SERUM XANTHINE OXIDASE STUDIES ON MINIATURE PIGS

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ABSTRACT

Miniature pigs were fed a commercial diet supplemented with homogenized bovine milk and xanthine oxidase to determine whether active xanthine oxidase is absorbed from the gastrointestinal tract into the bloodstream. No increase in xanthine oxidase activity, compared to the controls, occurred. Had even 0.001% of the ingested xanthine oxidase been absorbed in an active form, it could have been detected with the sensitive radiochemical assay. In pig serum studies, the half-time for elimination of intravenously injected bovine milk xanthine oxidase was 122 to 161 minutes.

INTRODUCTION

Xanthine oxidase (XO; EC 1.2.3.2), an enzyme capable of catalyzing the oxidation of numerous purines and aldehydes, is present endogenously in humans, particularly in liver and intestinal mucosa (1). The molecular weight of bovine milk xanthine oxidase is about 300,000 (2). According to Nagler and Vartanyan (2), the native enzyme consists of two polypeptide chains with molecular weights of 150,000 daltons each. Following limited proteolysis of xanthine oxidase and electrophoresis in the presence of sodium dodecyl sulfate, three fragments were produced with molecular weights of 92,000, 42,000, and 20,000 (2). Although there was no information that any of these three fragments was enzymatically active, activity was reported for the enzyme (isolated from whole milk) having a molecular weight in the range of 50,000 to 75,000 daltons as determined by ultrafiltration (3).

Oster postulated that xanthine oxidase in ingested homogenized bovine milk is absorbed from the intestines as an active enzyme, is carried by the lymph into the bloodstream, and injures the cell membranes in the myocardium and aorta by oxidizing the aldehyde moiety of the phospholipid plasmalogen (4). Based on detection of antibodies to

xanthine oxidase in human sera, Oster et al. (5) concluded that bovine milk xanthine oxidase must have been absorbed through the intestine. However, the xanthine oxidase used as antigen was only partially purified, and bovine milk and human xanthine oxidase cannot be distinguished immunologically (6). According to Oster's theory the resulting pathological processes eventually lead to the development of atherosclerosis, but there is no solid evidence to support this controversial hypothesis (7).

Recently, Clark et al. (8) studied serum xanthine oxidase activity in rats intubated with homogenized bovine cream (half cream and half milk). They found that the XO activity increased at 2 hours after treatment and suggested that absorption of active xanthine oxidase from the gastrointestinal tract had occurred or that cream may have stimulated endogenous xanthine oxidase activity. However, over the 6 hour test period, the mean activities for treatments and controls were the same. Normal variations in serum xanthine oxidase activity might explain the slight increase in activity observed initially.

In rat studies, Zikakis et al. (9) found that a portion of ingested bovine milk xanthine oxidase remained active after gastric digestion and entered the intestine. In in vitro studies, when milk was incubated with simulated gastric juice followed by pancreatin for 0.5, 3.5, and 7.5 hours, the recovered activities were 11.9, 7.2, and 6.1%, respectively (10). The enzyme in processed milk was completely inactivated below pH 3.9, but 35% of the activity was recovered by adjusting the pH to 7.3 (10). Ho et al. (11) reported that 27% of the original enzyme remained active after homogenization, pasteurization, and incubation of milk with human gastric juice and pancreatin for periods of time comparable to transit times in the gastointestinal tract of man.

Since absorption of active xanthine oxidase is a crucial part of Oster's hypothesis, we used a very sensitive assay for the enzyme to determine if any evidence could be found that an active form of xanthine oxidase from ingested homogenized bovine milk was absorbed into the blood serum of miniature pigs. In addition, we determined the biologic half-life of bovine milk xanthine oxidase in the pig serum.

MATERIALS AND METHODS

Xanthine oxidase (grade 1, from buttermilk) was purchased from Sigma,[1] and [8-^{14}C]xanthine (specific activity, approximately 62.8 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc. Pasteurized, homogenized bovine milk was purchased from a local market.

For use in one feeding experiment, partially purified bovine milk xanthine oxidase was isolated by the following procedure. Freshly collected raw milk from the University of Delaware Guernsey herd was

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[1]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

mixed (1:1) with 0.2 M phosphate buffer, pH 7.8, and incubated at 40°C for 2 hours. Triton X100 was added (1%), the solution was stirred for 15 minutes, and cooled to 4°C. A two-step $(NH_4)_2SO_4$ precipitation was conducted (20%, then 7% w/v), and the enzyme was dissolved in a minimum volume of 0.1 M tris-$CaCl_2$ buffer, pH 7. The crude preparation was used without desalting.

Eight miniature pigs[2] (Hormel strain) were removed from the mother at 5 days of age, divided randomly and by sex into two groups, and placed in a 4 x 8 ft. pen, divided into 2 sections. Three pigs received a commercial stock diet (Pig Primer, Southern States), and five were given the same feed plus pasteurized, homogenized bovine milk. Water was available ad libitum. When the pigs were 8 weeks of age, their stock diet was changed to Pig Developer. The average volumes of milk given and consumed per day by each pig at 30, 40, 45, and 59 weeks of age were 760, 1200, 900, and 1100 ml, respectively, and the average xanthine oxidase activity of the milk was 77.5 units/liter.

Prolonged Absorption Studies. After the pigs had been maintained on the experimental diets for 28 weeks, one pig from each group was cannulated through the jugular vein, and one week later, blood samples were drawn over a 200-minute period following feeding, and the serum xanthine oxidase activity was determined. The control pig (No. 54) consumed about 750 g of the commercial diet and the test pig (No. 55) ingested 200 g of the commercial diet plus 400 ml of pasteurized homogenized bovine milk. Five days later the procedure was repeated, except that the 400 ml of milk given pig 55 was supplemented with the partially purified bovine milk xanthine oxidase.

Half-time Determination. Two pigs from the milk supplemented diet were used to determine the biologic half-life of xanthine oxidase in serum (the time necessary to reach half the initial calculated zero-time activity). At 40 and 45 weeks of age pig 55 was fasted overnight and intravenously injected with 1004 and 918 milliunits of buttermilk xanthine oxidase (Sigma), respectively. Pig 56 (59 weeks old) was injected with 16 milliunits of bovine xanthine oxidase. The enzyme (in 3 ml of 1% albumin in 0.9% NaCl) was infused within 30 seconds into the anterior vena cava of the pigs. Blood was drawn periodically from the anterior vena cava into 10 ml vacutainers without additives. Immediately following collection and clotting, each blood sample was centrifuged and the serum was removed, stored at -20°C and analyzed within 8 hours after collection. Xanthine oxidase levels of 1 mU/liter remained stable when stored at -20°C for 3 days. The serum enzyme activities for each pig were plotted on semilogarithmic paper to give a curve showing the disappearance of the xanthine oxidase with time. The zero-time intercept was determined for each experiment, and from this value, the approximate volume of distribution and the half-time for elimination were calculated by standard methods (12).

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[2]Additional studies on these pigs will be described in another report.

Enzyme Assay. Xanthine oxidase activities were measured periodically from about 2 months after initiation of the test diets, using a sensitive radiochemical assay (13). Serum samples were filtered through Sephadex G-25 before assay in order to remove an apparent low molecular weight inhibitor. A unit (U) of xanthine oxidase activity was defined as the amount of enzyme which catalyzed the formation of 1 μmol of uric acid/min at 25°C (pH 8.3) at saturation levels of xanthine.

RESULTS

In a study conducted 12 weeks after initiation of the test diets, the serum xanthine oxidase activity of the milk group pigs was <0.4 mU/liter when measured 1 hour after the pigs consumed 600 ml of milk. During the course of our 14-month study, the activities usually ranged from 0.2 to 0.6 mU/liter for both groups. While the highest xanthine oxidase activity observed in the milk-supplemented group was 0.6 mU/-liter, about 3% of the control serum samples contained activities of 1.0 to 3.9 mU/liter.

Table I shows results of feeding studies conducted after the pigs had been maintained on the diets for 28 weeks. Compared with the control, the pig fed the stock diet plus homogenized milk showed no increase in serum xanthine oxidase activity. Five days later, after the

TABLE I. SERUM XANTHINE OXIDASE ACTIVITY FOLLOWING FEEDING

	Serum XO activity (mU/liter)		
Minutes after feeding	Pig 54 Stock diet[1]	Pig 55 Stock diet + bovine milk (36.8 U XO)	Pig 55 Stock diet + milk + purified XO[2] (184 U XO)
0	0.4	0.2	0.2
35	0.3	0.4	0.4
50	0.8	0.4	0.3
65	0.5	0.3	0.2
80	0.5	0.3	0.2
95	0.4	0.4	0.2
110	0.6	0.5	0.1
140	0.4	0.5	0.5
170	0.4	0.5	0.2
200	0.4	0.5	0.1

[1]Average values of two experiments, one week apart.

[2]Partially purified from bovine milk.

pig ingested a total of 184 units of bovine milk xanthine oxidase, the highest serum xanthine oxidase activity observed was 0.5 mU/liter. In the latter experiment, had 0.001% of the active enzyme been absorbed and remained in the circulation, the serum xanthine oxidase could be expected to reach about 3 mU/liter above normal. The approximate serum volume was 500 ml (14).

The half-time for elimination of intraveneously injected bovine milk xanthine oxidase was 122 to 161 minutes (Table II). Mammalian erythrocytes are virtually free of xanthine oxidase, and the calculated volume of distribution corresponds to the expected serum volume based on body weight (14). Figure 1 displays the rate of disappearance of bovine milk xanthine oxidase from the serum. After a phase of rather rapid decline (the mixing phase), serum xanthine oxidase activity declined in a fairly linear manner from about 30 minutes to the end of the initial 3-hour observation period. The possibility of activation or synthesis of endogenous enzyme was noticed at about 60 minutes following injection of 16 mU of xanthine oxidase (Fig. 1B); 7.5 hours after the administration of this low level of enzyme, the serum xanthine oxidase level was 3.9 mU/liter, which was higher than would have been expected had the activity continued to decrease in a linear manner. The high level of enzyme injected in the studies shown in Figure 1A would prevent detection of small increases in endogenous serum xanthine oxidase. Twenty-four hours after the infusion of 1004 and 16 mU of xanthine oxidase, serum activities of 9.8 and 1.3 mU/liter were present.

TABLE II. BIOLOGIC HALF-LIFE AND VOLUME OF DISTRIBUTION OF INTRAVENOUSLY INJECTED BOVINE MILK XANTHINE OXIDASE

Pig	Amount XO injected (mU)	Body weight (kg)	Zero-time XO activity (mU/liter)	Half-time (min)	Calculated volume of distribution (ml)
55	1004	30.8	1200	155	837
55	918	31.2	1070	161	858
56	16	24.5	22.1	122	724

DISCUSSION

The level of xanthine oxidase we found in the serum of miniature pigs was similar to that reported by Shamma'a *et al*. (1) for normal humans. Considering the estimated serum volume and the amount of xanthine oxidase consumed, it is possible to conclude that not even 0.001% of active xanthine oxidase was absorbed from the gastrointestinal tract into the blood serum of the pigs. This assumes that any xanthine oxidase absorbed would remain in the circulation and not be deposited in

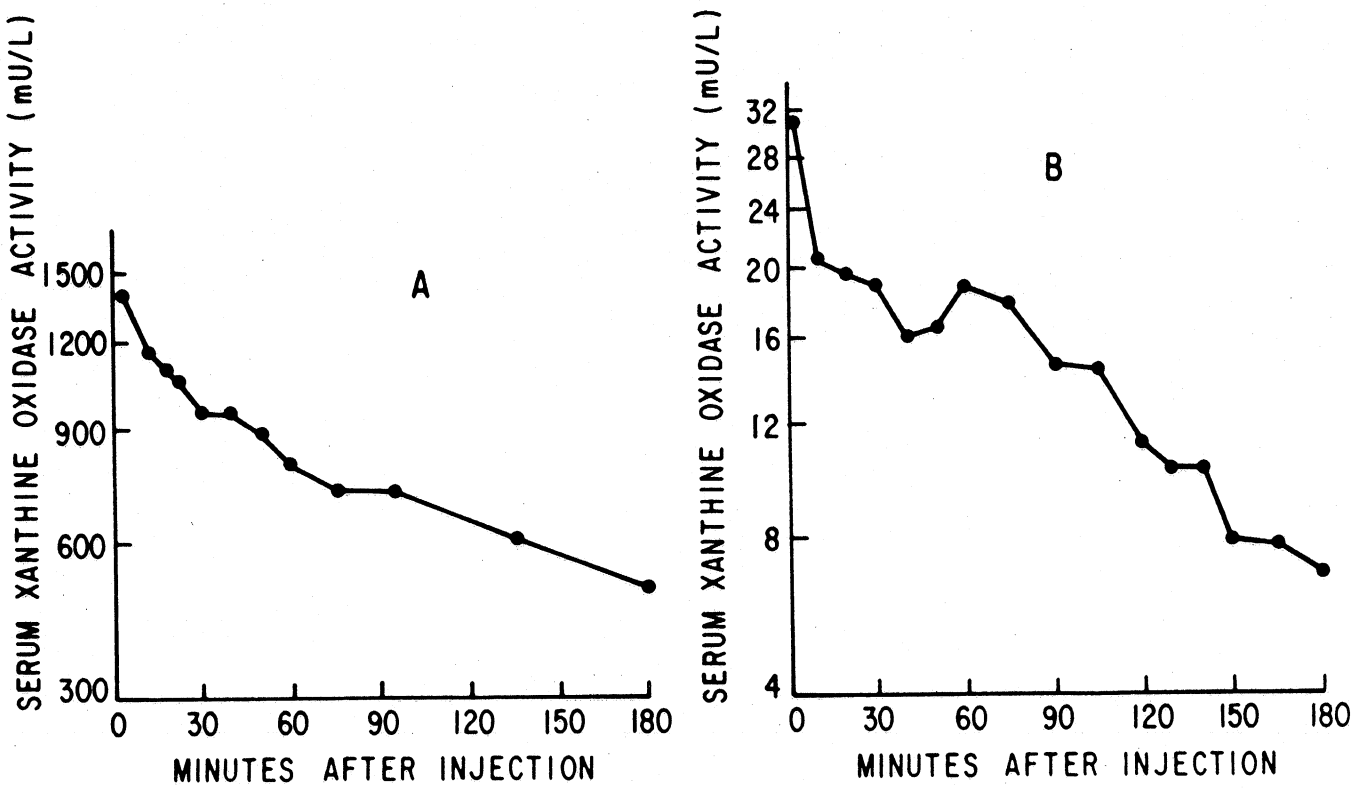

Figure 1. The disappearance of xanthine oxidase activity from serum after intravenous injection of 918 (A) and 16 mU (B) of bovine milk xanthine oxidase

the tissues. The data from the half-time studies confirmed that when the enzyme was injected, it remained in the serum for sufficient time to permit evaluation of data from the feeding experiments.

McCarthy and Long (15) studied the relationship of bovine milk intake to xanthine oxidase activity in pig and human sera. They found no correlation between average daily milk intake and serum xanthine oxidase in humans; however, no information was given regarding milk intake prior to blood collection. If the half-time of xanthine oxidase in human and pig sera is similar, it would not be possible to draw conclusions about absorption of low levels of the enzyme if the milk had been consumed several hours before blood was obtained.

McCarthy and Long (15) found no xanthine oxidase activity in pig sera. Using a similar assay procedure, we could not detect activity in pig serum prior to gel filtration of the samples. However, we observed an eleven-fold increase in activity of spiked serum following removal of low molecular weight components with Sephadex (13). We do not know whether the effect was due to an inhibitor or an endogenous substrate for xanthine oxidase.

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