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Liposomes as a Proposed Vehicle for the Persorption of Bovine Xanthine Oxidase (40736)

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Oster and Mulinos (1) demonstrated the depleting action of bovine milk xanthine oxidase (BMXO) (EC 1.2.3.2) on tissue plasmalogens which are phosphoglyceride analogs of the alkyl-ether acylglycerols. Subsequently, Buddecke (2) demonstrated a definite diminution of plasmalogen in the atherosclerotic plaques of human autopsy material. Similar plasmalogen depletion was found in human myocardial tissue in a case of nonnecrotic myocardial infarction (3). Since plasmalogens constitute 30% of the phospholipids of the human myocardium, Oster hypothesized that such a plasmalogen depletion could initiate a pathological chain reaction leading to cell membrane damage in plasmalogen-rich cardiovascular and nerve tissue (4). Because of the importance that various epidemiological studies (5) ascribe to a dietary cause for atherosclerotic diseases, Oster suggested the possibility that BMXO could represent one such dietary factor in atherogenesis. Experimental evidence for this hypothesis was provided by the discovery of xanthine oxidase in both atherosclerotic plaques and pathological myocardial tissues of human autopsy specimens but not in a normal cardiovascular tissue from the same source (6). Others have demonstrated the absence of xanthine oxidase in normal plasmalogen-rich tissues (7).

That BMXO could be absorbed from the gut, despite its high molecular weight (290,000), is suggested by the presence of serum antibody titers to BMXO in clinically confirmed atherosclerosis patients, as well as in a few "normal" individuals who consumed large amounts of milk (8). The contention of absorption of xanthine oxidase from the gut is further supported in a study in which a population, apparently free of atherosclerotic disease, also showed a range of serum antibody titers to BMXO (9). This work was an extension of Davies' observation that bovine milk antibody titers are significantly increased in the serum of male patients with ischemic heart disease (10).

Although it has been frequently argued that macromolecules are not absorbed by the gastrointestinal tract, it has recently been demonstrated that such molecules as IgG immunoglobulins (MW 188,000) contained in liposomes could enter a cell (11). Liposomes are tiny membranous vesicles formed from various combinations of lipids resembling those found in natural cell membranes. Weissman has demonstrated that liposomes may be formed so as to encapsulate enzymatically active proteins or other macromolecules. Moreover, he has shown that *in vitro* and *in vivo* administration of enzymes by means of liposomes increases the uptake of the liposomes by cellular lysosomes (12). Accordingly, we postulated that the homogenization of whole milk would generate digestion-resistant liposomes with characteristics suitable for transporting BMXO through the intestinal mucosa with eventual deposition on target cell membranes.

Bailie and Morton (13) isolated a milk fraction high in xanthine oxidase activity which was subsequently shown by Hayashi and Smith (14) to possess a high phospholipid-to-protein ratio similar to that found in experimentally generated liposomes. We adapted the procedure of Hayashi and Smith for isolating milk lipoproteins to obtain a fraction from both raw

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and homogenized, pasteurized milk that satisfied the criteria for a liposome, viz., (i) vesicular structure; (ii) isolation through a Sephadex column; (iii) release of the contents of the isolated liposomes by a surface-active agent.

Materials and Methods. Xanthine oxidase assays were performed with a Gilson Differential Respirometer, using the method of Ball (15). Enzyme activity was expressed as microliters of O_2 consumed per milliliter sample per minute at 20°C. The reaction mixture consisted of 1 ml of a 5×10^{-3} *M* xanthine solution, 2 ml of a 0.2 *M* phosphate buffer, pH 7.4, and 2 ml of fraction sample obtained during the purification procedure. Readings, taken at 2-min intervals for 20 min, exhibited a linear relationship between O_2 consumption and time.

The fractionation procedure for both fresh, raw (control), and commercial homogenized, pasteurized cow's milk is outlined in Fig. 1. One part of a stock solution of 10% (w/v) sodium deoxycholate in 0.25 *M* sucrose buffered at pH 8.5 with 0.25 *M* Tris–chloride was added to 9 parts of a pooled commercial whole milk sample. The mixture was stirred for 1 hr at 37°C to release water-soluble lipoprotein from the fat membranes. Samples were centrifuged at various speeds and temperatures as indicated, and the supernatant J was passed through a Sephadex column (40 cm length, 2.6 cm i.d.) packed with Sephadex G-100. The eluant employed was 0.2 *M* phosphate buffer, pH 7.4. The resulting eluate contained the purified liposomes. All flotation layers, supernatant J, and pellets obtained during the course of fractionation were assayed for xanthine oxidase activity which was expressed as a percentage of the original whole milk activity. The lipoprotein-releasing surfactants employed were 3% (w/v) solutions of sodium taurocholate and sodium deoxycholate dissolved in a 0.25 *M* sucrose–0.25 *M* Tris–chloride at pH 8.5.

Visual identification of the vesicular structure of the purified lipoprotein (PLP) fraction was made with an RCA EMU-3H transmission electron microscope at a magnification of 30,000 X. The fraction was negatively stained with a phosphotungstic acid preparation.

Results. The BMXO activity of whole

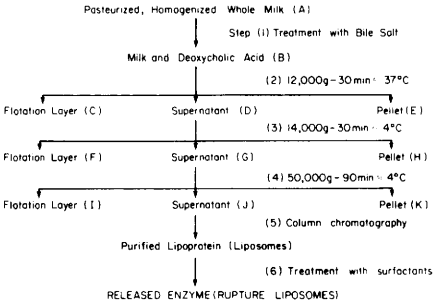

FIG. 1. Isolation and purification procedure for the separation of liposomes from homogenized, pasteurized bovine milk. Whole milk samples were stirred for 1 hr at 25°C in a 1% solution of sodium deoxycholate to release water-soluble lipoprotein from the fat membrane. Centrifugation forces, times, and temperatures are indicated.

milk fractions illustrated in Fig. 1 is reported in Table I as a percentage of the original whole milk sample activity which is assigned a value of 100%. A 70% reduction of activity resulted from both a dilution effect and a partial inhibition of BMXO by the bile salt solution. Small amounts of free enzyme activity accompanied all subsequent fractions. Following the third centrifugation, supernatant J contained 120% whole milk enzyme activity. Approximately a fourfold increase in concentration of water-soluble lipoprotein and free enzyme was found in supernatant J, when compared with xanthine oxidase activity in whole milk treated with bile salt. The lipoprotein component of supernatant J was isolated

TABLE I. BOVINE XANTHINE OXIDASE ACTIVITIES OF THE DIFFERENT FRACTIONS ILLUSTRATED IN FIG. 1[a]

Fraction	BMXO activity (% of whole milk)
Whole milk (A)	100
Whole milk plus bile salt (B)	30
Pellet (E)	Trace
Flotation layer (C)	3
Pellet (H)	Trace
Flotation layer (F)	Trace
Pellet (K)	Trace
Flotation layer (I)	10
Supernatant (J)	120
Purified lipoprotein (PLP)	8
PLP plus Tween 80	92

[a] Activities of each fraction are expressed as the percentage of the original whole milk activity. Supernatants D and G were not examined for BMXO content because they were preparative fractions.

TABLE II. XANTHINE OXIDASE ACTIVITY OF BOVINE MILK FRACTIONS

Fraction	*n*	Activity (mean ± SD)	95% C. I. (a)
Lipoprotein	15	118 ± 109.7	118 ± 61.4
Purified lipoprotein	17	8 ± 4.3	8 ± 2.2
Purified lipoprotein + Tween 80	13	91 ± 46.4	91 ± 28.2

from any remaining free enzyme and bile salts by passage through the Sephadex column. Although the final PLP fraction possessed 8% of the original activity, elimination of this residual externally bound enzyme activity can be achieved by a second passage through the column. Addition of either Triton X-100,[3] Tween 80,[3] or both surfactants to the final PLP fraction produced a dramatic increase in BMXO activity which is ascribable to the rupture of the liposomal membrane and subsequent release of the entrapped enzyme (13). Neither of these detergents were found to affect the enzyme activity. Table II shows that the increase in activity was statistically significant ($p \leq 0.05$). An F test showed no significant difference in variance for any of the samples. Commercially pooled milk samples frequently exhibit large variations in their BMXO content. Others have reported similar findings (16).

Two sets of experiments were performed to demonstrate the effects of gastric digestion on the purified liposomes containing BMXO. In the first instance, 100 cc of pasteurized, homogenized milk (pH 6.5) was treated for 2.5 hr at 37°C with 10 cc of artificial gastric juice (pH 1.6) prepared by adding 750 mg of Pepsin USP (Merck) to 100 ml of 0.1 *N* hydrochloric acid. The resultant mixture, 10:1 (v/v) had a final pH 6. The resulting digest was subsequently subjected to the liposome isolation and purification procedures as outlined in Fig. 1. In the second case, a purified liposomal fraction obtained from pasteurized, homogenized milk was similarly treated (Table III). Significant BMXO activity was retained in both samples, indicating that the gastric juices failed to completely digest the milk, the PLP, and the liposome-entrapped BMXO.

[3] Obtained from Sigma Chemical Co., St. Louis, Mo.

Figure 2 demonstrates the vesicular structure of the PLP, a morphology characteristic of liposomes. The vesicles range in size from 0.2 to 1 nm. Addition of the surfactants, Triton X-100 or Tween 80, produced the same increase in enzyme activity ascribable to the rupture of the liposomal membrane and subsequent release of the entrapped enzyme (13). Only rare liposomes were observed by electron microscope examination of the raw milk control fractions, and no significant increase in enzyme activity was measurable in these fractions following treatment with the surfactants.

Discussion. An extensive literature on intestinal absorption argues against the possibility that a molecule as large as xanthine oxidase can survive digestion in the stomach and small intestine. However, experimentally produced insulin-containing liposomes, when administered intragastrically, can transport the hormone into the circulation of diabetic rats, lowering the blood sugar (17). Recently, Zikakis *et al.* have shown that BMXO survives the digestive action of the gastrointestinal tract of the rat (18). Ferritin (MW 750,000) can be absorbed from the gut of the adult rat and subsequently detected in various organs but not in blood serum (19). Modern pharmacological studies have repeatedly demonstrated the absorption of liposome-entrapped enzymes, drugs, and other macromolecules. Such liposomal vehicles enhance the bioavailability via the digestive tract of substances which are ordinarily inactivated by this route (20). As far as we know, milk is the only food which, by homogenization, artifactually creates enzymes in liposomal form capable of being persorbed by the intestinal mucosa.

In contrast to homogenized, pasteurized milk, raw milk contained only minute amounts of liposomes as demonstrated by measurement of enzyme activity and elec-

TABLE III. XANTHINE OXIDASE CONTENT OF PURIFIED LIPOPROTEIN (PLP) EXPOSED TO GASTRIC JUICE (GJ)

Fraction + GJ	*n*	Activity (mean ± SD)	95% C. I. (a)
Whole milk			
Purified lipoprotein	4	7 ± 0.21	7 ± 0.35
Purified lipoprotein + Tween 80	4	54 ± 3.2	54 ± 5.4
Isolated PLP			
Purified lipoprotein	3	13 ± 0.4	13 ± 1.0
Purified lipoprotein + Tween 80	3	27 ± 1.35	27 ± 2.7

tron micrographic visualization.

Poste *et al.* conclude that since liposomes are incorporated into the cells as intact structures, their uptake must occur either by endocytosis or by fusion with the cellular plasma membrane or both (21). With this suggestion in mind, we looked for BMXO in the particulate structure of human blood. Preliminary milk-loading experiments conducted in our laboratory have indicated that habitual milk drinkers do indeed absorb liposome-bearing BMXO. The enzyme was detectable in the leukocyte fraction of the blood 2 hr after ingestion of 1 liter of homogenized, pasteurized milk. No xanthine oxidase activity was detected in the serum. This is consistent with the observation by others that liposome-entrapped enzymes can be incorporated into other cells (22, 23). Perhaps there is

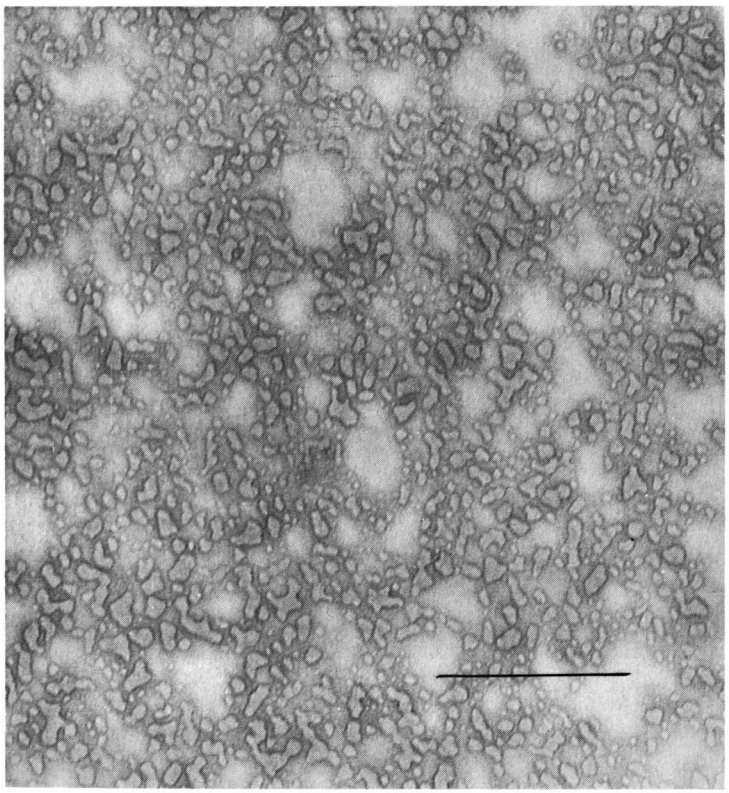

FIG. 2. Electron micrograph of a phosphotungstic acid preparation of the purified liposome fraction of bovine homogenized milk. The scale bar represents 5 μm. (Photographs by Dr. M. Somers, Department of Biology, University of Bridgeport, Bridgeport, Conn.)

some similarity here to the aforementioned case of ferritin absorption (19). Furthermore, absorption and targeted deposit of milk liposomes can be facilitated by the presence of immune globulins which normally accompany them in mammalian milk (23, 24).

Summary. We present experimental evidence for the hypothesis that bovine milk xanthine oxidase (BMXO) is entrapped in liposomal form by the milk homogenization process. In this form, it will resist gastric digestion and become biologically available. This is the first demonstration of the presence of a liposome-sequestered enzyme in a widely consumed food. The results indicate that a major physical alteration by a technological manipulation of a basic food may have far-reaching biological significance.

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