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Studies on the Intestinal Absorption of Bovine Xanthine Oxidase[1] (39700)

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Xanthine oxidase is a two-subunit (mol wt 150,000/subunit) enzyme that catalyzes the conversions of hypoxanthine to xanthine and xanthine to uric acid. It occurs in several organs of the body, especially the liver and small intestine (1). Xanthine oxidase is found in high concentrations in bovine milk, and it has been suggested that this enzyme from homogenized milk is absorbed intact from the small intestine and is instrumental in the development of atherosclerosis (2, 3). Evidence presented has included a correlation, in some of the countries considered, between homogenized milk consumption per capita and the incidence of coronary artery disease (4). Also, xanthine oxidase activity has been found in human atherosclerotic tissues obtained at autopsy (5). It is purported that homogenization of milk reduces the size of the fat particles, which contain the xanthine oxidase, to such a degree that they are readily absorbed without being hydrolyzed (2, 4). Many objections to this hypothesis exist (6); the purpose of this work is to investigate the possibility that xanthine oxidase may be carried through the stomach and absorbed intact by the small intestine.

If xanthine oxidase is to be absorbed by the small intestine, it must first pass through the stomach. This would require that the enzyme resist hydrolysis when held at the pH of the stomach for a time comparable to its transit time. If the xanthine oxidase gets through the stomach, it faces a barrier to absorption in the small intestine because of its large size; macromolecules are not routinely absorbed (7). There is, however, some evidence for absorption of intact pancreatic enzymes (mol wt 25,000) (8, 9).

We have investigated the fate of xanthine oxidase at the pH of the stomach using a low pH buffer, and we have studied the absorption of the enzyme by the everted rat intestine.

Materials and methods. Xanthine oxidase was assayed as follows (10, 11). An aliquot of 100 μl of the solution or suspension to be assayed was combined with 100 μl of 0.5 *M* Trizma buffer (pH at 37°, 8.31; Sigma Chemical Co., St. Louis, Mo.) and 100 μl of [8-^{14}C]xanthine solution (2.22×10^8 dpm/ml; ICN Pharmaceutical, Irvine, Calif.). The radioactive xanthine was diluted with 0.025 *M* xanthine to different specific activities in the different experiments: It was diluted to 3.6×10^4 dpm/μl in the acid denaturation experiment, to 4.5×10^4 dpm/μl in the everted gut experiment, and to 3.3×10^4 dpm/μl in the homogenized gut experiment. The following volumes of milk were assayed for xanthine oxidase activity: 0, 15, 45, and 150 μl. The first three volumes were made up to 100 μl with Krebs buffer (millimolarities: NaCl, 128; KCl, 5.1; $CaCl_2$, 2.3; KH_2PO_4, 1.3; $MgSO_4$, 1.4; $NaHCO_3$, 25.6; D-glucose, 9.0), and then combined with 100 μl of Trizma buffer and 100 μl of [^{14}C]xanthine solution for the assay. The 150 μl of milk was made up to 200 μl with Trizma and combined with 100 μl of [^{14}C]xanthine solution.

These solutions were shaken at 37° for 30 min in a Dubnoff metabolic shaking incubator. The incubation was stopped by the addition of 50 μl of 2.3 *M* $HClO_4$. Samples were centrifuged at 1500 rpm for 20 min. A 140-μl aliquot was taken and added to 20 μl of 1:1 solution of 0.025 *M* xanthine and 0.05 *M* uric acid. Separation of xanthine from uric acid was accomplished by spotting 10 μl of this solution, in five increments, on a cellulose thin-layer chromatogram (No.

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13254, Eastman Kodak, Rochester, N.Y.). Assays were prepared by adding 10 μl of each suspension to 15 ml of Aquasol liquid scintillation cocktail (New England Nuclear, Boston, Mass.) and counting as described below. The chromatogram was developed for 2–2.5 hr in 2:1:1 *n*-BuOH, CH_3COOH, and H_2O, and allowed to dry overnight. Xanthine and uric acid spots were visualized under ultraviolet light and scraped into centrifuge tubes. Scrapings were shaken with 5 ml of 30% NH_4OH, centrifuged at 1500 rpm for 2–3 min and the supernatant was decanted into liquid scintillation vials. A total of three extractions of each sample was performed. This procedure routinely recovers 90% of the radioactivity from the chromatogram. The NH_4OH solutions were evaporated at 70° in a Brinkmann (Westbury, N.Y.) sample concentrator to a volume of less than 1 ml. Aquasol (15 ml) was added and the samples were counted three times for 5 min in a Packard (Downers Grove, Ill.) Model 3330 liquid scintillation spectrometer. Disintegration rates (dpm) were calculated by correcting for the efficiency of the instrument (86%) and for quench through the use of an external standard.

Xanthine oxidase activity is expressed as nanomoles of uric acid formed per hour and microliters of incubation medium. The percentage of spotted activity recovered from the xanthine and uric acid spots was 80% or greater. Ten percent of that not recovered was lost in the extraction process. It is likely that the remainder was elsewhere on the chromatogram. Other regions of the chrmatogram were not routinely assayed.

University of Wisconsin homogenized vitamin D milk and pH 1.75 buffer (0.108 *M* in HCl, 0.050 *M* in KCl) were used in the acid denaturation experiment. Eight milk–buffer suspensions were held at 37° for 15 min. Four of these were composed of 1 ml of milk and 9 ml of buffer and the remaining four were composed of 5 ml of milk and 5 ml of buffer. Aliquots of 1.5 ml from each suspension were neutralized with either 1.5 or 3 ml of Trizma buffer (pH 8.31).

The pH of all eight milk–buffer suspensions, the eight aliquots neutralized with 1.5 ml of Trizma, and the eight aliquots neutralized with 3 ml of Trizma was measured with a Corning (Corning, N.Y.) Model 10 pH meter. Xanthine oxidase activity of all neutralized samples was determined as described above.

For the everted intestine experiment, four adult male Sprague–Dawley rats (Holtzman Farms, Madison, Wis.) were starved for 18 hr, then killed by cervical dislocation. The small intestine was removed and flushed with five 10-ml portions of cold Krebs buffer and everted over a glass rod. The intestine was cut into three 10-cm strips, which were filled with Krebs and tied at both ends. Two of the strips were incubated in 10 ml of Krebs and the third was incubated in 5 ml of Krebs plus 5 ml of milk. The assignment of oral, middle, and caudal strips to the incubation flasks was randomized among the different animals. The strips were incubated at 37° for 1 hr with 95:5 O_2–CO_2. After incubation, the strips were cut and the serosal fluid was withdrawn. The fluid from one of the two strips incubated in Krebs alone was combined with 50 μl of milk. All samples were dialyzed for 2 hr against 0.15 *M* KCl and assayed for xanthine oxidase activity. Also included in the xanthine oxidase assay were controls consisting of 100 μl of nonincubated Krebs.

Homogenized gut experiments followed the same procedure as everted gut experiments through the incubation of the intestinal strips, except that the strips were 15 cm long and six rats were used. After incubation the strips were weighed and homogenized in nine times their weight of Krebs, first in a chilled Waring Blendor for 1 min, then with a Potter-Elvehjem Teflon and glass homogenizer held in ice. A 50-μl aliquot of the homogenate of one of the two strips incubated in Krebs alone was combined with 50 μl of milk. Xanthine oxidase activity was determined.

Statistical analysis was done utilizing an analysis of variance and the Student–Newman–Keuls test to determine differences between the means (12). The significance level selected was $p < 0.05$.

Results. The xanthine oxidase activity of milk was determined by using three different volumes of milk and assaying for conversion of xanthine to uric acid. These results

showed that the enzyme activity was proportional to the volume of milk up to approximately 45 μl of milk. The amount of uric acid formed per hr per μl of milk was 7.75 nmole.

For the acid denaturation experiment, the xanthine oxidase activity for the milk–buffer suspensions is shown in Table I. For both mixtures and both Trizma volumes, the conversion was less than 1.5 nmole of uric acid/hr/μl of milk. The last column of the table indicates the expected xanthine oxidase activity if the enzyme activity of the milk present in the assay were fully expressed. The latter values were obtained from the aforementioned assay of xanthine oxidase activity of milk. None of the samples showed as much activity as expected if the enzyme were intact.

The results of the everted gut experiment are shown in Table II. There was no significant difference between those strips incubated in Krebs alone and those incubated in Krebs plus milk, nor was there a difference between either of these and the control. There was a difference between those samples with added milk and the control, indicating that the milk used did contain xanthine oxidase.

The purpose of the homogenized gut experiment was to investigate the possibility that xanthine oxidase is absorbed by the intestinal mucosa but does not traverse the intestinal wall and appear in the serosal medium. Results are shown in Table II. Note that any activity may have been due not only to the presence of the enzyme in the wall, but also to its presence in the serosal fluid. This accounts for the possibility that the enzyme may be present in both locations in amounts too small to be detected, but when enzyme activity from both locations is combined, it may be measurable. Again there was no significant difference between those strips incubated in Krebs and those incubated with milk. The differences of these values from the controls reflect endogenous xanthine oxidase activity in the intestinal wall.

Discussion. The results presented in Ta-

TABLE I. XANTHINE OXIDASE ACTIVITY OF MILK AFTER TREATMENT WITH ACID.[a]

Buffer/milk ratio	pH of buffer/milk suspension	Volumes of Trizma	Assayed suspension pH	Xanthine oxidase activity (nmole of UA/hr/μl of milk)	Expected xanthine oxidase activity (nmole of UA/hr/μl of milk)
9:1	1.78 ± 0.11	1	2.23 ± 0.01	0.75 ± 0.05	8.1
		2	3.18 ± 0.14	1.30 ± 0.23	8.1
1:1	2.71 ± 0.02	1	6.05 ± 0.16	0.11 ± 0.0	7.1
		2	7.56 ± 0.02	0.26 ± 0.04	7.1

[a] Buffer/milk suspensions were held at low pH, neutralized with Trizma, and assayed for xanthine oxidase activity at the pH indicated in column four. Values are means ± SE for four determinations. UA, uric acid.

TABLE II. XANTHINE OXIDASE ACTIVITY OF EVERTED RAT INTESTINE SEROSAL FLUID AND OF HOMOGENIZED EVERTED RAT INTESTINE.[a]

Incubation medium	Krebs	Krebs plus milk	Krebs; milk added	Control (no incubation)
Xanthine oxidase activity nmole of UA/hr/μl of serosal fluid	0.25 ± 0.05†	0.16 ± 0.04†	0.90 ± 0.05*	0.08 ± 0.01
Xanthine oxidase activity nmole of UA/hr/μl of milk–homogenate	1.08 ± 0.12*,†	0.95 ± 0.05*,†	4.87 ± 0.83*	0.09

[a] Values for serosal fluid were obtained as follows: Everted rat intestinal strips were incubated in Krebs buffer with or without milk. After incubation, milk (50 μl) was added to the serosal fluid of one of the strips incubated in Krebs. Serosal fluid of all strips was assayed for the xanthine oxidase activity. Values are means ± SE for four rats.

Values for milk–homogenate mixtures were obtained as follows: Everted rat intestinal strips were incubated in Krebs buffer with or without milk. Strips and contents were homogenized 1:10; milk (50 μl) was added to 50 μl of the homogenate of one of the strips incubated in Krebs alone. All homogenates were assayed for xanthine oxidase activity. Values are mean ± SE for six rats. UA, uric acid.

* Significantly different from appropriate control at $p < 0.05$.

† Significantly different from Krebs; milk added at $p < 0.05$.

ble I show that xanthine oxidase is inactivated upon treatment with acid for 15 min at a pH of less than 3. Two recent abstracts have reported that some xanthine oxidase activity persists after *in vitro* incubation with artificial gastric juice (13) and aspirated human gastric juice (14). In both cases the volume of milk used was sufficient to raise the pH above 3.5. Ho *et al.* (14) state that complete inactivation of xanthine oxidase occurs below pH 3.35. These reports are consistent with the data presented herein and indicate that xanthine oxidase activity would persist in the stomach only after larger volumes of milk are consumed.

Table II shows that there is no difference in xanthine oxidase activity of serosal fluid from everted intestinal strips incubated with and without milk, and that there is no difference in intestinal homogenates incubated under identical conditions. These results suggest that the enzyme does not enter the intestinal wall in active form within the limits of detection of the assay.

The minimum amount of milk detectable is small: 0.160 nmole of uric acid/hr/μl of serosal fluid (Table II, Krebs + milk) corresponds to 1.0 μl of milk in the 100 μl of serosal fluid assayed. This value was obtained from the slope of a plot of xanthine oxidase activity in milk vs the volume of milk.

Previous objections to the xanthine oxidase theory of atherosclerosis have not been based on experimental evidence. Presented here is evidence to substantiate two of these objections: (i) that xanthine oxidase does not retain its enzymatic activity at the pH of the stomach in the presence of homogenized milk, and (ii) that the large xanthine oxidase molecule is not detectably absorbed by the small intestine of the rat.

(i) The H^+ present in high concentration in the stomach is known to protonate several amino acid groups and catalyze the digestive action of pepsins (7). These two actions result in conformational and covalent changes in proteins. It is likely that xanthine oxidase is no exception, since when exposed to low pH and returned to neutrality, the enzyme from milk showed no catalytic activity.

(ii) The intestinal epithelium constitutes a barrier to absorption for large molecules; macromolecules that are not digested are not absorbed, e.g., cellulose (7). If xanthine oxidase were to pass intact through the stomach, it probably would also pass intact through the intestine.

One further point not considered here is that xanthine oxidase would be subject to the action of pancreatic enzymes in the intestine. These enzymes split certain peptide bonds in proteins and polypeptides. Xanthine oxidase, whether inert to gastric digestion or not, would have to encounter these enzymes as well.

Although the evidence presented here indicates that xanthine oxidase is not absorbed from the small intestine, the possibility remains that it is absorbed in active form in amounts too small to be detected by this assay.

Summary. Certain aspects of the xanthine oxidase hypothesis of atherosclerosis were studied. Xanthine oxidase activity was determined by liquid scintillation counting of [^{14}C]uric acid formed by the action of the enzyme on [^{14}C]xanthine. Results show that the proposal that xanthine oxidase passes through the stomach and is absorbed intact by the small intestine is implausible. The treatment of milk with a low pH buffer resulted in irreversible loss of xanthine oxidase activity. The incubation of everted rat intestine in buffer and in buffer-milk mixtures showed no difference in enzyme activity in serosal fluid or in intestinal homogenates.

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