 2.45×10^{-5} mg of XO protein (assuming all of the protein in the purified XO is XO). This corresponds to 4.93×10^{10} molecules of XO

With the IXP assay the limits of detection were 3.17×10^{-6} mg XO protein, which corresponds to 6.17×10^{8} molecules of XO. The IXP assay was found to be in the range of 100 fold more sensitive than the urate assay.

Product Inhibition of IXP Assay

IXP assay for XO was performed both in the presence and absence of 3nM IXP using substrate concentrations from 1 μM to 25 μM. 3 nm of IXP was shown to inhibit the enzymatic rate of reaction in a competitive manner as shown in a Lineweaver-Burke Plot (Figure 11). The Km for the reaction was 3.4×10^{-3} M. The Ki for the reaction was 4.4×10^{-10} M.
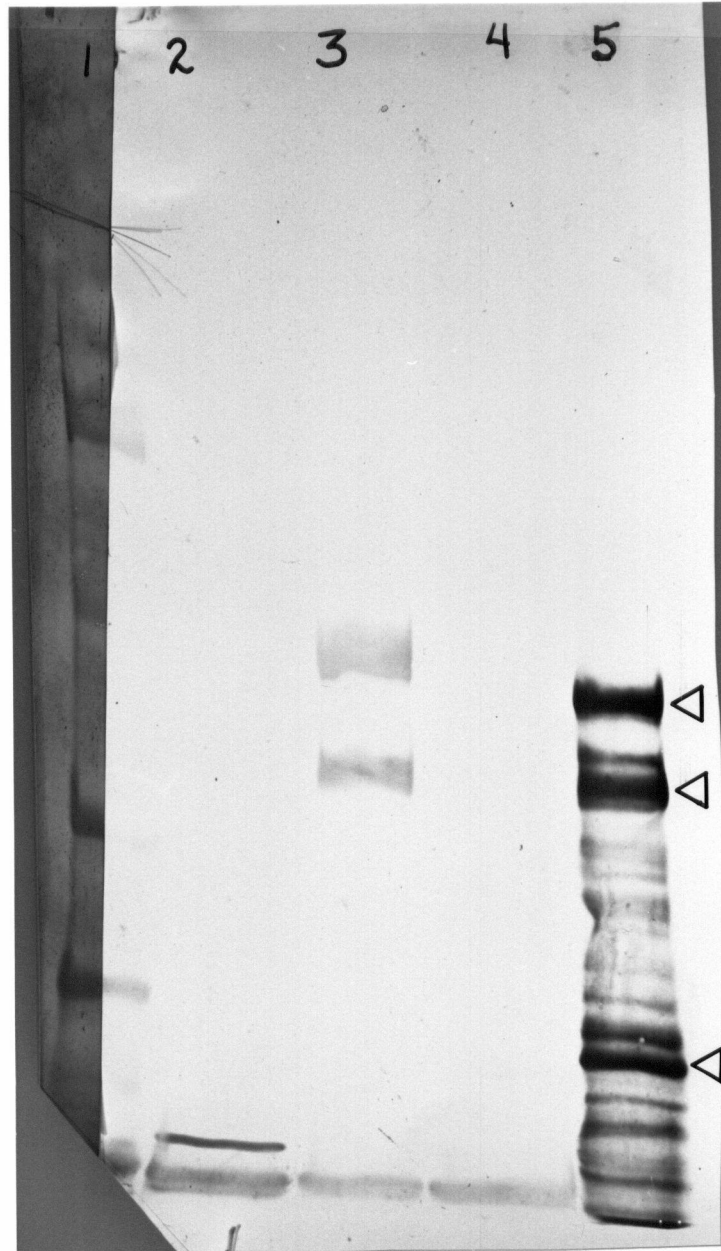
Figure 9.   Western blot of porcine tissues.   100 µg of redissolved
             protein   precipitates   were   electrophoresed,   blotted   and
             visualized as previously described (Figure 6).   Lane 1 high
             molecular weight markers stained with amido black, top to
             bottom; myosin 205,000 Da, β-galactosidase 116,000 Da,
             phosphorylase b 97,400, BSA 66,000, ovalbumin 45,000 Da.   Lane 2
             pig lung homogenate.   Lane 3 contains pig liver homogenate.
             Lane 4 contains pig kidney homogenate.   Lane 5 contains purified
             bovine milk XO showing the 150 KDa, 135 KDa and 90 KDa subunits
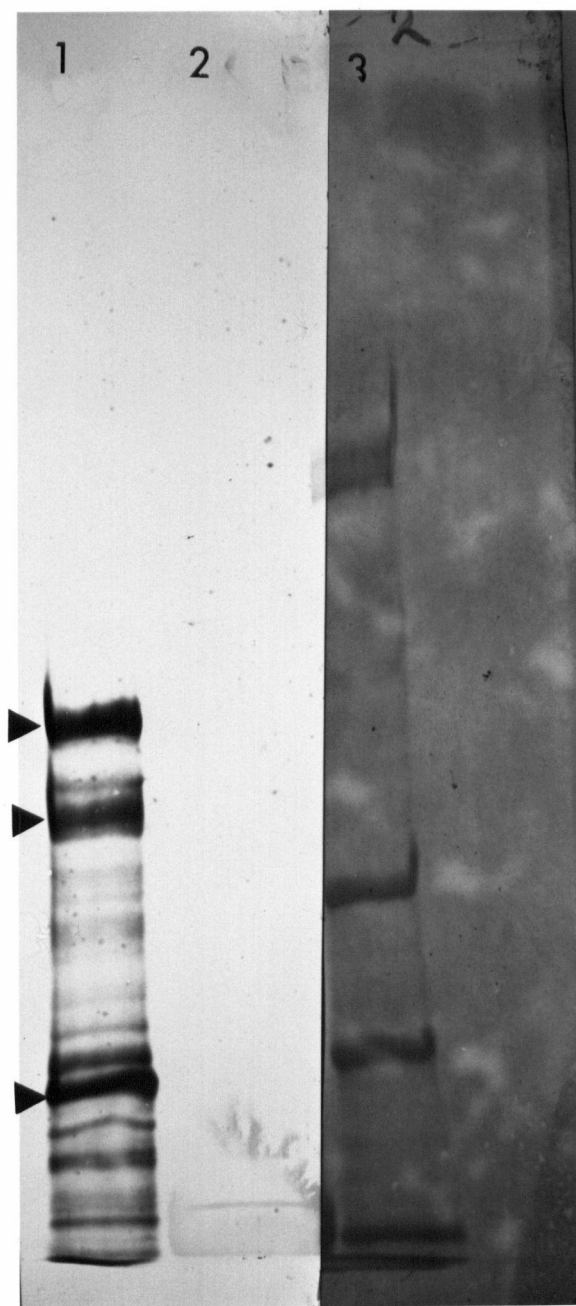   (arrows).

Figure 10. Western Blot of human lung tissue. 100 µg of redissolved protein precipitates were electrophoresed, blotted and visualized as described (Figure 6). Lane 1; purified bovine milk XO showing 150 KD, 135 DK and 90 KD subunits (arrows), lane 2, human lung homogenate, lane 3, high molecular weight markers stained with amido black (Top to bottom) myosin 205,000 Da, β-galactosidase 116,000 Da, phosphorylase b 97,400 Da, BSA 66,000 Da, ovalbumin 45,000 Da.
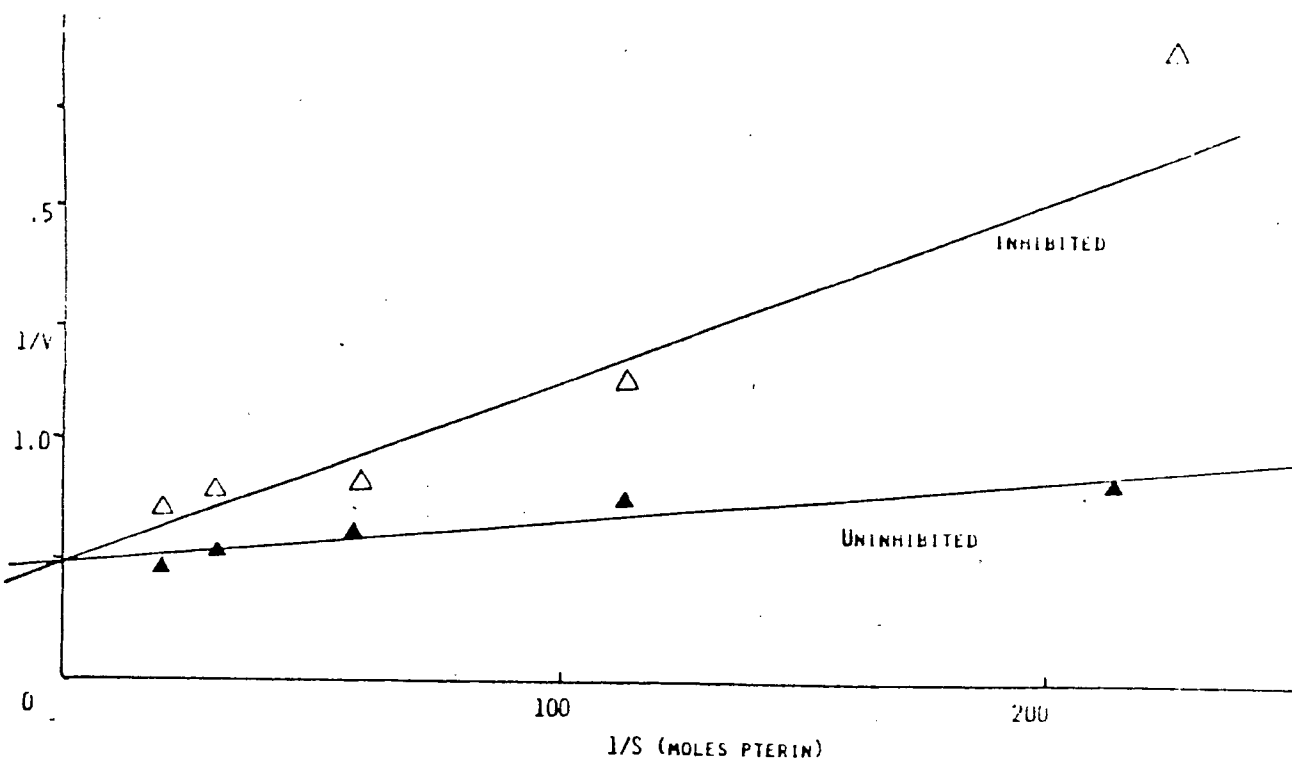
Figure 11. Lineweaver-Burke plot showing the competitive inhibition of purified XO by 3 nm IXP; $K_m = 3.4 \times 10^{-3}$ M). $K_i = 4.4 \times 10^{-10}$ M.

EXPERIMENTS IN PORCINE TISSUE

Assay for XO XDH Activity in Pig Tissue

No enzyme activity was detected in pig kidney, whole lung or peripheral
lung homogenates from 6 different pigs, even when most sensitive
spectrofluorimeter settings were used. Representative tracings taken from
data obtained on the enzymatic activity in pig lung homogenates (Figure 13)
and pig kidney homogenates (Figure 14) revealed a decreased rather than the
increased fluorescent intensity as a function of time which would be
expected for the enzymatic conversion of pterin to fluorescent product,
IXP. Pig liver contained XO XDH activity as determined by IXP assay, with a
specific activity of 1.1×10^{-3} μg IXP/min/mg protein (Figure 12).

Inhibition of Purified XO Activity by Pig Extracts

Based on the previous observation that pig kidney and lung caused a
decrease in fluorescence in the IXP assay, an inhibitor for XO was sought.
The rate of IXP production by purified XO in buffer was compared to that of
the same amount of purified XO in pig lung extract. Enzyme activity in
buffer was found to be 7.5 μg IXP/mg protein/min. Enzyme activity in pig
lung extract was found to be 1.98 μg IXP/mg protein/min showing a 73.5%
inhibition in enzyme rate of activity (Figure 15).

Heat Stability of Active Factors in Pig Tissues

In order to test whether an enzyme or protein might be involved in the
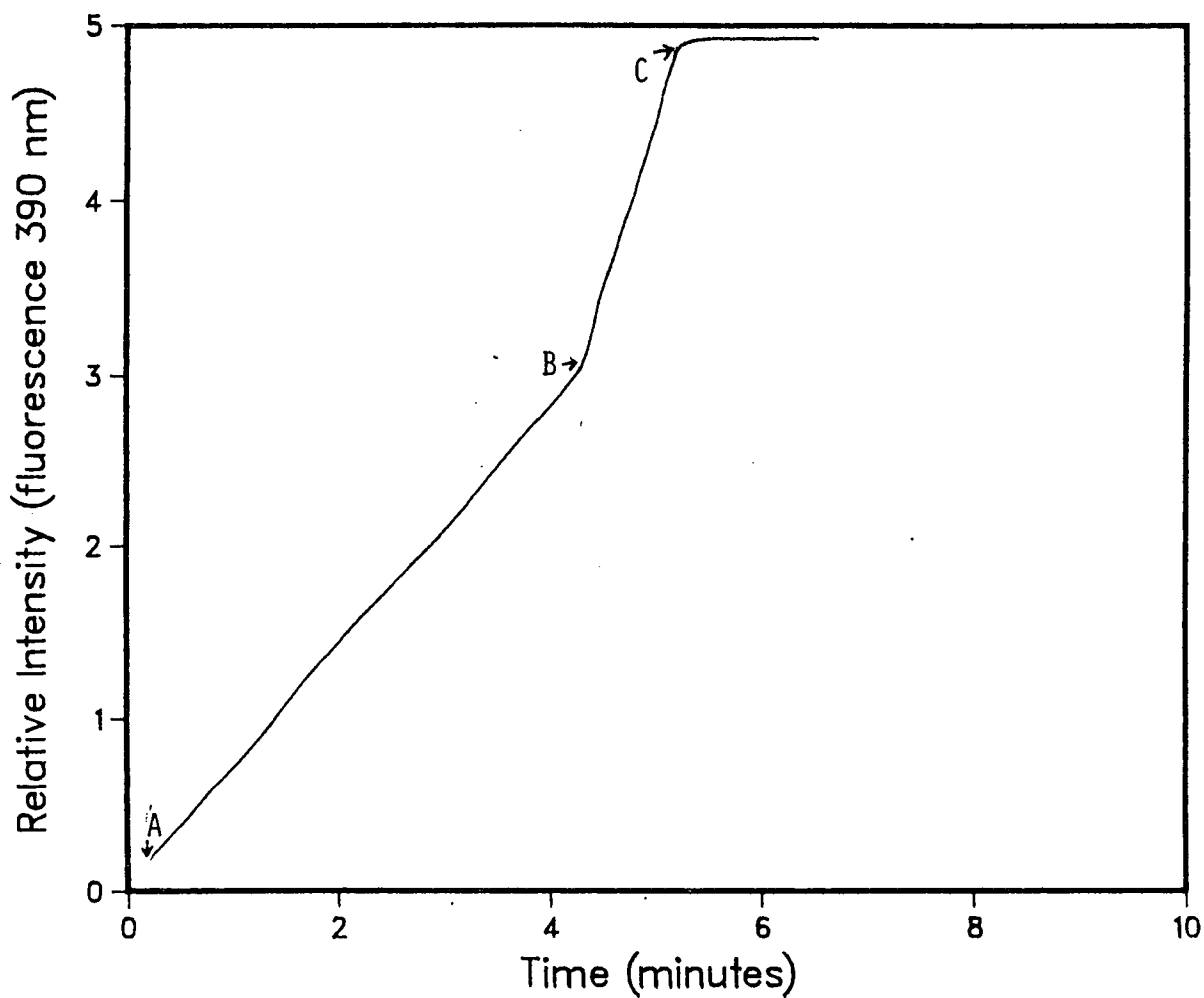inhibition of XO activity by pig lung extract, we examined the effect of

Figure 12. Representative tracing of XDH XO activity in pig liver
homogenates from n=3 samples. A. Addition of 25 μ moles
pterin to initiate the aerobic reaction. B. Addition of 5 μ
moles methylene blue to measure the total XDH + XO activity.
C. Addition of 20 μ moles allopurinol to inhibit the reaction.
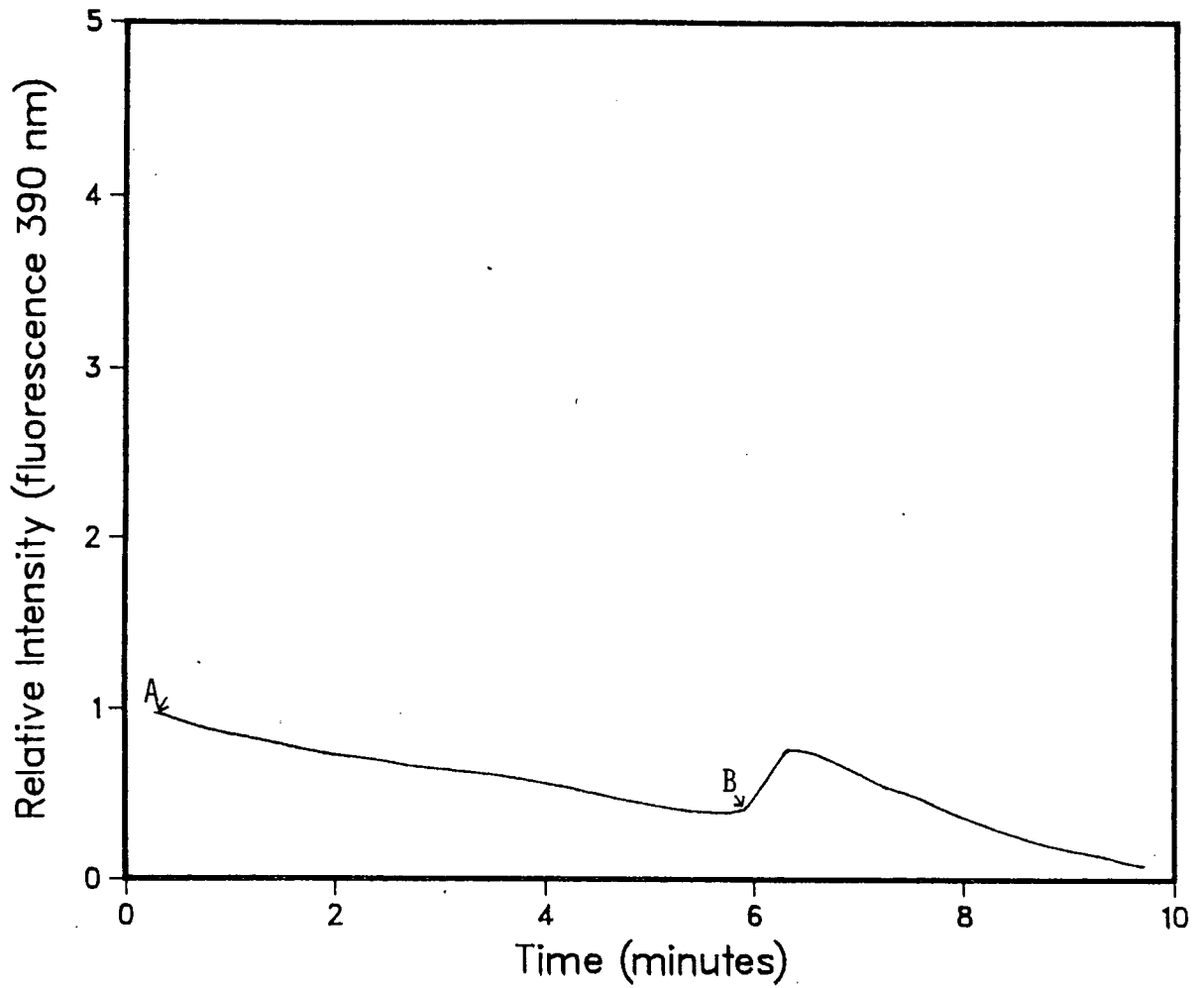
Figure 13. Representative tracing of XDH XO activity in pig lung from n=6
            samples. A. Addition of 25 μ moles pterin to initiate the
            aerobic reaction. B. Addition of 5 μ moles methylene blue to
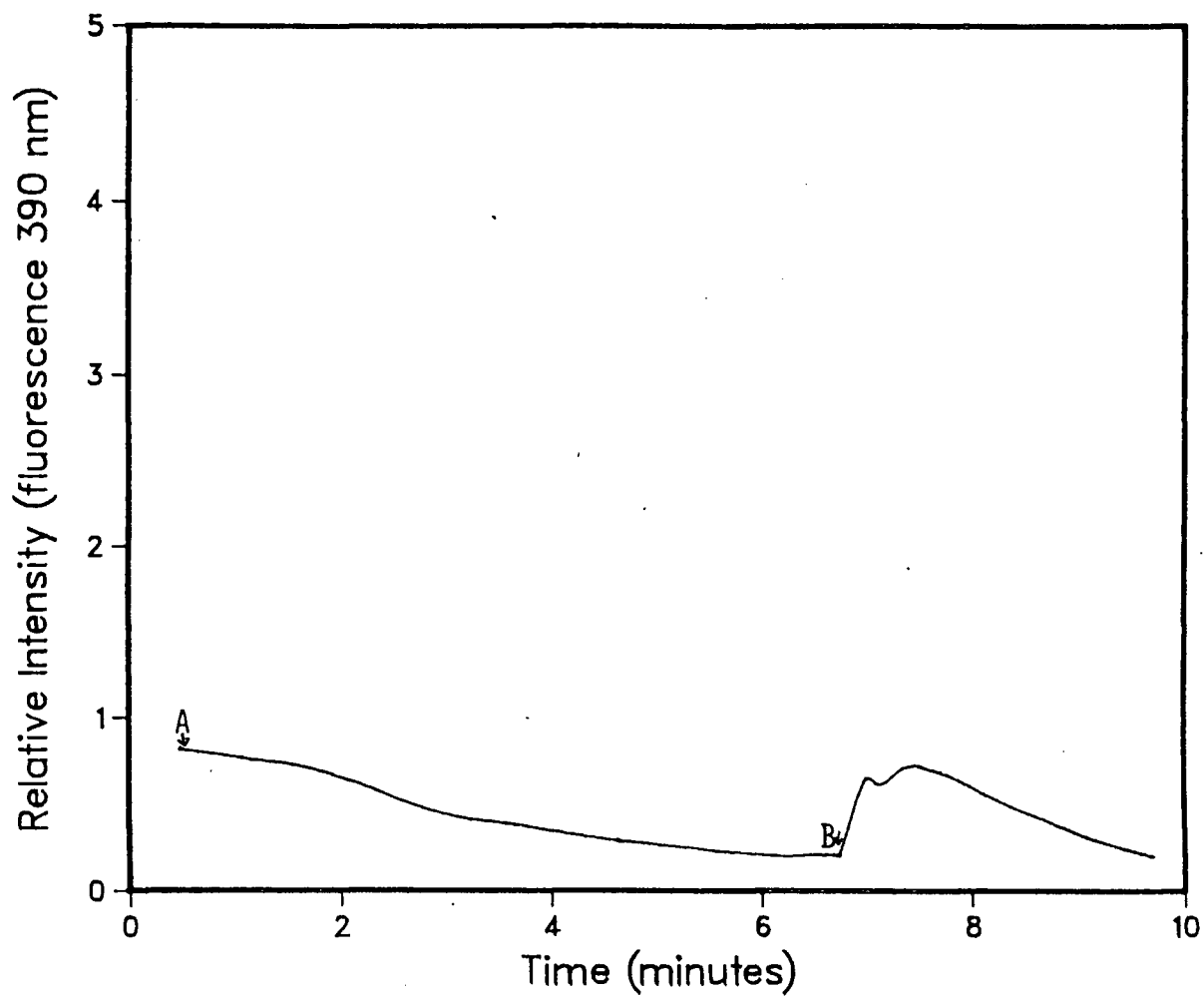   measure total XDH + XO activity.

Figure 14. Representative tracing of XDH-XO activity in pig kidney from n=6
samples. A. Addition of 25 µ moles pterin to initiate the
aerobic reaction. B. Addition of 5 µ moles methylene blue to
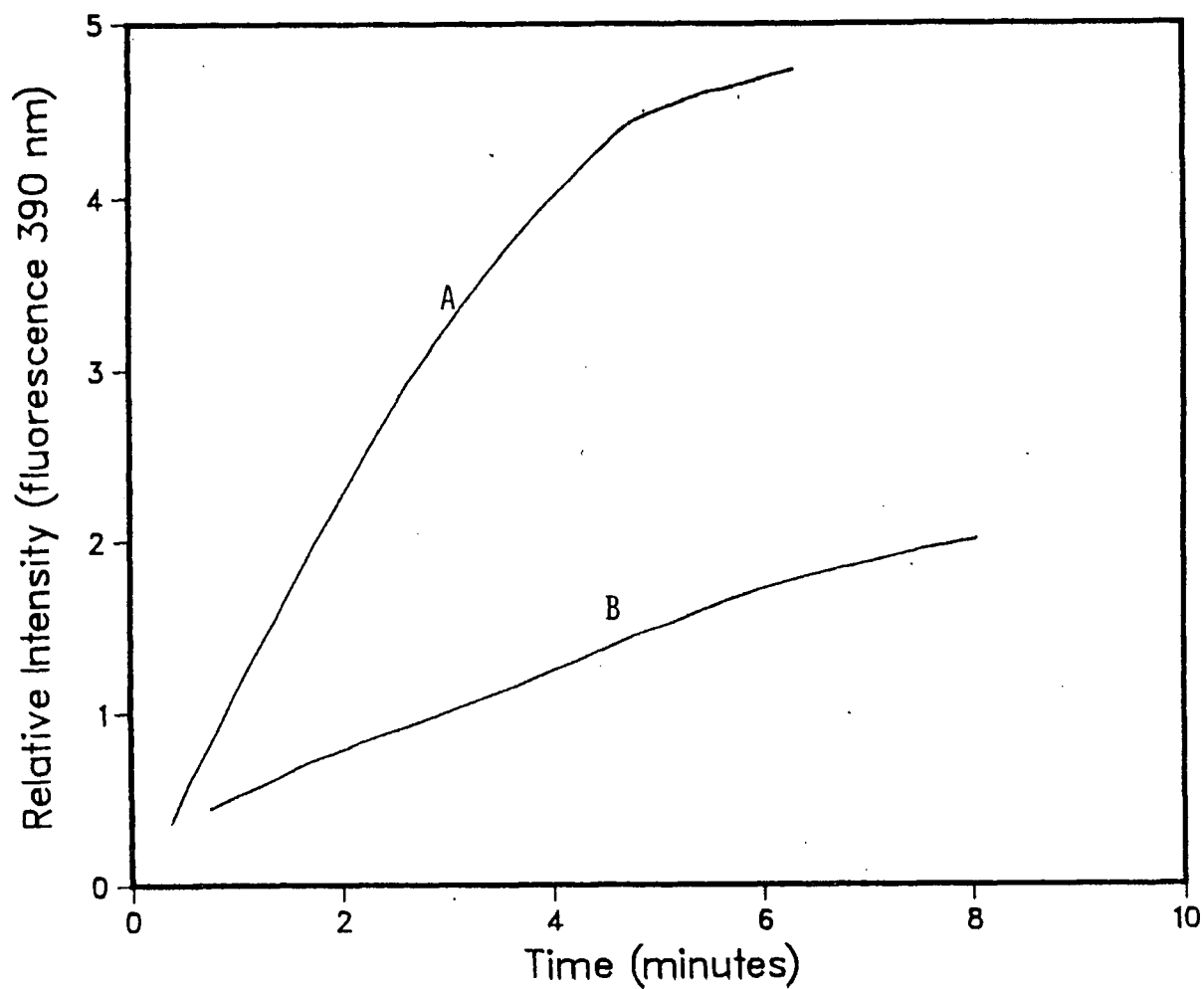measure total XDH + XO activity.

Figure 15. Inhibition of purified XO activity by pig lung homogenate. A.
Reaction rate of purified bovine XO in 50 mM KPi + mM EDTA with
25 µM pterin. B. Reaction rate of same amount of purified
bovine XO in pig lung homogenate in 50 mM KPi + 0.1 mM EDTA with
25 µM pterin showed a 73.5% inhibition of reaction rate.

incubation at 100°C for 20 min on ability of pig tissue extract to inhibit

XO activity. Purified XO activity in buffer was found to be 3.6 µg IXP/mg

protein/min while purified XO activity in boiled pig tissue extract was

found to be 2.25 µg IXP/mg protein/min. This represented a 37% inibition

of activity, as compared to a 73.5% inhibition in non-denatured pig

homogenate (Figure 16).

Effect of Pig Tissue Extract on IXP Relative Intensity

Because boiling of pig tissue extract did not totally ablate inhibition

of XO activity, it was necessary to investigate whether pig tissue extract

could quench IXP fluorescence or degrade IXP. The fluorescence intensity of

3nM IXP in buffer decreased immediately by 8% when measured in the same

amount of pig lung extract. Following the reaction for 10 minutes showed an

additional 3% decrease in IXP fluorescence (Figure 17).

Measurement of XO-XDH Activity in Cultured Porcine Pulmonary Artery
Endothelial Cells

Cellular extract from seven T-80 flasks of porcine pulmonary artery

endothelial cells were assayed for XO-XDH activity. This corresponds to

cell numbers ranging from 7.8 x 10^7 to 1.3 x 10^8 cells. No XO-XDH

activity was detected in this extract, or in extract which had been

concentrated threefold by removal of H_2O therefore activity, if present,

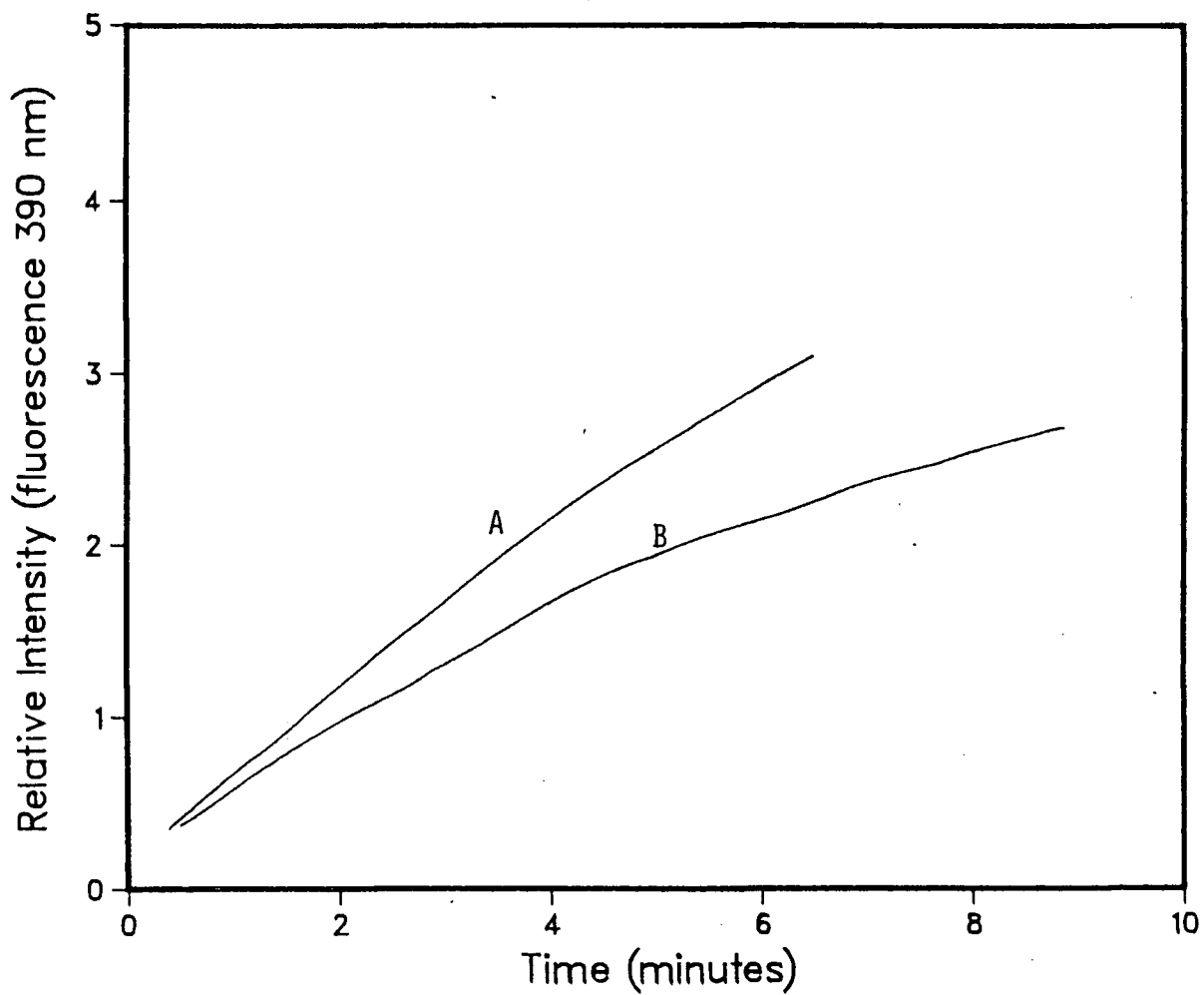would be less than the level of detection for this assay.

Figure 16. Heat stability of active factors in pig tissue. A. Reaction rate of purified bovine XO in 50 mM KPi + 0.1 mM EDTA. B. Reaction rate of purified bovine XO in pig lung homogenate denatured by incubation in boiling water for 20 min, showing 37% inhibition of XO activity.
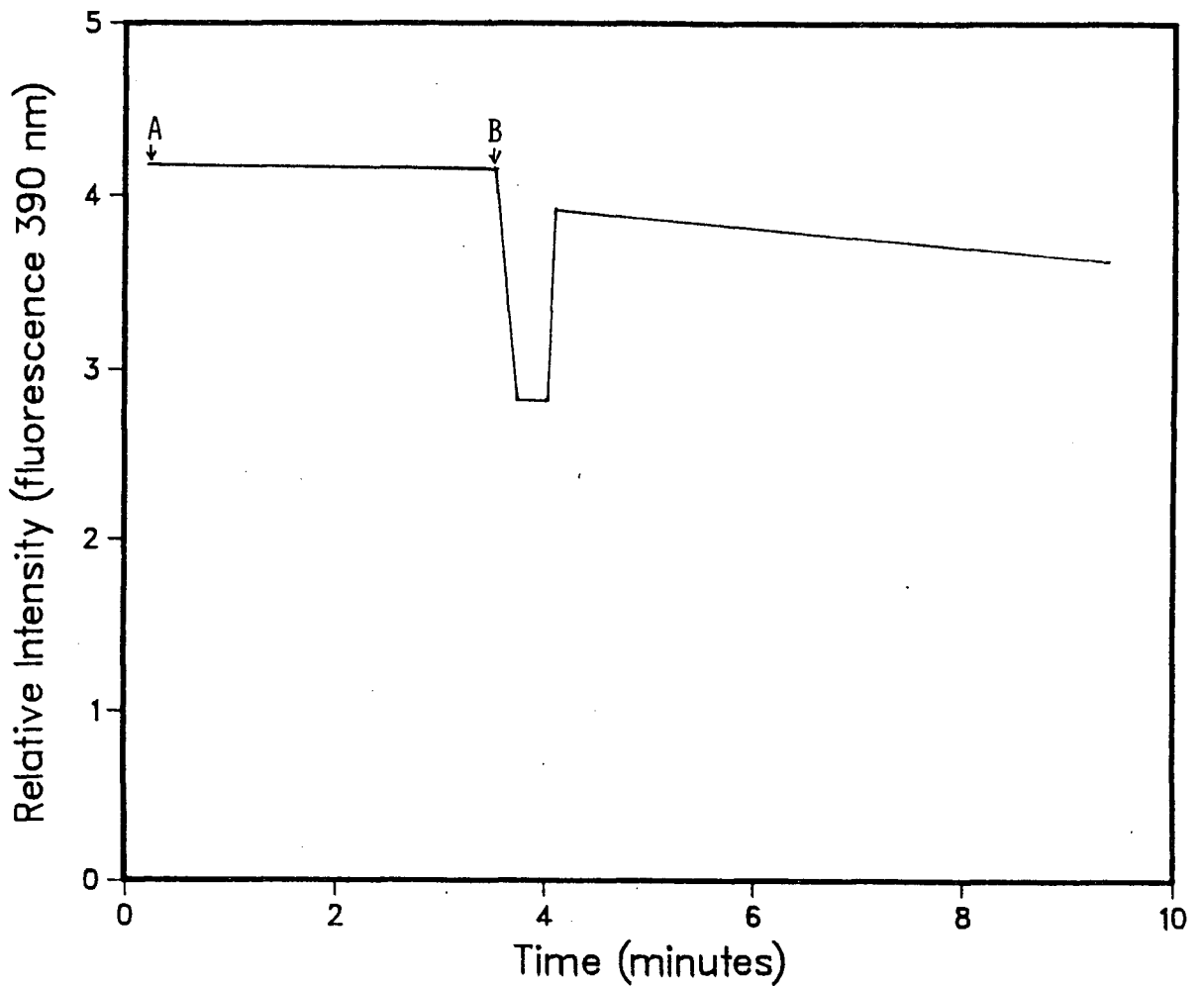
Figure 17. Effect of Pig Tissue Extract of Relative Fluorescent
Intensity of IXP. Averaged curve from points obtained for
three replicates of representative traces. A. Relative
intensity of 3 nM IXP dissolved in 900 ul 50 mM KPi. B.
Relative intensity of 3nM IXP dissolved in 900 ul pig lung
homogenate.

Isolation of Porcine Pulmonary Microvascular Cells

Endothelial cells were determined to be separated from parenchymal cells in elutriation Fraction 2. These cells were followed with phase microscopy. After initial seed of adult cells, clumps of 4-6 cells adhered to the gelatin-coated culture flask. By 5 days later these had grown into patches of polyhedral cells interspersed with spindly open networks of cells which had the appearance of newly isolated large vessel endothelium in culture. By 10 days many healthy patches of polyhedral cells with oval nuclei were apparent growing in cobblestone arrangements typical of large vessel endothelium in culture. These were interspersed with "networks" of spindle shaped cells which gave the appearance of capillary networks. By 14 days large patches of subconfluent polyhedral cells growing with a characteristic cobblestone monolayer appearance were apparent. All colonies were found to be infected with a mycotic contamination at this point and were thus discarded. The cells were not passaged.

Isolation of microvascular endothelial cells from newborn piglet provided a very low initial number of cells. Elutriator fraction 2 was cultured for 28 days. Patches of cells growing with the morphological appearance of fibroblasts were scraped from the culture surface and rinsed from the flask. After 56 days the T-25 flask of newborn piglet cells was confluent with polyhedral cells in a cobblestone monolayer, similar to large vessel cells in culture (Figure 18). The cells were passaged into two flasks. One flask was supplemented with newborn piglet serum, the other supplemented with commercial adult pig serum. Cells grown in newborn pig serum grew slightly faster; i.e. they reached confluence one day sooner than cells
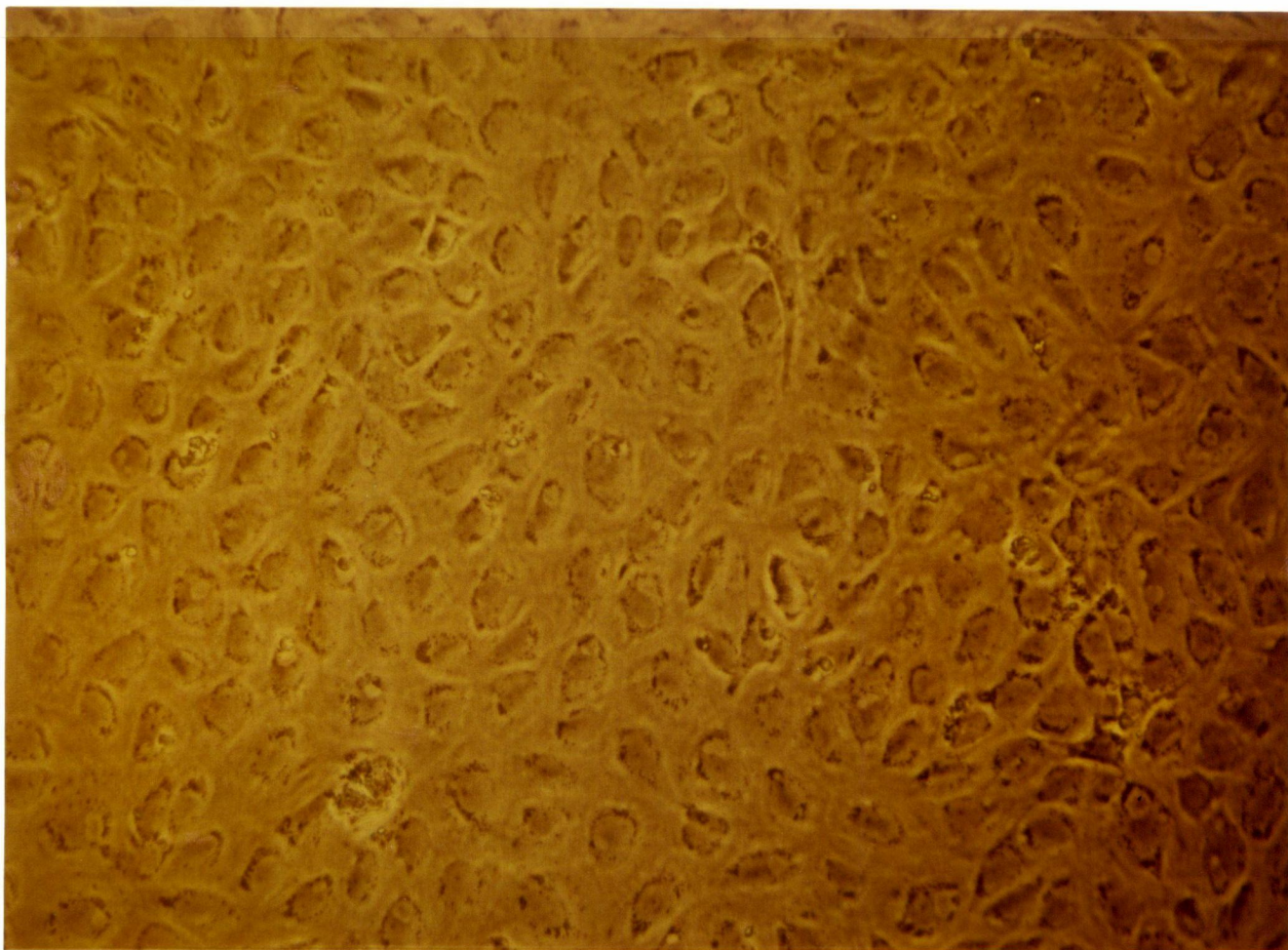
Figure 18. Microvascular endothelial cells isolated by centrifugal elutriation shown in culture. Cells show morphological appearance characteristic of cultured endothelial cells with cobblestone monolayer arrangement and ovoid nucleus. (Magnification 142 x)

grown in adult pig serum. Despite manual removal of fibroblastic appearing patches of cells, the cultures were contaminated by fibroblasts by the time second confluence was reached. There was no evidence of contamination by bacteria or fungus. Because of this impurity colonies were discarded.

Neither adult pig nor newborn piglet cells could be subcultured onto glass coverslips in order to perform the assay for Factor VIII immunolocalization in order to confirm that they were indeed endothelial cells. It was therefore necessary to rely on morphological appearance in order to identify them.

In addition, neither adult pig nor newborn piglet cells were cultured in large enough quantity use for biochemical analysis of XO-XDH activity.

EXPERIMENTS IN RAT TISSUE

Characterization of IXP Assay in Tissue

Whereas use of the IXP assay showed XO activity in purified milk, the same assay did not detect XO activity in pig lung and kidney homogenates. This raised the question of whether the assay system was effective in crude tissue homogenates. To determine the effectiveness of this assay in tissue homogenates, the enzyme was assayed in rat kidney homogenates since this tissue is know to contain the enzyme.

Rat kidney was excised and immediately homogenized in ice cold 50 mM Kpi + 0.1mM EDTA. The total enzyme activity (XDH + XO) was 1.5×10^{-2} µg IXP/min/mg protein ($\pm$ SE 3×10^{-3}). XO activity was 3.9×10^{-3} µg IXP/min/mg protein ($\pm$ SE 1×10^{-3}) on n=5 animals. IXP production was shown to be inhibited by the addition of 20 µ moles of allopurinol (Figure 19).

Determination of XDH-XO Level in Normal Rat Lung by IXP Assay

Normal rat lung was quickly excised and homogenized in ice cold 50 mM KPi + 0.1 mM EDTA. Total enzyme activity (XDH + XO) was 1.7×10^{-2} µg IXP/min/mg protein ($\pm$ SE 4×10^{-3}). XO activity was shown to be 9.3×10^{-3} µg IXP/min/mg protein ($\pm$ SE 3×10^{-3}). n=3 animals. Conversion of pterin to IXP was inhibited by the addition of 20 µ moles of allopurinol (Figure 20).

Studies of the Conversion of XDH to XO

Effect of Trypsin Treatment on XDH to XO Conversion

Based on studies in liver showing that XDH is irreversibly converted to XO by limited proteolysis (86) the effect of incubating 100 µg trypsin per ml of rat lung extract was studied and compared to control rat lung extract. Both trypsinized and control extracts were also incubated with 1mM DTT in order to test for the reversible conversion involving oxidation of thiol groups. Conversion is expressed as the ratio of total enzyme activity to oxidase activity (XDH+XO/XO). A ratio of (XDH + XO)/XO = 1 implies that all enzyme has been converted to the oxidase form. In control rat lung XDH+XO/XO was 1.05. Control extract + DTT showed (XDH + XO)/XO of 4.92. Trypsinized extract showed XDH+XO/XO of 1. Trypsinized extract incubated with DTT showed (XDH + XO)/XO of 1.73 (Figure 21).

Effect of Entended Incubation at 15°C on Conversion of XDH to XO

Rat lung extracts were incubated without EDTA at 15°C for 16 h. Analysis of (XDH + XO)/XO ratio show complete conversion (XDH + XO)/XO = 1). Further incubation of these extracts with 1mM DTT for 60 min at 37°C showed a (XDH + XO)/XO ratios of 3.59 (Figure 22).

Figure 19. Representative tracing of XDH-XO activity in rat kidney
homogenate, from n=6 samples. A. Addition of 25 μ moles
pterin to initiate the aerobic reaction. B. Addition of 5 μ
moles methylene blue to measure the total XDH + XO activity. C.
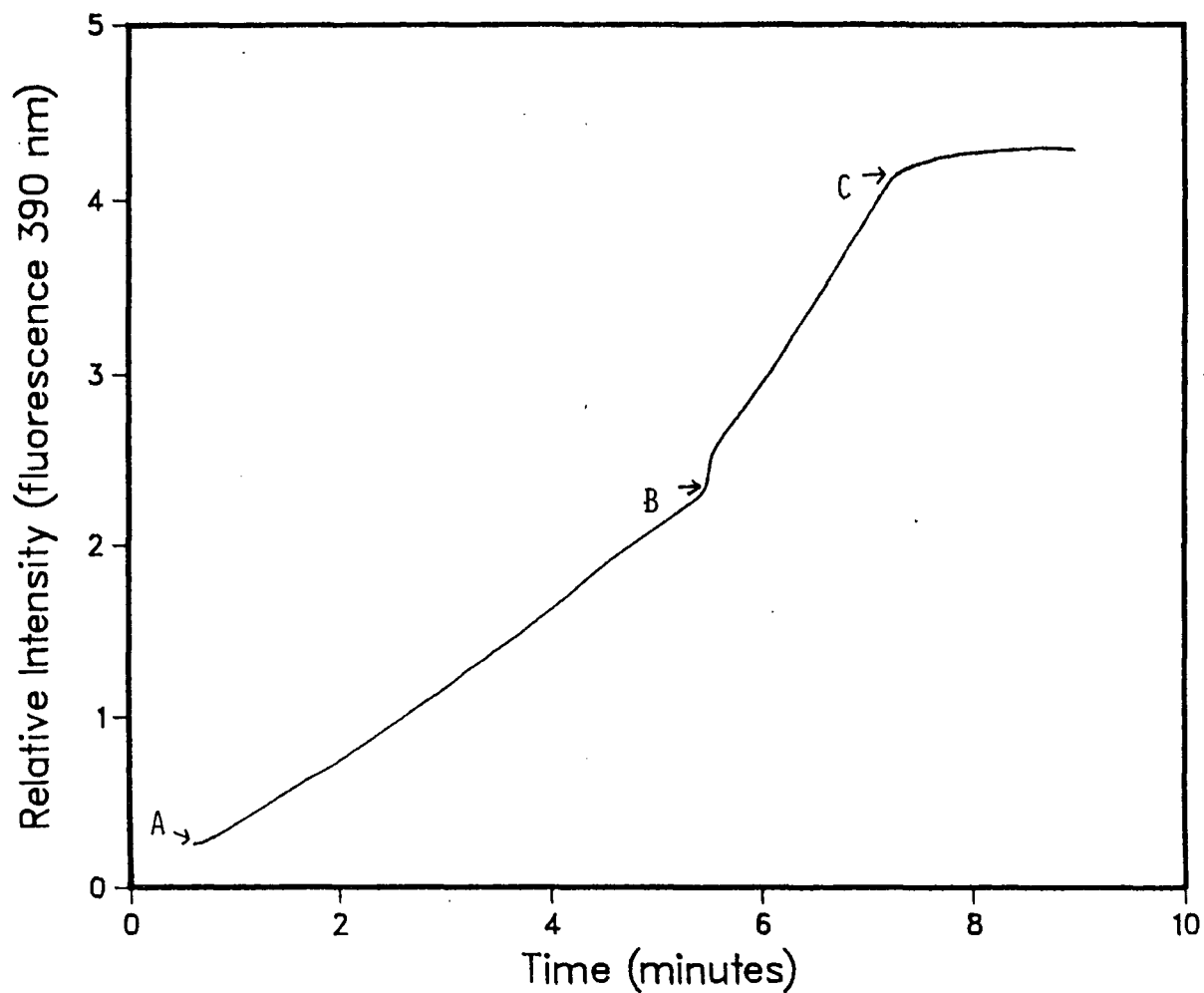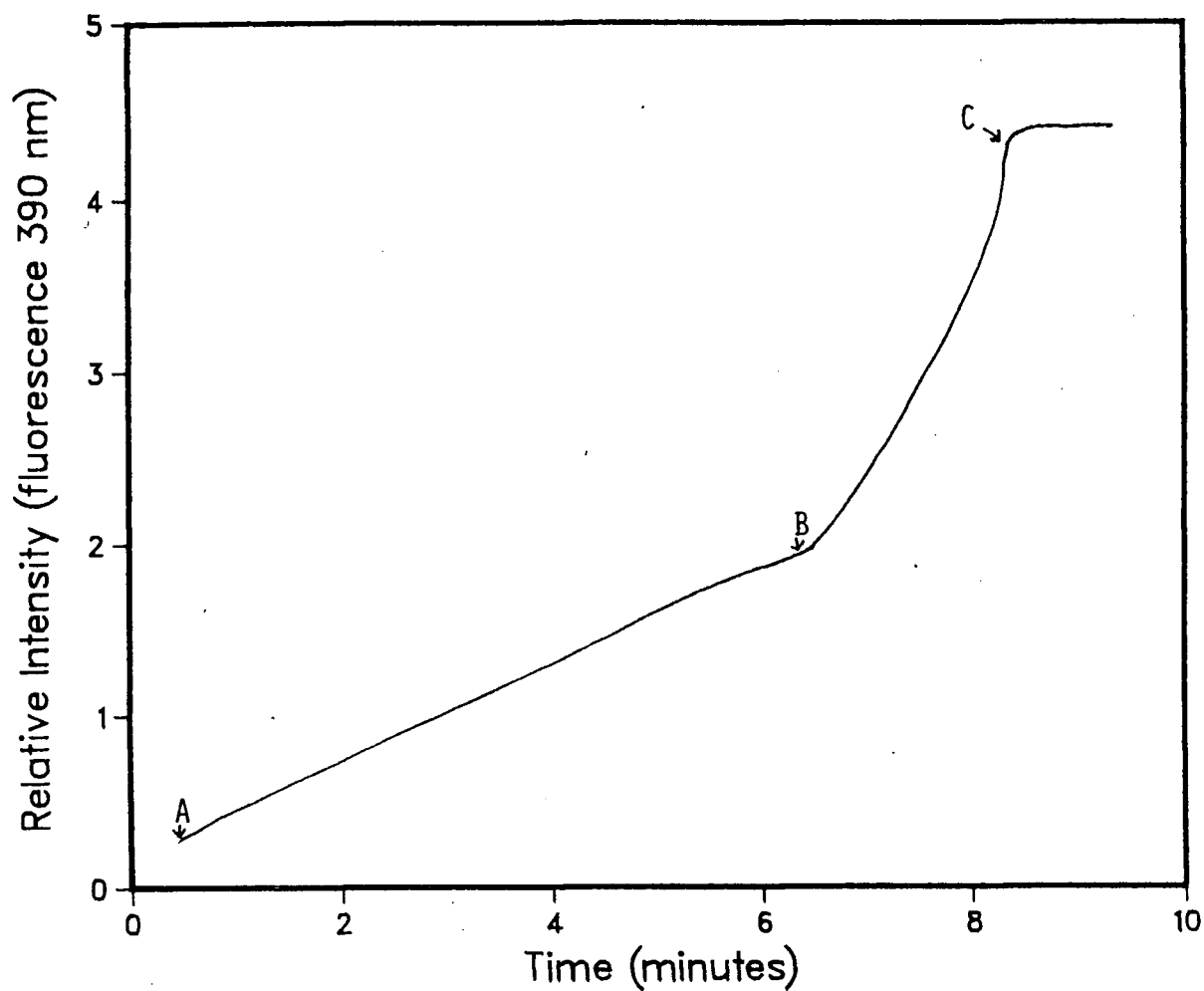Addition of 20 μ moles allopurinol to inhibit the reaction.

Figure 20. Representative tracing of XDH-XO activity in rat lung from n=6
samples. A.   Addition of 25 μ moles pterin to initiate the
aerobic reaction. B.   Addition of 5 μ moles methylene blue to
measure XDH + XO activity. C.   Addition of 20 μ moles
allopurinol to inhibit the reaction.

Effect of Calcium on Conversion of XDH to XO in Normoxic Lung

In order to test the effects of calcium on XDH to XO conversion, the effect of EDTA as a chelator of divalent cations was compared with EGTA, a more specific chelator of calcium, using samples prepared in the absence of chelator as controls. The reversibility of the conversion was tested by incubation with 1mM DTT. Experiments were performed on normoxic rat lung quickly excised and homogenized in the appropriate buffer. (Table II).

For purposes of comparison, (XDH + XO)/XO of normoxic rat lung in the absence of calcium chelators was 1.45. With the addition of 1 mM EDTA to all buffers used for tissue homogenization, chromatography or sample preparation, the ratio calculated for the tissue samples, (XDH + XO)/XO was 1.8. When EGTA was used as more specific chelator of calcium, the ratio of (XDH + XO)/XO was 1.1 $\pm$ SE 0.33 (Figure 23).

Incubation of the above described extracts with 1mM DTT for 60 min at 37°C to reduce thiol groups gave the following (XDH + XO)/XO ratios:

```
        Control 4.74
        EDTA    2.8
        EGTA    3.25
```

Effect of Calcium on XDH to XO Conversion in Ischemic Lung

Rat lung was excised, rinsed, and incubated in PBS at 37°C to reproduce the conditions of warm ischemia as described (132). Lungs were then blotted to remove excess PBS and homogenized in iced 50 mM KPi containing either 1mM EDTA, 1mM EGTA or no calcium chelator as a control. Three animals were studied for each experiment (Table III).

The ratio of (XDH + XO)/XO obtained from ischemic rat lung in the absence of calcium chelators was 1.5. Ratio of (XDH + XO)/XO for ischemic

TABLE II

Comparison of XDH and XO Activities in Normoxic Rat Lung
in the Absence and Presence of Calcium
Chelators During Sample Preparation

Treatment	XDH + XO ($x\ 10^{-3}$)	XO ($x\ 10^{-3}$)	$\dfrac{XDH + XO}{XO}$
Control	7.1 ± 0.1	5.0 ± 1.0	1.45
Control +1mM DTT	7.0 ± 0.1	$1.5^{*} \pm 0.4$	4.74
1mM EDTA	16.7 ± 0.4	9.3 ± 0.3	1.8
1mM EDTA +1mM DTT	6.7 ± 0.2	$2.4^{*} \pm 0.1$	2.8
1mM EGTA	6.0 ± 0.2	5.2 ± 0.1	1.1
1mM EGTA +1mM DTT	7.7 ± 0.3	$2.4^{*} \pm 0.7$	3.25

Values given are expressed as mean $\pm$ SE µg IXP/min/mg protein. Rat lungs
were assayed by the fluorometric method of Markley (113). Experiments were
carried out at 37°C. Experiments with 1 mM DTT were incubated at 37°C
for 60 minutes.
n=3 for each group.
* = $p<0.05$ for comparisons between XO activity with and without DTT for
each calcium chelating agent.
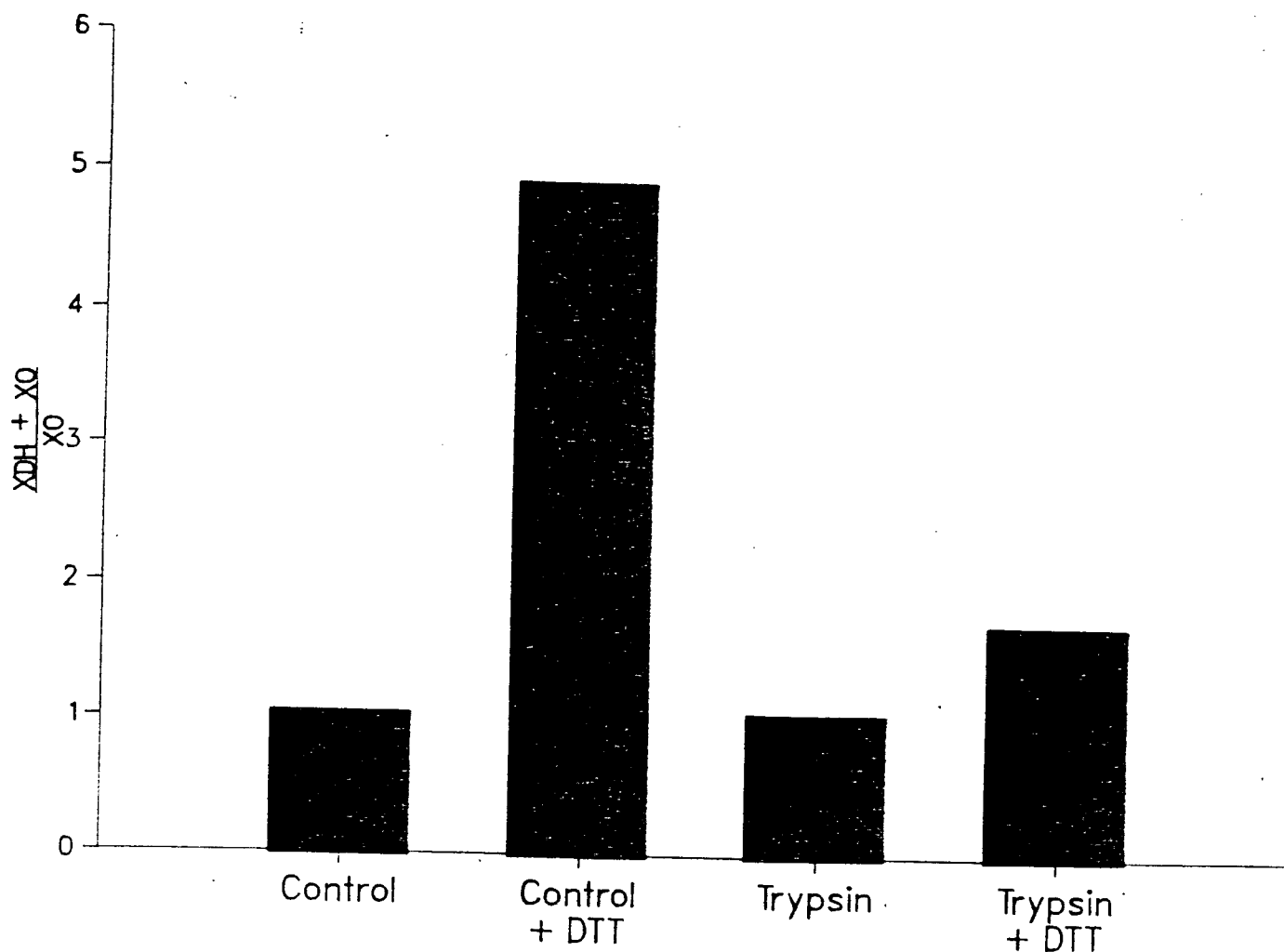
Figure 21. Effect of Trypsin Treatment on the (XDH + XO)/XO Ratio. Conversion of XDH to XO was tested by incubation of one ml of rat lung extract with 100 μg trypsin as compared to control. Conversion to XO in the control preparation was 80% reversible to XDH by incubation with DTT. Conversion to XO in trypsin-treated preparation was reversible to XDH to only a slight extent. Enzyme activity was irreversibly converted to XO by 95%.
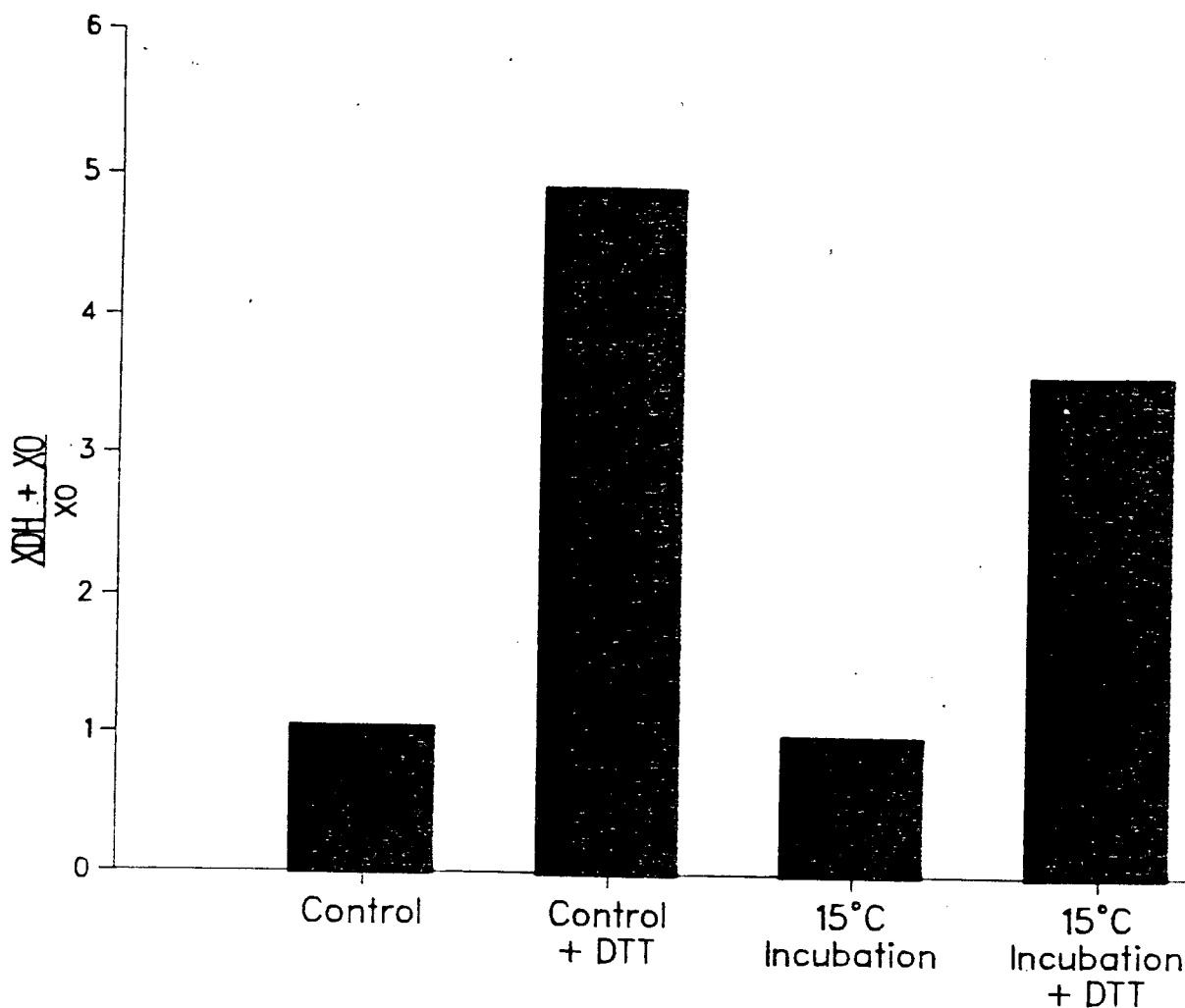
Figure 22. Effect of Extended Incubation of $15^{\circ}C$ on the (XDH + XO)/XO Ratio. Conversion to XO in control lung homogenate was reversible by 80% to XDH after incubation with DTT. Conversion to XO after 16h incubation of rat lung extract at $15^{\circ}C$ was reversible by 75% to XDH with only a small proportion of the enzyme activity being irreversibly converted.
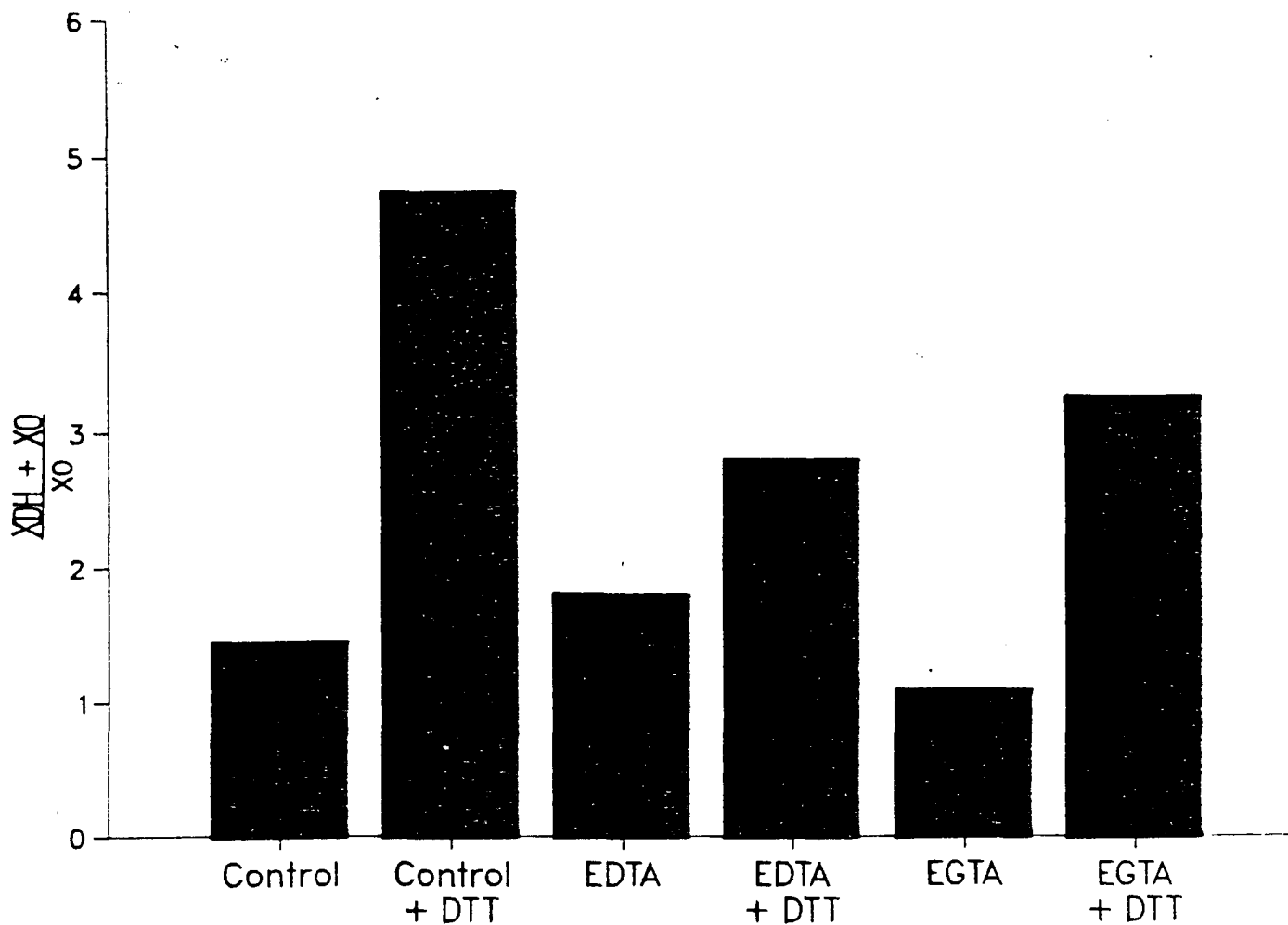
Figure 23. Effect of Calcium on Reversibility of the conversion of XDH to XO in Normoxic Rat Lung. Treatment of rat lung extracts with EDTA or EGTA did not affect reversibility of XDH to XO conversion tested by incubation with DTT as compared to control.

TABLE III

Comparison of XDH and XO Activities in Ischemic Rat Lung
in the Absence and Presence of Calcium Chelators
During Sample Preparation

| Treatment	XDH + XO (x 10^{-3})	XO (x 10^{-3})	$\dfrac{XDH + XO}{XO}$
Control	24.3 $\pm$ 10.0	15.9 $\pm$ 6.0	1.5
Control +1mM DTT	24.4 $\pm$ 10.0	7.5* $\pm$ 3.0	3.2
1mM EDTA	7.0 $\pm$ 3.0	3.1 $\pm$ 0.4	2.24
1mM EDTA +1mM DTT	8.3 $\pm$ 4.0	2.2* $\pm$ 1.0	2.3
1mM EGTA	7.5 $\pm$ 2.0	5.7 $\pm$ 1.0	1.28
1mM EGTA+ +1mM DTT	8.4 $\pm$ 3.0	2.0* $\pm$ 0.6	4.2

Values given are expressed as means $\pm$ SE of µg IXP/min/mg protein. Rat lungs were assayed by fluorometric method of Markley (113). Experiments were carried out at 37°C. Experiments with 1 mM DTT were incubated at 37°C for 60 minutes.
n=3 for each group.
* = $p < 0.05$ for comparisons between XO activity with and without DTT for each calcium chelating agent.
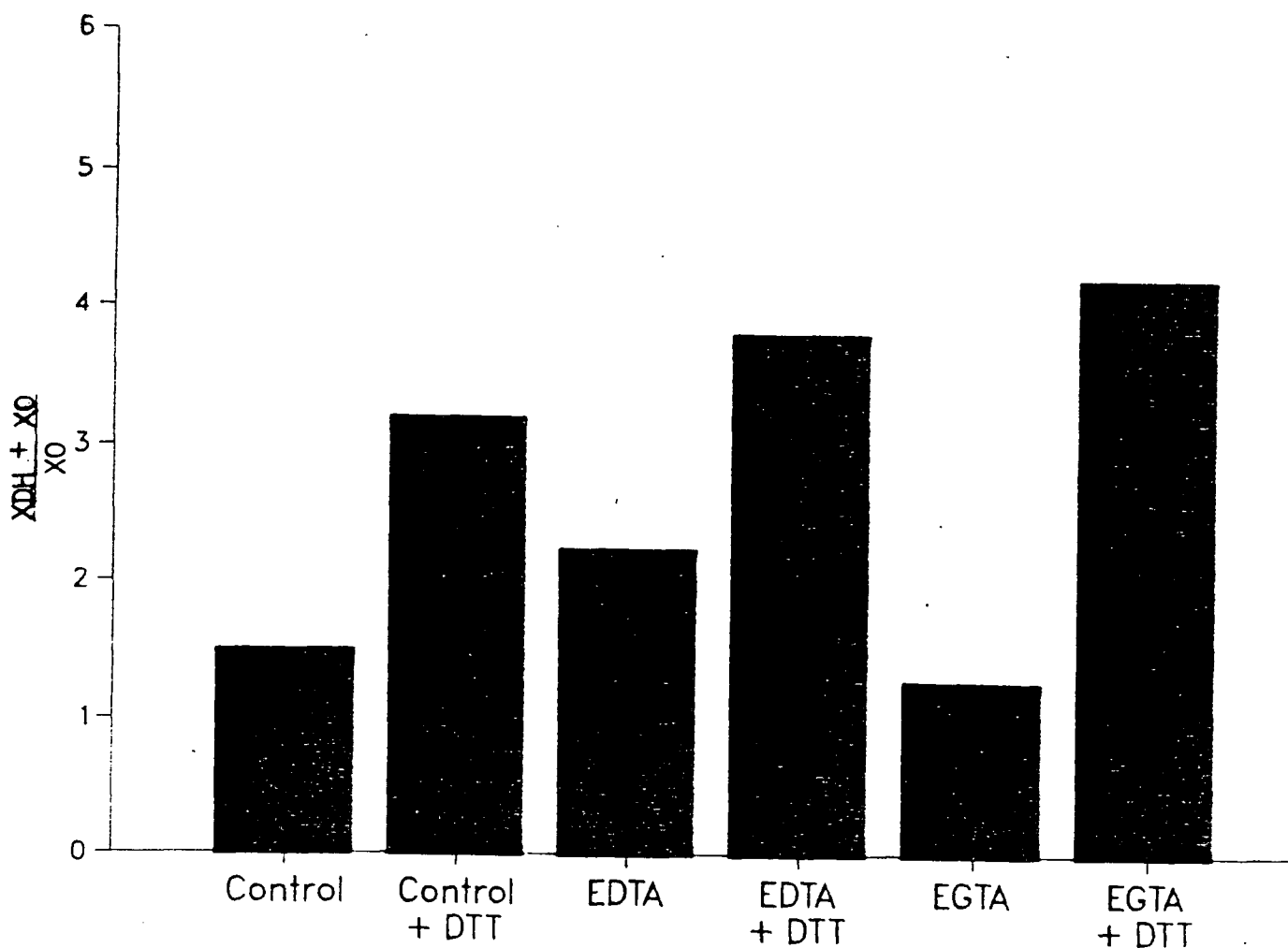
Figure 24. Effect of Calcium on Reversibility of the conversion of XDH to XO in Ischemic Rat Lung. Treatment of rat lung extracts with EDTA or EGTA showed a slight increase in reversibility of the conversion as measured by incubation with DTT, but this was not significant as determined by XO values.

rat lung with 1 mM EDTA in all homogenizing, chromatography and sample
buffers was 2.24. Ratio of ischemic rat lung extract with EGTA was 1.28
(Figure 24).

Homogenates prepared from ischemic rat lung were then incubated with 1mM
DTT for 60 min at $37^{o}C$. This treatment resulted in reversal of enzyme
conversion from XO to XDH such that in ischemic lung extract ratio of (XDH +
XO)/XO was 3.2. Ratio of (XDH + XO)/XO was 3.8 in rat lung homogenized with
1mM EDTA. In rat lung homogenized with 1 mM EGTA, ratio of (XDH + XO)/XO
was 4.2.

Effect of Perfusion with EDTA and DTT Prior to Homogenization

Because the effects of EDTA as a chelator of divalent cations on the
enzyme converting activity might be masked by the conversion of enzyme
proceeding before the protective agents reached all cells during
homogenization, the tissue was perfused with the chelator before excision.
The pulmonary artery of rat lungs was cannulated with PE tubing via the apex
of the right ventricle and the lungs were perfused with iced 50 mM Kpi pH
7.4 + 1 mM EDTA + 1 mM DTT + 1 mM PMSF immediately after the chest cavity
was opened. The lungs were then removed and rapidly homogenized in 2 ml
more of the same buffer and processed as usual (113).

In n=5 rat lung extracts treated, EDTA and DTT were present in all
homogenizing, chromatography and sample buffers. After this method of
preparation the ratio of (XDH + XO)/XO was 3.53 (Table IV)

Effect of Perfusion with Oxygen Depleted Buffer and 60' Ischemia on XDH to XO Conversion

In order to maximize the effect of ischemia rat lungs were cannulated and

perfused with a mixture of 50 mM KPi pH 7.4 at 37^OC with no DTT, EDTA or PMSF in order clear blood from the lungs. Blanched lungs were then clamped at the pulmonary artery and kept warm in the rats chest cavity for 60 minutes. After this period of time, clamp was removed and lungs homogenized in iced 50 mM Kpi pH 7.4 and processed as usual. Three animals were studied. Ratio of (XDH + XO)/XO in rat lung extracts treated in this manner was 1.32. After extracts from these experiments were further incubated with 1mM DTT at 37^OC for 60 min ratio of XDH + XO/XO increased to 2.53 (Table IV).

Experiments in Human Lung Tissue

Six human lung biopsy samples were assayed for XDH-XO activity. Of these, only three were found to be capable of oxidizing NADH. The other three samples contained no XDH-XO activity. Of the three which redox cycled NADH, only one showed measureable XO activity of 5.35 x 10^{-6} μg IXP/mg protein/min. No XDH activity was found. Because of unknown variability in tissue removal and transport it was impossible to determine whether DTT would be effective in reversing conversion to XO (Figure 25).
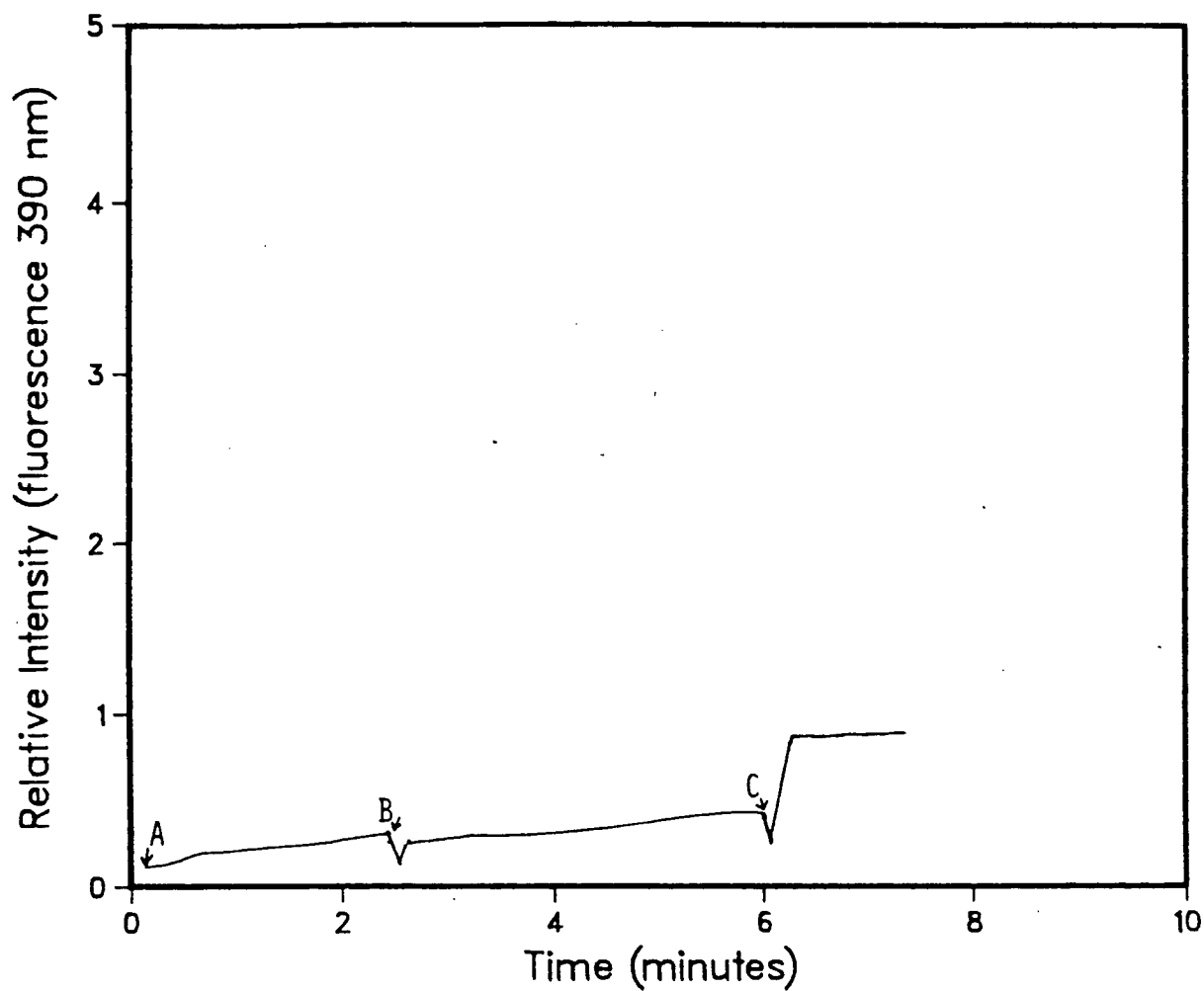
Figure 25. Trace of XO activity in human lung sample found to have enzyme
activity. A. Addition of 25 μ moles pterin to measure the
aerobic rate of reaction. B. Addition of 5 μ moles methylene
blue. C. Addition of 20 μ moles allopurinol to inhibit the
reaction.

TABLE IV

Effect of Perfusion of lung with EDTA and DTT on Preventing
Conversion of XDH to XO

Treatment	XDH + XO (x 10^{-3})	XO (x 10^{-3})	$\dfrac{XDH + XO}{XO}$
Perfusion with 4°C 1 mM EDTA at 4° + 1 nM DTT in 50 mM KPi. N=5	9.9 ± 0.3	13.4 ± 0.1	3.53
Perfusion with 37° C 50 mM KPi + 60 min ischemia N=3	18.0 ± 10.0	13.6 ± 0.7	1.32
Perfusion with 37° C 50 mM KPi + 1mM DTT X 60 min N=3	27.4 ± 15.0	10.9 ± 0.6	2.53

Enzyme activities are expressed as mean ± SE of μg IXP/min/mg protein.
Rat lungs were assayed by fluorometric technique of Markley (113). Assays
were conducted at37°C.

DISCUSSION:

The goal of these studies was to assess XDH to XO activities in lung tissues of several species, to compare the activity in lungs to that in other tissues and to examine the relationship of XDH to XO in the lung with a view to understanding the implications of such changes in inflammatory lung disease. It was of particular interest to examine the hypothesis that calcium-calmodulin mediated proteolysis may cause irreversible conversion from XDH to XO, and to relate the findings from the study of other organ systems such as gut (57)(58) and heart (55) (Figure 26) to the conversion of XDH to XO in the lung. If this were so, the importance of XO activity in contributing to free radical mediated damage in pathologic conditions could be applied to the proposal that oxygen free radicals generated by XO could be important in the etiology of ARDS by acting on cellular components to produce chemotactic factors thus causing PMN activation (71) in the lung .

Use of Purified Bovine Milk XO in These Studies

Based on high yield, activity and apparent purity the use of purified XO from our own laboratory by the method of Waud (91) was judged to be superior to use of commercially available XO for characterization of our assay system and preparation of a polyclonal antibody. Because purified XO had appeared as a single band at 135,000 Da upon post-isolation electrophoresis it was surprising to find strong reactivty against albumin at 67,000 Da. Since the 5 to 50 mM KPi buffer gradient on the DEAE Sephadex A-50 column was not conducted due to technical problems, the reactivity of the anti-XO antisera was attributed to the fact that trimers of albumin

which have the the same molecular weight as XO subunits were possibly contaminants the XO preparation. Thus antibodies were raised in the rabbit to the highly immunogenic BSA as well as to the XO. This would be a more reasonable explanation of the results rather than the possibility that the antibody to XO was cross reactive with BSA. Antigen-antibody precipitation of the polyclonal antisera with BSA would help to eliminate some of the antibody to BSA. This purification not being possible, the dual reactivity of the antisera was noted to provide a convenient molecular weight marker for Western blotting.

Use of this antisera in Western blotting of SDS-PAGE of redissolved protein precipitates from bovine, porcine rat and human tissues indicated that whereas all bovine and rat tissues tested showed immunoreactivity at 150,000 and 130,000 Da with anti-XO, only pig liver, but not heart, lung or kidney was immunoreactive with anti-XO. An extensive literature search indicates that while pig liver is a known source of the enzyme (72) there is no reliable information available on other porcine tissues. It is not known if these tissues have been unsuccessfully assayed, or if XO is found in porcine milk. It is clear, however, that bovine and porcine XO cross-react as confirmed by the observed reactivity of the enzyme in porcine liver homogenates with the antisera to the bovine enzyme. Although it has been reported that XO in human lung cross-reacts with anti sera specific to bovine XO (73), no human reactivity with the anti bovine XO was observed in these studies.

Use of IXP Assay in these Experiments

Purified bovine XO was used to characterize the fluorescence assay system of Markley (113) for the purpose of these studies. Although this

assay has been reported to be 1000 fold more sensitive than the conventional urate assay (115), in this laboratory it was only 100 fold more sensitive when assayed at the limits of detection. This difference could be attributable to the use of different fluorimeters, since all reagents purchased were the same as those prescribed by the protocol (116).

An interesting finding from these studies was that a certain percentage (10%) of purified bovine XO could be converted back to XDH by the use of thiol reducing agents (96). This was fortuitous since it made possible the characterization of the assay for use with XDH in tissue as well.

The efficiency of the fluorometric IXP assay for XDH-XO has been recently questioned for application ot human tissues (90). Although pterin is a synthetic substrate for XO (77), IXP catalytically produced from pterin was similar to the normal product of the degradation of hypoxanthine, urate in two ways. First, the IXP product inhibited the catalytic action of XO at high concentrations as the product accumulated during catalysis similar to the product inhibition of XO by urate. Secondly, IXP is degraded in pig tissue. Uricase which degrades urate into allantoin is found in pig tissues. While it is unknown whether it is indeed uricase which is acting on the IXP, this could be tested by investigating the effects of uricase inhibitors on the degradation of IXP by pig tissue extracts. It should be noted that the effects of heat denatured pig tissue homogenate on IXP were not investigated in these studies.

Using the IXP assay to determine XDH-XO levels in various tissue homogenates gave results similar to those from SDS-PAGE Western blotting.

Nevertheless, it was surprising to note the apparent total lack of enzyme activity in pig lung. This is certainly the exception to all other mammalian species studied to date, which show at least some level of activity (72). It has been hypothesized that subhuman species, which are capable of the further degradation of urate to allantoin do not have the enzyme strictly compartmentalized in the endothelial cells, but rather show a more ubiquitous distribution of the enzyme throughout the tissues (88). The finding that XO is apparently localized only in the liver of the pig appears to contradict this hypothesis. Because of the extremely low levels of activity found in human lung extracts, this finding was not seen to be in conflict with results from gel electrophoresis but rather that it was below the level of sensitivity for that method. Based on the overall lack of XDH-XO activity in porcine lung, along with data suggesting that XO is localized only in microvascular endothelium XDH-XO activity was not expected to be found in cultured porcine pulmonary artery endothelial cells. This was confirmed by analysis. It would be of interest to pursue this line of investigation using cultured bovine or lamb pulmonary artery cells in order to see if this can be confirmed as well in species which do show XO activity in the lung.

Despite lack of porcine pulmonary XO activity it was unfortunate that porcine microvascular endothelium could not be cultured in sufficient number to assay for XDH-XO activity. The author is optimistic that this method of isolating pulmonary microvascular cells could prove to be a profitable tool for investigation with a few more trials. A larger initial seed of cells would be most beneficial. Based on casual observation of cells in culture,

cells from neonatal tissue did show a greater propensity to divide and reach confluence than did cells from adult tissue. The question remains unanswered as to whether colonies of cells originating from neonatal pigs were initially contaminated with fibroblasts or if cells in culture pleomorphically transformed into fibroblast-like cells. Since distinctly fibroblastic patches did not appear until 4 to 6 weeks after the initial seeding the second explanation may be more reasonable since neonatal and fetal cells are known to show more pluripotentiality than adult cells and fibroblasts normally grow much more rapidly than endothelial cells. Overgrowth would occur much more rapidly than was observed, therefore. The use of sera from neonatal pigs was not found to have enough observable benefit in these experiments as observed by time required to reach confluence to merit its separate harvesting as it is not commercially available. However, greater benefits may be present for more established cell colonies, since fetal bovine serum is known to be more beneficial than adult bovine serum as a cell culture supplement. Again, it would be of interest to pursue this technique of microvascular endothelial isolation in neonatal peripheral lung of other species such as calf or lamb. Rat lungs are deemed to be too small for the peripheral dissection necessary to eliminate contamination by large vessel endothelium. Rat pulmonary endothelium also does not grow readily in culture (134). It would be of great morphological interest to determine if microvascular endothelium in culture will form monolayers. From our observations they appear to have a propensity to grow in regularly spaced open networks reminiscent of the microvasculature in vivo. It may well be that the contact inhibition of

large vessel endothelium is exaggerated in the microvasculature, causing them to branch out in new directions before central confluence is reached.

Comparison of Results from These Studies with Those Previously Cited in the

Literature

The detrimental effects of exogenous perfused xanthine oxidase plus xanthine on the pulmonary circulation are well documented (33), (60). An attempt has been made to examine the effects of allopurinol, a xanthine oxidase inhibitor on preventing the development of free radical—mediated pulmonary edema with both collapsed lung (63) (74) and embolic models. Results from these studies showed that allopurinol does have a slightly beneficial effect, however, the efficacy of the collapsed lung model in causing significant edema has been called into question, and thus only a slight effect of allopurinol would be seen if the baseline edema was minimal. Nevertheless, relatively few studies have addressed the function of endogenous xanthine oxidase in the lung, despite the fact that it has been known since 1935 to be present in the lung of rat and cow as detected by the urate assay (110). In this early comparative work by Booth (110), a reference is given to the enzyme being unsuccessfully assayed in the lung, but the author indicates his uncertainty as to whether the enzyme is present or not by the notation, "?", opposite that tissue in his table listing XO activities for various species and organs. The results reported in this study using a more sensitive enzyme assay and immunolocalization indicate that if present, the enzyme was below the level of detection in pig lung.
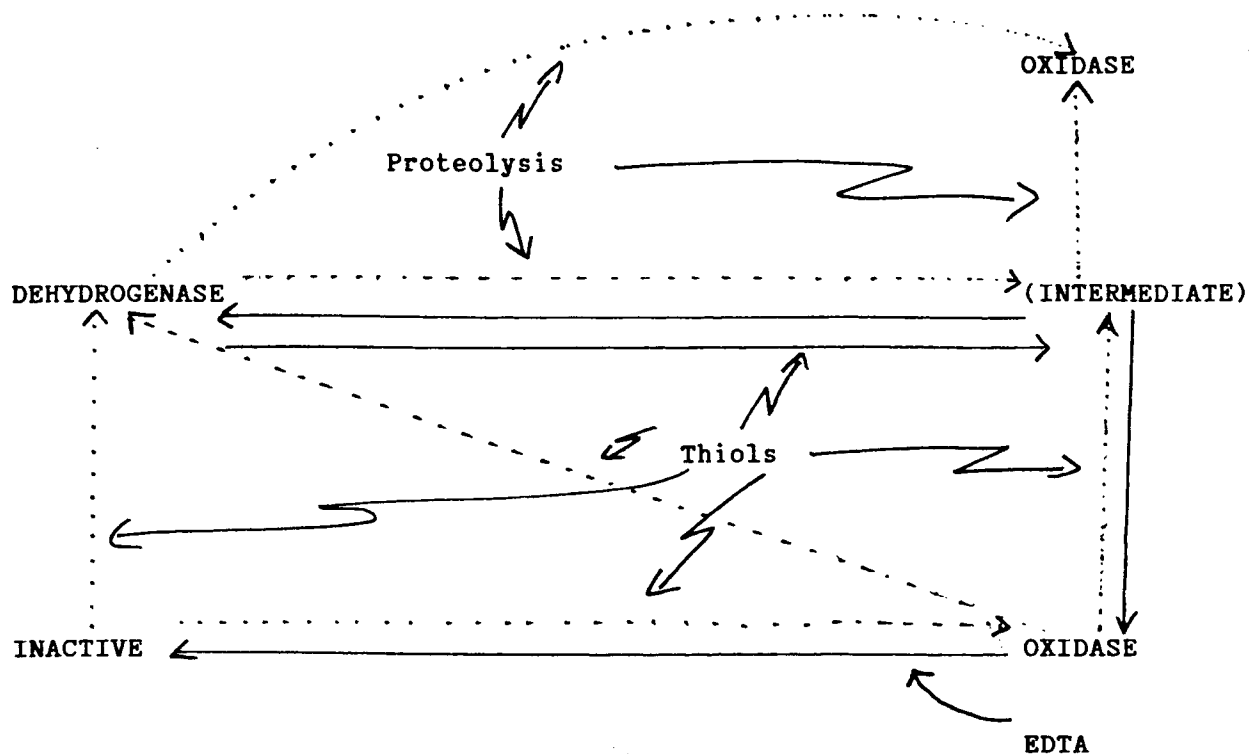
Figure 26. Conversion of Rat Liver Xanthine Oxidase. This schema shows the interconversion of xanthine oxidase among its various possible forms. Broken arrows indicate uncertain or alternative pathways (87).

In the present study 85-90% of the enzyme was found to be reversible to the dehydrogenase form. This is in accordance with the work of McCord and Roy (100), who found 85-90% of the enzyme to be in the dehydrogenase form when ileal tissue was frozen and processed immediately in liquid nitrogen to prevent proteolysis. Interestingly in the present study, freezing was not necessary to prevent proteolytic conversion in the lung. These results are also in conflict with McCord and Roy who found that 30 min of ischemia could irreversibly convert XDH to XO (100). Our findings indicate that up to 60 min of tissue ischemia or 10 hours of incubation designed to activate endogenous proteases in the lung did not cause irreversible enzyme conversion. It was found that while the enzyme appears to be predominantly in the oxidase form after standard isolation procedures from the lung even when EDTA and EGTA are employed as calcium chelators, thiol reduction by DTT will convert it to the dehydrogenase. In agreement with this observation is the work of Della Corte and Stirpe (94) who noted that while the rat lung appears to be in the oxidase form it may be converted back to dehydrogenase by DTT.

The work of Jarash et al. (73) used an immunolocalization technique to visualize XO in frozen sections examined by light microscopy and electron microscopy. These authors found that the enzyme was localized in lactating bovine epithelial cells and microvascular but not large vessel endothelium. An initial objective of this thesis project was to assay porcine peripheral lung sections, which are known to contain only vessels less than 50 μm (128) in comparison with central lung homogenates. If indeed XDH-XO is only found in microvascular endothelium, an enrichment of enzyme activity would

be expected in the peripheral lung homogenate. Due to a lack of XDH-XO activity in porcine lung this question remains unanswered. Further work by Bruder and Jarash (87) involved the use of immunoaffinity chromatography isolated XO from various bovine tissues. SDS-PAGE of XO purified from bovine lung was shown to separate on gel electrophoresis as a single band on SDS-PAGE, with molecular weight 155 KDa. Since no anti-proteolic precautions were taken with their preparation, it can be interpreted that proteolytic activity in the lung does not act on XDH to any great extent. This work is in agreement with our findings on conversion of XDH to XO in the rat lung.

Conversion of XDH to XO in the Rat Lung

While one of the initial goals of this project was to study XDH to XO in an easily manipulatable endothelial cell culture system, difficulties in obtaining viable microvascular cultures in a species exhibiting pulmonary XO made it necessary to study enzyme conversion in rat lung extracts. Using trypsin derived from bovine pancreas on lung extracts with no EDTA it was shown that (Figure 21) proteolysis of XDH will indeed cause conversion from XDH to XO which cannot be reversed by incubation with thiol reducing agents, in agreement with the multiple conversion scheme of Stirpe (Figure 26).

In an attempt to employ endogenous proteases in the lung extracts to effect enzyme conversion by overnight incubation at 15°C it was found that while 100% of the enzyme was in the oxidase form after 16 h, incubation of these extracts with 1 mM DTT returned most of the enzyme to its dehydrogenase conformation. Thus, endogenous proteases in the lung do not

appear to be capable of affecting the irreversible conversion of XDH to XO.
Since low molecular weight substances had been removed from the lung extract
by separation on chromatographic columns the possibility of small molecular
weight protective compounds are present. The lung is known to be protected
by α-1-antitrypsin (63), which has itself been shown to be degraded by
oxygen free-radicals. Soybean trypsin inhibitor has also been used as a
control for the proteolytic activity of trypsin on rat liver XO (93),
leading to the hypothesis that α-1-antitrypsin may serve more than one
protective function in the lung.

Studies with the use of different calcium chelators on enzyme conversion
in both normoxic and ischemic lung tissue indicated that neither chelation
of divalent cations with 1 mM EDTA nor more specific chelation of calcium
with 1 mM EGTA had any significant effect on preventing conversion of XDH to
XO. This conversion was however reversible with the thiol reducing agent
DTT and thus proteolysis of the enzyme by calcium-mediated proteases (100)
did not account for irreversible conversion of the enzyme. It is possible
that the calcium effect is not an important factor in control of XDH/XO
conversions in lung tissue if the lung is indeed protected against
proteolysis as was suggested earlier.

The length of time which was required for tissue to be excised, weighed
and homogenized before assay was of some concern since presumably the rats
would by ischemic thus allowing the conversion process to proceed from the
time the diaphragm was opened. A series of experiments were were conducted
which employed rapid cannulation of the pulmonary artery and perfusion of
the lung vasculature with a homogenizing buffer containing 1 mM EDTA and 1

mM DTT was used in order to investigate whether having calcium chelators and thiol reducing agents perfused into the lung as rapidly as possible might result in some slight difference in irreversible and reversible conversion. When compared to perfused lung without protective agents this was found to be true. Levels of XDH in lung perfused with DTT and EDTA were significantly higher than XDH levels in lungs which had no DTT and EDTA present from the onset of ischemia. When compared with results from previous experiments using calcium chelators, it is possible to speculate that there is a small initial pool of XDH which will convert very rapidly and irreversibly to XO in the lung. Studies using Granger's model of excision "ischemia" (58) on the lung do not take this into consideration and only account for conversion which may take place after the initial five minutes. This is, however, a small fraction of total enzyme activity. XDH which is not irreversibly acted on during the initial period of ischemia to be remarkably resistant to proteolytic conversion even up to 10 hours later, as shown by incubation of lung extracts at $15^{o}C$ overnight. Thus oxidation of thiol groups appears to be a more important mechanism in the lung (93) (96).

No success was achieved upon attempts to separate the effects of DTT and EDTA in the perfusate to determine which has the greater effect on maintaining XDH both conformationally and structurally. Further studies are necessary on both of these factors during the initial period of ischemia in order to draw any firm conclusions as to whether thiol redox status or Ca^{++}-mediated proteolysis is more important for maintaining the enzyme in the dehydrogenase form (XDH).

Consequences of Conversion of XDH to XO for the Organism

Regardless of whether XDH is permanently or reversibly converted to XO, XO is capable of producing superoxide free radicals. Indeed, while the bulk of XDH in the lung appears to be subject only to reversible conversion to XO, this conversion appears to take place very easily and rapidly. If microemboli cause a focal area of ischemia within the lung one could speculate that rapid yet reversible conversion to XO might take place. The same would hold true for microthrombi which are known to be cleared from the circulation in the pulmonary microvasculature.

What the role of this rapid conversion might be remains unclear. Jarasch, Bruder and Heid have speculated that XDH may convert rapidly to XO when the endothelium is subject to bacterial attack causing endothelial injury (Figure 27) (132). Xanthine oxidase may thus form a first line of oxidant defense for the body against microorganisms in the blood. In addition superoxide generated by XO could elicit a chemotactic response for neutrophils through the generation of lipid radical chemoattractants (71). This group supports this hypothesis with the interesting observation that while XO was virtually non-existent in the serum, even in patients with extensive vasculopathy, extremely high levels of endogenous antibodies to xanthine oxidase were found in the serum of all animals studied. Up to 7% of all IgG specificity was directed against XO in one study of human samples (133). It is felt that these autoantibodies serve to protect the organism from massive oxygen free radical damage, precipitating the enzyme as soon as it is released from the cell. However, since one of the most important by-products of the generation of superoxide, H_2O_2, is freely diffusable
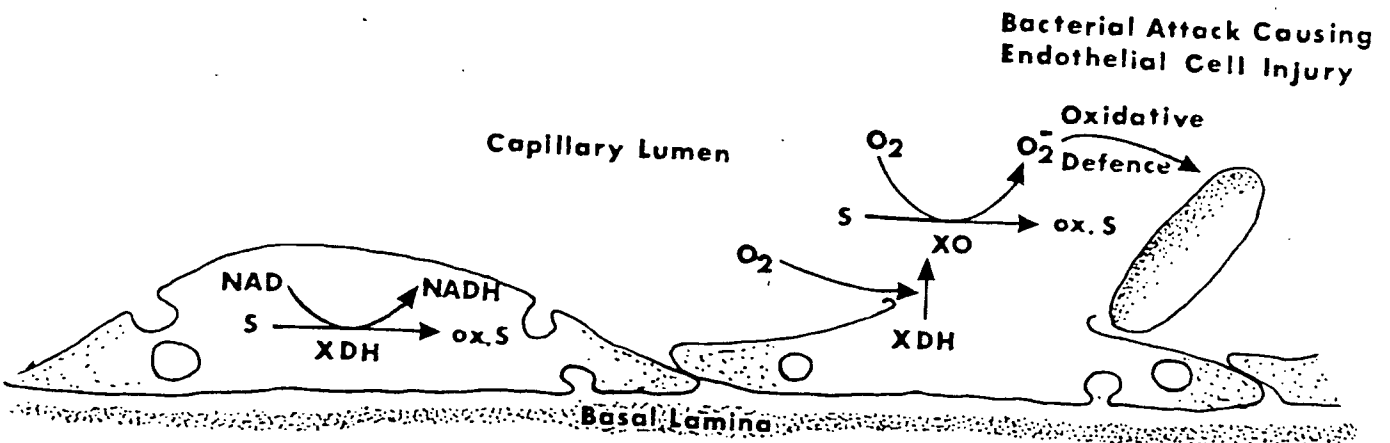
Figure 27. Hypothetical scheme of a role of xanthine oxidase in defence mechanism of capillary endothelial cells against bacterial attack. As per Jarash, Bruder and Heid (122).

across cellular membranes it seems unnecessary for endothelial lysis to occur for oxygen free radicals to be released into the circulation. Oxygen free radicals generated by activated neutrophils have been shown to lyse endothelial cells (28), leading to the question of whether it is XO or activated neutrophils which act as the proverbial chicken or egg.

Recent commentary on the role of oxygen free radicals in causing protein oxidation which facilitates normal proteolytic breakdown and protein turnover (14, 17) gives rise to another interesting approach. It is possible that focal patches of ischemia in the lung due to clearance of microthrombi might cause rapid conversion of XDH to XO, with subsequent generation of oxygen free radicals causing modifications in clotting proteins, thus initiating the first stage in fibrinolysis. Whether this is a realistic hypothesis and how the reversibly converted enzyme would return to its reduced state remains to be seen.

Another area yet to be explored is whether oxygen free radicals and proteases generated from activated neutrophils could cause conversion of XDH to XO, either reversibly or irreversibly. While some form of hypofusion appears to be required along with the presence of neutrophils in the etiology of ARDS, it remains unclear whether it is indeed endothelial changes which activate neutrophils or vice versa. Many studies have been published showing the protective effects of inhibition of XO by allopurinol in various ischemic and hypoxic lung models. The interaction between neutrophils and the endothelium are certain to be of continuing interest to free radical biologists and clinicians alike. The identification of XO activity in one human lung sample in this study confirming immunoreactive

findings (73) indicates that this avenue of thought should be further investigated for whatever role it might play in the etiology of ARDS.

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