Absorption of Enzymatically Active ^{125}I-Labeled Bovine Milk Xanthine Oxidase Fed to Rabbits

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Rabbits fed a regular laboratory diet supplemented with a high-fat milk containing xanthine oxidase (XO) were studied to determine the presence of active XO in the blood. A pilot feeding study, where rabbits consumed a high-fat diet containing xanthine oxidase, showed a correlation between dairy food consumption and XO activity in the blood. Antibody to dietary XO was also found. In a second study, rabbits were fed ad libitum the high-fat milk and blood serum samples were tested weekly for XO activity. No elevation in serum XO activity was found. A third study showed that serum XO activity was increased when rabbits were force fed the high-fat milk. The final study consisted of force feeding ^{125}I-labeled XO to one rabbit to ascertain whether the observed increase in serum XO was due to dietary or endogenous XO. Isoelectric focusing of sera collected from the test rabbit strongly suggested that at least a portion of the serum XO contained the radioactive label. This is the first direct evidence showing the uptake of dietary active XO from the gut.

Xanthine oxidase (XO; EC 1.2.3.2) is present in high concentration in cow's milk (Ball, 1939; Greenbank and Pallansch, 1962; Zikakis and Wooters, 1980), whereas the milk of sheep and goats contains low levels of XO activity (Crosland et al., 1958; Modi et al., 1959; Owen and Dundas, 1969; Zikakis et al., 1983).

In the early 1970s a hypothesis was proposed (Oster, 1971, 1972; Ross et al., 1973; Oster et al., 1974) associating the initiation of the atherogenic process with ingestion of bovine milk XO. With its broad specificity, XO oxidizes purines, pyrimidines, other heterocyclic compounds, and most known aldehydes including the arterial cell membrane based plasmalogens as they are metabolically turned over (Oster, 1972; Morpeth, 1983). This causes the initial damage to the cell membrane of the arterial intima and the myocardium. The resulting histochemical injury is followed by cell proliferation and scar formation in the affected cardiovascular tissues, local deposition of cholesteryl esters, and ultimate development of typical atherosclerotic lesions in the arteries (Oster and Mulinos, 1944; Oster, 1971).

In the mid 1970s this topic received considerable publicity in the popular press (Michelini, 1975; Von Hoffman, 1974; Webb, 1975). In 1975 the Food and Drug Administration commissioned the Life Sciences Research Office of the Federation of American Societies for Experimental Biology (FASEB) to review the evidence and evaluate the potential role of XO in the etiology of atherosclerosis. The same year, FASEB's evaluators issued a technical report (Carr et al., 1975) concluding that the available evidence for or against Oster's hypothesis is inconclusive. Due to the gap in knowledge in certain areas and the urgency of elucidating heart disease, FASEB evaluators recommended additional research to be done in specific areas. Subsequently, a considerable number of studies have been carried out, most of which support the concept (Oster et al., 1974; Zikakis and Rzucidlo, 1976; Mangino and Brunner, 1976; Clark et al., 1976; Ho and Clifford, 1976; Zikakis et al., 1977; Gandhi and Ahuja, 1979; Rzucidlo and Zikakis, 1979; Zikakis and Wooters, 1980; Povoa et al., 1984; Bruder et al., 1984; Zeise and Zikakis, 1987; partial list).

In order for XO to interact with the arterial cell membrane, the enzymatically active enzyme must enter the bloodstream intact. Therefore, one important factor that had to be determined was whether dietary, enzymatically active XO could be absorbed or persorbed (Volkheimer, 1972). Because of the nature of the association of XO with the milk fat globule membrane (Zikakis, 1974), which has been reported to resemble a liposome in structure (Ross et al., 1980), the passage of enzymatically active XO into the blood is believed to be possible (Kabacoff et al., 1963; Warsaw et al., 1971; Volkheimer, 1972; Walker et al., 1972; Walker and Isselbacher, 1974; Ross et al., 1980; Rzucidlo and Zikakis, 1988).

Animal studies have shown that serum levels of XO activity increased following ingestion of processed cow's milk or purified milk XO (Clark et al., 1976; Gandhi and Ahuja, 1979). In vitro studies supported these results (Gandhi and Ahuja, 1979). Furthermore, Gandhi and Ahuja (1979) found that XO decreased the plasmalogen content of the heart muscle after daily oral and intravenous administrations of purified XO to rabbits. Moreover, the presence of antibody titers to purified XO has been found in man (Oster et al., 1974; Rzucidlo and Zikakis, 1979). Although the aforementioned studies support the hypothesis, the evidence is indirect and presently no one has directly identified dietary XO in the blood.

The objective of this study was to find out whether dietary enzymatically active XO could be detected and identified in the serum of rabbits.

MATERIALS AND METHODS

Materials. Bovine milk was obtained either from the Guernsey herd of the University of Delaware Agricultural Experiment Station (for enzyme purification) or from local supermarkets (for feeding studies). Xanthine oxidase purified by the

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method of Zikakis (1979) was iodinated at Meloy Laboratories by a modified chloramine T procedure (Hunter and Greenwood, 1962). The enzyme was assayed for activity before and after labeling with ^{125}I (Zikakis et al., 1983). 2-^{14}C-Labeled uric acid was purchased from Amersham Searle (Arlington Heights, IL), and [8-^{14}C]xanthine was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Tissue culture reagents were purchased from Flow Laboratories or GIBCO. A-Gent uric acid kits were purchased from Abbott Laboratories, and Monofluor liquid scintillation fluid was purchased from National Diagnostics. Other reagents and solvents were of reagent grade.

New Zealand albino rabbits were purchased from HARE (Hewitt, NJ).

Methods. *Study 1.* Four New Zealand Albino rabbits (3.8–4.6 kg) were housed individually under laminar flow conditions with the temperature and humidity at 23 ± 5 °C and 50 ± 10%, respectively. A 12-h light/dark cycle was observed. Each rabbit was identified with a three-digit ear tattoo. All rabbits received Purina rabbit chow pellets. Control rabbits received tap water, and treated rabbits received only a high-fat milk, ad libitum, as their source of fluid. The high-fat milk consisted of 50% commercial milk and 50% commercial half and half. XO activity in all dairy preparations was measured by the method of Zikakis and Treece (1971) before feeding. Two rabbits (379, 373) received the supplemented milk, and two rabbits (387, 390) received tap water. Food and liquid consumption were measured daily.

Rabbits were bled daily, prior to treatment for the first 7 days and on days 10, 24, 45, 46, and 47 of the study. Blood was drawn from the marginal ear vein after the ear was swabbed with isopropyl alcohol. Serum was harvested and assayed for XO activity with use of an A-Gent uric acid kit.

At necropsy, mesenteric lymph nodes were harvested from one treated and one control rabbit and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 10^{-5} M dexamethasone, and 25 μg/500 mL porcine pancreas insulin (25.2 U/mg), and the pH was adjusted to 7.4. The cultures were challenged with purified XO, washed, and cultured for several days. Each day the medium was removed, harvested, and replaced with fresh culture medium. The harvested samples were assayed for XO antibodies by a passive hemagglutination procedure (Garvey et al., 1977).

Study 2. A group of 18 male New Zealand albino rabbits was randomized by weight and housed individually under conventional conditions. Purina rabbit chow was available ad libitum as were the water and high-fat milk (50% whole milk and 50% half and half). Body weights were taken weekly, and food and liquid consumptions were measured daily. All other conditions were the same as described in study 1. Rabbits were bled weekly, and the sera were assayed for XO activity by a modified radiochemical procedure (Dougherty, 1976). Prior to assay all sera were chromatographed in micropipet columns packed with Sephadex G-25 (fine) with 1% albumin as the eluant.

Serum samples were incubated with 2.22×10^5 dpm [^{14}C]xanthine for 60 min to allow conversion of [^{14}C]xanthine to [^{14}C]uric acid. A 1-mL portion of acetonitrile was added to each tube to stop the reaction, and the precipitate was centrifuged for 10 min at 800*g* in an IEC HN-SII table-top centrifuge. Supernatants were harvested and evaporated to dryness under nitrogen. The residues were reconstituted with 0.3 mL of 20 M NaOH and filtered through 0.45-μm Acro LC-13 filters with 1-mL disposable syringes. Ten microliters (7400 dpm) of each sample was applied to reversed-phase TLC plates and chromatographed in closed TLC tanks containing 100 mL of 10 mM sodium acetate buffer (pH 7.0) (Kiser et al., 1978). This procedure separated [^{14}C]xanthine from [^{14}C]uric acid. Plates were allowed to dry overnight and were scrapped in 1-cm sections into mini scintillation vials. Five milliliters of Monofluor scintillation cocktail was added to each vial. Appropriate blanks and standards were also prepared. All samples were counted in an ISOCAP 300 table-top scintillation counter, and the data were statistically analyzed by analysis of variance.

Study 3. Beyond the 4-week feeding study, the rabbits were maintained on their respective treatments. Four pairs of rabbits from the ad libitum study were chosen to be fed by gavage. One pair at a time was tested on different days. Two of the pairs (065, 055; 052, 082) were fasted prior to treatment. The other two pairs (061, 078; 069, 067) were allowed food and their treatment regimen ad libitum. Each pair was bled prior to gavage and then given three 50-mL doses of their respective treatments at 30-min intervals. Each rabbit was bled 30 min after each dose and at 3 h after the first dose. Sera were assayed for XO activity by the method of Dougherty (1976). All analytical procedures were the same as mentioned earlier.

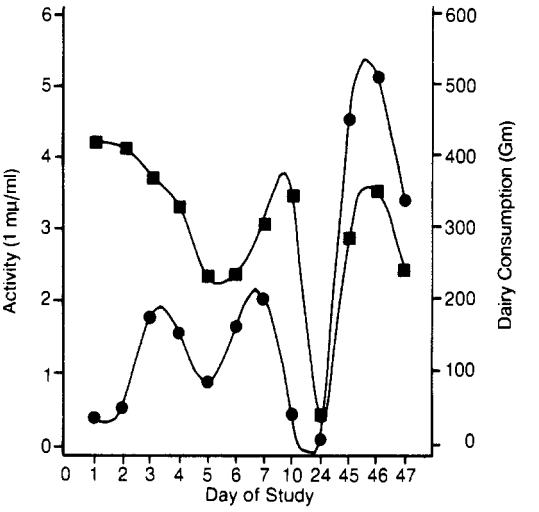

Figure 1. Correlation between dairy food consumed (■) by the treated rabbit (379) and its serum XO activity (●).

Study 4. Rabbit 061 was chosen to receive the dose of radioactive XO. This rabbit was fasted from midnight on the day of dosing. A pretreatment blood sample was taken, and the rabbit was given a 50-mL dose of dairy mixture containing 5.25 mCi of ^{125}I-labeled XO. Blood samples were drawn at 2 and 3.5 h after dosing and the sera harvested and frozen. A pH profile was performed on isoelectric focusing gels to confirm the presence of a linear gradient. The sera were isoelectrofucused (Zikakis and McGinnis, 1988) by combining 150 μL of serum with 4.5 μL of sucrose and 15 μL of Pharmalyte. Seventy microliters of the sample was applied to each gel. Duplicate gels, one for protein and one for enzyme activity, were run for each sample and standard. Gels were prepared and prerun to establish the pH gradient before samples were added. Samples were isoelectrofocused for 3 h at 9-W constant power with the temperature maintained at 16 °C. When completed, the duplicate gels were placed either in trichloroacetic acid/5-sulfosalicylic acid for 2 h and then 0.1% fast green stain for 30 min to stain for protein or in a stain solution of neotetrazolium/xanthine to stain for XO activity (Zikakis, 1981). After being stained, the protein gels were placed into a destaining solution of 10% acetic acid/30% ethanol to remove excess stain from the gels. This destain solution was changed once or twice a day until gels were clear. The protein gels were scanned in a gel scanner at 625 nm and then sliced in 0.5-cm sections and counted in a γ counter (Tracor Model 1185) for ^{125}I. The XO activity gels were scanned at 550 nm with a gel scanner and also counted for ^{125}I.

RESULTS AND DISCUSSION

Study 1. The serum levels of XO activity increased in rabbits fed the high-fat milk. In fact, Figure 1 shows a clear correlation between dairy food consumption and XO activity in the blood of this treated rabbit. Figure 2 shows that water consumption produced no increase in XO activity in the blood. The data are in agreement with the findings of Clark et al. (1976) with rats and Gandhi and Ahuja (1979) with rabbits.

Table I indicates that the serum of treated rabbits showed a time-dependent increase in antibody production to XO starting around day 24 of the study. Control

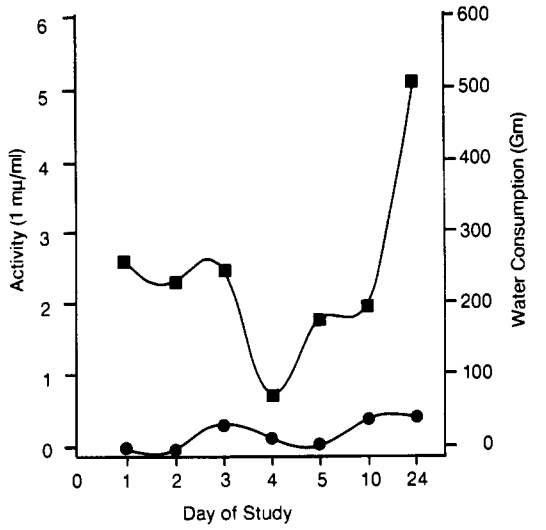

Figure 2. Control rabbit (390) consuming water (■) showed no elevation in serum XO activity (●).

Table I. Antibody Titers to Bovine Milk Xanthine Oxidase (XO) in Sera Collected Periodically throughout a 47-Day XO Feeding Study in Rabbits

	antibody titer[a-c]					
day of study	387 control	390 control	373 control	379 XO treated	GP12[d] XO treated	GP19[d] control
pretreat	0	0	0	0		
1	0	0	0	0		
2	0	0	0	0		
3	0	0	0	0		
4	0	0	0	0		
5	0	0	0	0		
6	0	*e*	0	0		
7	0	*e*	0	0		
10	0	0	0	0		
24	0	0	$10^{2.9}$	$10^{2.9}$		
45	0	*f*	$10^{4.1}$	$10^{3.5}$		
46	0		$10^{4.1}$	$10^{3.5}$		
47	0		$10^{4.1}$	$10^{4.1}$		
week 17					$10^{5.5}$	0

[a] Titers are expressed as the log of the reciprocal of the dilution to the base 10. [b] All assays were done in duplicate. [c] Starting dilution for the four-rabbit sera was 1:100. [d] Starting dilution for the two guinea pig sera was 1:10 000. These were sera of known Ab titers from immunized guinea pigs and were done as controls. [e] No serum samples could be obtained. [f] Died.

rabbits produced no antibody to XO. Control guinea pig serum and serum from guinea pigs immunized with XO were also tested to confirm that the test system was working properly.

Table II shows that mesenteric lymph node cultures from a rabbit treated with XO containing dairy food produced antibody to XO while similar lymph node cultures from a control rabbit did not produce antibody to XO. Although this is the first study where lymph nodes of animals consuming dairy foods produced antibody to XO when challenged, our results corroborate previous findings with human subjects (Oster et al., 1974; Rzucidlo and Zikakis, 1979).

Study 2. Because it was necessary to bleed this large number of rabbits weekly rather than daily, and because of the sporadic feeding habits of these animals, it was impossible to determine the optimal bleeding time for each rabbit. As a result, no statistically significant difference between sera of treated and control rabbits was discovered. It was concluded that better control of the feeding and bleeding times was necessary, so the same

Table II. Antibody Titers to Bovine Milk Xanthine Oxidase (XO) Derived from Mesenteric Lymph Node Cultures from a Rabbit Treated with Bovine XO and Control Rabbit

			antibody titers[a,b]	
rabbit	treatment	days in culture	culture 1	culture 2
379	XO	1	0	0
		2	0	0
		3	0	0
		4	$10^{0.9}$	10^{0}
		5	$10^{1.2}$	$10^{0.6}$
		6	$10^{1.8}$	$10^{1.2}$
		7	$10^{1.8}$	$10^{1.8}$
		8	$10^{1.8}$	$10^{2.1}$
		9–10	$10^{2.1}$	$10^{2.4}$
387	control	1	0	0
		2	0	0
		3	0	0
		4	0	0
		5	0	0
		6	0	0
		7	0	0
		8	10^{0} [c]	0^{0}
		9–10	0	0

[a] Antibody titers are expressed as the reciprocal of the dilution of the culture medium to the base 10. [b] The culture medium in well 1 of each microtiter plate was undiluted. [c] The positive response is attributed to a nonspecific reaction.

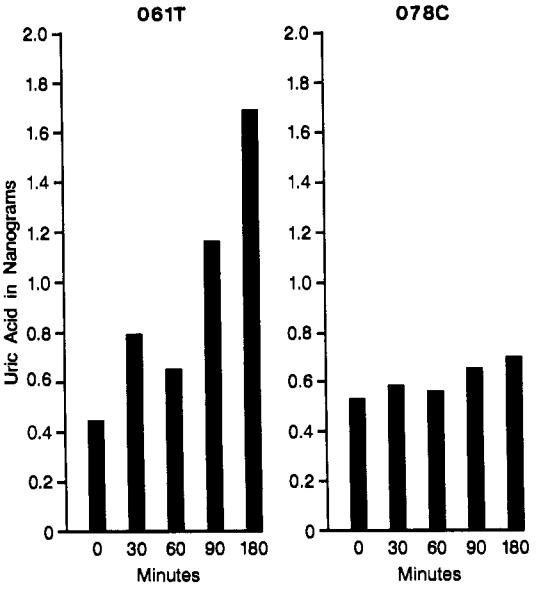

Figure 3. Levels of serum uric acid found in rabbits 078 (control) and 061 (treated). Both rabbits were unfasted prior to intubation of three 50-mL doses of a dairy preparation (treated) or water (control), given at 30-min intervals. Blood samples were taken prior to dosing (0 min), 30 min after each dose, and 180 min after the first dose.

rabbits were used in a paired forced feeding experiment (study 3).

Study 3. Results represented in Figure 3 are from unfasted rabbits. The treated rabbit (061T) shows increased uric acid production at 90 and 180 min, suggesting an increased concentration of XO in the blood at the later sampling times. Since the rabbits were unfasted and food was present in the gastrointestinal tract, it appears to have taken 1.5 h for XO to be detected in the blood. Uric acid levels of the control rabbit (078C) remain constant around 0.6 ng.

Results represented in Figure 4 are from fasted rabbits. The treated rabbit (052T) shows increased uric acid production at 30 and 60 min. The presence of XO in the blood at the earlier sampling times is probably due to the lack of food in the gastrointestinal tract after fast-

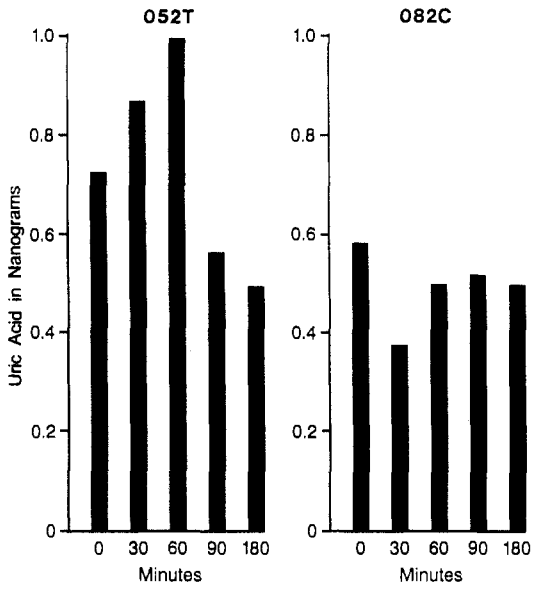

Figure 4. Levels of serum uric acid found in rabbits 082 (control) and 052 (treated). Both rabbits were fasted prior to intubation of three 50-mL doses of a dairy preparation (treated) or water (control), given at 30-min intervals. Blood samples were taken prior to dosing (0 min), 30 min after each dose, and 180 min after the first dose.

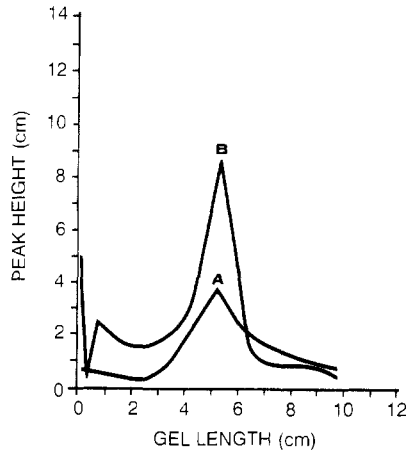

Figure 5. Xanthine oxidase standards. Peak A represents unlabeled xanthine oxidase while peak B is [^{125}I]xanthine oxidase.

ing. Again, uric acid levels for the control rabbit (082C) are around 0.6 ng.

Study 4. Standard gels were prepared by isoelectrofocusing radioactive and nonradioactive XO. These gels were stained for enzymatic activity and then scanned in a gel scanner, the results of which are shown in Figure 5. When the resulting profiles for these gels were superimposed on each other, the peaks representing XO overlapped. This confirms that both labeled and unlabeled XO migrated similarly in the gels.

Serum taken from the treated [^{125}I]XO rabbit 3.5 h after dosing was also isoelectrofocused in duplicate gels. One gel was stained for protein and the other for XO activity. When the gel stained for enzymatic activity was scanned densitometrically and the resulting profile was overlayed on the profile of the radioactive XO standard gel (Figure 6), the XO peaks coincided, indicating identity.

The gel that was applied with the radioactive XO standard and stained for protein was sliced in 0.5-cm sections and counted for ^{125}I in a γ counter. The radioactive profile of this gel (Figure 7) showed a large radioactive peak representing XO at about the midpoint of the

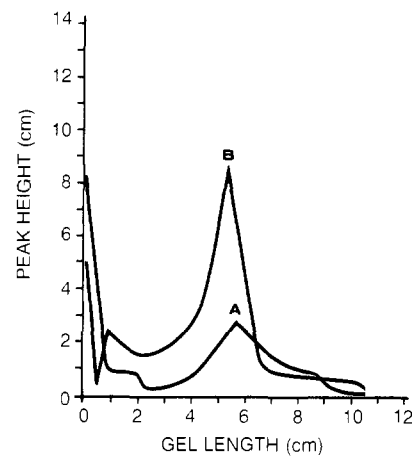

Figure 6. Densitometric scans of isoelectrofocused protein gels. Peak B represents [^{125}I]xanthine oxidase standards. Peak A is rabbit serum 3.5 h after dosing with [^{125}I]xanthine oxidase (5.25 mCi).

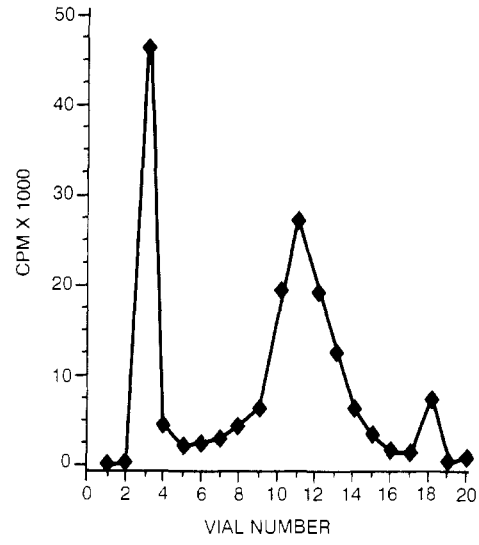

Figure 7. Radioactivity scans of isoelectrofocused protein gels containing [^{125}I]xanthine oxidase standards.

gel. The large initial peak is probably due to some polymerized XO that could not migrate into the gel. The small trailing peak is due to free ^{125}I that attached to the albumin which was added to the labeled XO as a stabilizer, after iodination. A similar radioactive profile was also prepared from the gel applied with treated rabbit serum and stained for protein. In Figure 8, this profile was overlayed on the profile of the radioactive XO standard. A small radioactive XO peak produced by the rabbit serum corresponds to the larger XO peak produced by the radioactive standard.

Contrary to popular belief, the results of these studies strongly suggest that dietary bovine milk xanthine oxidase was taken up (persorbed) into the blood and was detected in its enzymatically active form up to 3 h after its consumption. This is the first direct evidence to date that indicates the presence of enzymatically active XO in the serum taken up from the gut. These small-scale studies provide enough direct evidence to warrant further investigation to confirm the absorption of XO in various species, including man. Results from more extensive studies could help to elucidate the involvement of XO in the etiology of atherogenesis.

In related studies, it has been shown that oxygen-derived free radicals are the primary mediators of changes in vascular permeability associated with intestinal ischemia.

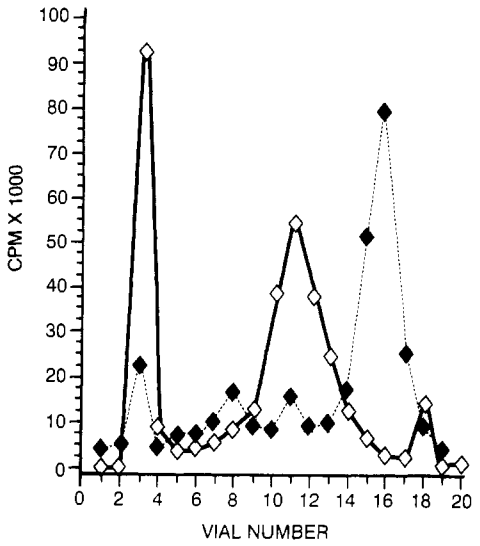

Figure 8. Radioactivity scans of isoelectrofocused protein gels: ◊—◊, [^{125}I]xanthine oxidase standards; ♦ - - -♦, rabbit serum collected 3.5 h after dosing with 5.25 mCi of [^{125}I]xanthine oxidase.

Roy and McCord (1983) reported that ischemia-induced conversion of xanthine dehydrogenase (EC 1.2.1.37) to XO. Park and Granger (1983) found XO to be the principal source of superoxide anions produced during intestinal ischemia. Superoxide radicals generate highly reactive hydroxyl radicals (McCord and Day, 1978; McCord, 1985), which, in turn, could alter the integrity of capillaries via lipid peroxidation. In this process, hydroxyl radical removes an allylic hydrogen from polyunsaturated fatty acid in the membrane of endothelial cells (Del Maestro et al., 1981). This could lead to a free-radical chain reaction, causing injury to cell membrane and subsequent cell death. More recently, it has been confirmed that XO is the generator of oxygen free radicals during reperfusion of ischemic hearts and plays a contributing role in the genesis of reperfusion injury (Lim and Kim, 1988). During ischemia, XO activity is increased with a concomitant decrease in superoxide dismutase and glutathione peroxidase activity, leading to an increase in the oxygen free radicals (Prasad and Kalra, 1988).

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Hydrolysis of Grape Monoterpenyl β-D-Glucosides by Various β-Glucosidases

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The efficiency of hydrolysis of monoterpenyl β-D-glucosides by various plant (grape and sweet almond) and fungal (*Aspergillus niger*) β-glucosidases was found to be dependent on the structure of the aglycon and the origin of the enzyme. Plant β-glucosidases hydrolyzed only β-D-glucosides of primary alcohols, such as geraniol, nerol, and citronellol. β-D-Glucosides of tertiary alcohols such as linalool and α-terpineol could be hydrolyzed efficiently only by one of the two fungal β-glucosidases studied. Diastereoisomeric monoterpenyl β-D-glucosides (linalool, α-terpineol, citronellol) and isomeric monoterpenyl β-D-glucosides (geraniol, nerol) were hydrolyzed at different rates.

The role of monoterpenes in Muscat grapes and wine flavors is now well established (Bayonove and Cordonnier, 1971; Ribereau-Gayon et al., 1975; Williams et al., 1980). A major fraction of these compounds is present in grapes under the form of diglycosides consisting of 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides and 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides. The corresponding aglycons are mainly geraniol, nerol, linalool, linalool oxides, 3,7-dimethylocta-1,5-diene-3,7-diol, and to a lesser extent α-terpineol and citronellol (Williams et al., 1982a; Günata et al., 1985). These precursors are unaffected during wine making and represent an important potential source of aroma (Günata et al., 1986).

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