Review

Effects of xenobiotics on milk secretion and composition

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The effect of xenobiotics on milk secretion and composition has received limited systematic study. This review highlights current understanding of the mechanisms and regulation of milk secretion and indicates potential sites of xenobiotic perturbation. Major emphasis includes review of the cellular architecture and biochemical mechanisms of the differentiated mammary alveolar cell in relation to secretion of lipid, lactose, milk proteins, electrolytes, trace elements, host defense systems, vitamins, hormones, growth factors, and antioxidant enzyme systems. The regulation of milk secretion by oxytocin, prolactin, steroid hormones, and thyroxine is reviewed in the context of potential or known modulation by xenobiotics. Lastly, the impact of xenobiotic-induced changes in nutrient delivery to the mammary gland from altered mammary blood flow or malnutrition are considered. This review also summarizes the limited current knowledge of the impact of selected xenobiotics of environmental concern on milk secretion and composition. Attention is directed to alcohol, polychlorinated biphenyls, phthalate plasticizers, pharmacologic agents, tumor promotors, insecticides, heavy metals, and vitamins. The critical need for additional research is addressed, with proposals for epidemiologic studies in the human population and further development of animal and tissue culture models for mechanistic investigations. (J. Nutr. Biochem. 5:418–441, 1994.)

Keywords: milk secretion; milk composition; lactation; mammary gland; xenobiotics

Introduction

Although the role of xenobiotics in development and differentiation of the mammary gland has been extensively studied in relation to mammary carcinogenesis,[1–3] the effects of toxic agents on the differentiated function of the mammary gland, that is, milk secretion, have received little systematic review. Xenobiotics have the potential of acting directly on the mammary gland to alter the various processes by which milk components are synthesized and/or secreted. They may also have systemic effects that alter either the hormonal milieu that supports lactation or the delivery of substrate to the mammary gland. To provide a logical framework for the examination of the myriad potential actions of xenobiotics on mammary function, the first and longest segment of this article deals with the mechanisms of milk secretion and the potential effects of xenobiotics on it. The second part of the article focuses on the toxins themselves, describing the mechanisms of action of those categories of xenobiotics that may or may not have the potential to alter mammary function. This approach is important in deciding which agents are likely to have mammary-specific actions. In the third part of the article, we outline epidemiologic and experimental approaches that are likely to fill the wide gaps in our current knowledge.

Many of the toxins that have the potential to disrupt milk secretion also act at other sites within the body, producing severe metabolic disruptions that cause maternal morbidity or even death. In lower doses, they may enter milk and be ingested by the infant with deleterious effects on infant growth and metabolism. Our task here is to identify substances whose primary effect is to alter either the rate of milk secretion or milk composition.

Unfortunately, most studies have not examined lactation per se, so there are few data to go by. Selective effects on mammary function might be expected from toxins that act on pathways unique to the mammary gland, such as lactose synthesis or milk fat secretion, or that interfere with hormones important to milk secretion or its initiation. Specificity is also possible if toxins are selectively delivered to or

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metabolized in the mammary gland, especially if the gland is subjected to chronic insult. Because many environmental toxins are lipophilic, they are stored in adipose tissue; during lactation they are found at high concentrations in milk fat,[4] implying systematic delivery to and uptake by mammary cells.[5] To what extent this process augments the potential effect of toxins on mammary cell activity is a question that has not, to our knowledge, been addressed.

Xenobiotics and mammary function

This section outlines both the morphologic requirements for and the biochemical mechanisms involved in milk secretion and describes those processes where existing data, primarily from experiments in other tissues and organs, suggest disruptive actions of xenobiotics. The hormonal signals that promote both lactogenesis and the maintenance of milk secretion are surveyed, with suggestions of agents that may interfere with these hormonal effects. Finally, a brief section suggests potential effects of xenobiotics on substrate delivery to the lactating mammary gland.

Direct effects of xenobiotics on milk secretion

The diagram in *Figure 1* shows both the major interactions of the mammary alveolar cell with its tissue environment and the major pathways of milk secretion. The secretory function of the mammary alveolar cells is crucially dependent on the establishment of a functional architecture, which includes the scaffolding for polarized secretion (the cytoskeleton), interactions with neighboring cells (tight junctions, gap junctions, and desmosomes), and cell-matrix interactions primarily mediated by a class of membrane proteins known as integrins. Five major pathways are responsible for secretion of the major milk components.[6] Most of the aqueous components of milk are secreted via the exocytotic pathway (pathway I in *Figure 1*) responsible for secretion of milk protein, lactose, calcium, phosphate, and other components. Milk lipid is secreted by a pathway (pathway II) unique to the mammary gland that results in the secretion of drops of lipid surrounded by a specialized membrane structure; this complex is known as the milk fat globule (MFG). Pathway III allows equilibration of certain milk components including Na^+, K^+, Cl^-, and glucose across the apical membrane of the mammary alveolar cell; it is probably a minor pathway and is poorly understood. Pathway IV is a transcytotic pathway responsible for endocytosis of certain proteins from the interstitial space at the basolateral surface, transport across the mammary alveolar cell, and exocytosis into the milk space. The transport of immunoglobulins via this pathway has been most extensively studied.[7] Pathways I through IV are mediated by the mammary alveolar cell. Pathway V, the paracellular pathway, circumvents the mammary alveolar cell, allowing passage of substances directly from the interstitial space to the milk. In full lactation, pathway V is tightly closed by gasket-like junctions around the apical border of the alveolar cells. These tight junctions, the *zonulae occludentes,* join each cell tightly to its neighbors, providing a seal that separates the interstitial space from the milk space. This pathway is open during pregnancy and in the involuted and mastitic gland, leading to the passage of both sodium and chloride from the blood into the milk and milk products, such as lactose, in the opposite direction.

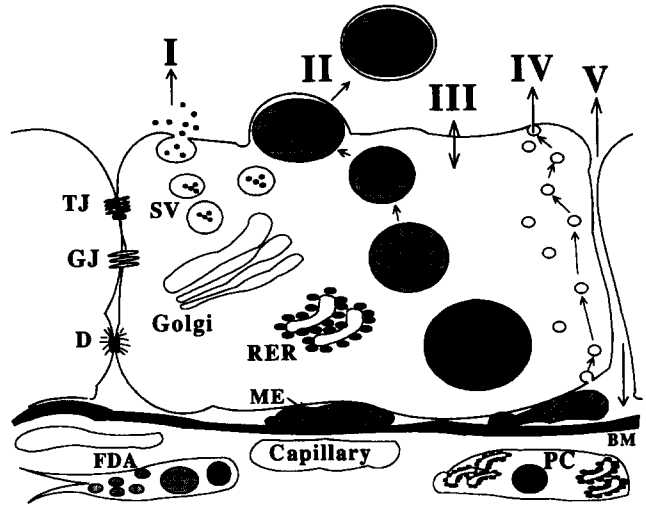

Figure 1 The function of the mammary alveolar cell. The cell in the center is attached to its neighbors by a variety of junctions: TJ, the tight junction or zonula occludens forms a gasket around each cell sealing it to its neighbor; GJ, the gap junction allows communication between alveolar cells; D, the desmosome or zonula adherens, provides structural support to the epithelium. These junctions are fully formed in the lactating mammary gland, as shown on the left. During pregnancy and mastitis and after involution, the junctions are less fully formed, and substances can pass by the paracellular route between the milk and the interstitial space. A basement membrane (BM) separates the epithelial cell layer from the interstitial components including capillaries, fat depleted adipocytes (FDA), and plasma cells (PC). Five pathways are involved in the secretion of milk. Pathway I, exocytosis, is responsible for most of the aqueous content of milk. It consists of the rough endoplasmic reticulum (RER), Golgi saccules, and secretory vesicles (SV). Lactose, most milk proteins, calcium, citrate, and phosphate are secreted via this pathway. Pathway II is a unique secretory mechanism responsible for the secretion of milk lipid. Pathway III represents small molecular weight substances (sodium, potassium, chloride, and glucose) that equilibrate across the apical membrane. Pathway IV is the transcytotic pathway that carries proteins and other interstitial substances from the interstitial space to the milk space. Pathway V is the paracellular pathway closed in the normal lactating gland.

Effects of xenobiotics on the secretory architecture of the epithelial cell

The proper maintenance of secretory architecture (often expressed more simply as cell shape) is critical to milk secretion. It has long been known that in vivo treatment of the mammary gland with the microtubule disrupting agent colchicine inhibits milk secretion.[8,9] In tissue culture, the seminal observation of Emerman and Pitelka[10] that primary cultures of mouse mammary gland could be induced to secrete milk components only if the cells assumed a rounded morphology provided striking evidence for the importance of cytoarchitecture in secretory activity. Secretory architecture is maintained by the cytoskeleton and is dependent on contacts between the cytoskeleton and certain classes of integral membrane proteins that provide attachments to both neighboring cells and the extracellular matrix.[2,11,12] Agents that alter these contacts, either intracellularly or extracellu-

larly, are likely to bring about cytoskeletal changes that will alter most or all the pathways of milk secretion.

Tumor promoters and the protein kinase C cascade. A number of tumor promoters, of which the best studied are the phorbol esters, have long been known to alter epithelial morphology.[13] They have been shown both in vivo and in vitro to interfere with the differentiated function of the mammary gland. In primary cultures of mouse mammary gland[14] the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), inhibited the synthesis of the milk proteins casein and α-lactalbumin with an ED_{50} of 0.1 ng/mL. Inhibition of casein synthesis by various TPA analogs correlated with their potency as tumor promoters. Prolactin binding was also inhibited. Martel and colleagues,[15] who studied the effects of phorbol esters on rabbit mammary explants, found that the induction of casein synthesis and mRNA by prolactin was inhibited. In vivo, injection into lactating mice of as little as 4 μg of TPA twice daily for 2.5 days completely inhibited pup growth.[16] There was no effect on maternal weight or secretion of growth hormone (GH) and prolactin by pituitaries isolated from the treated mice. Further, the ratio of RNA to DNA synthesis in the mammary gland declined. Although no attempt was made to measure the volume or composition of milk, these observations suggest that the locus of the inhibition was primarily the mammary gland, and that milk secretion may be exquisitely sensitive to the actions of compounds that act like tumor promoters.

Some components of the mechanism of action of tumor promoters are well understood. Studying MDCK cells, a kidney epithelial cell line that is the model system for most studies of epithelial cells in culture, Ojakian[17] observed that TPA at a concentration of 10 ng/mL reduced transepithelial resistance from 200 Ohm.cm^2 to 50 Ohms.cm^2 in 1 hour. Kellie et al.[18] observed that the shape change induced by TPA in these cells was accompanied by a redistribution of F-actin and a decrease in the number of vinculin-containing plaques. Protein synthesis was not essential for these rearrangements, but ATP was. Ben-Ze'ev,[11,13] studying MDCK cells, found a decrease in the expression of mRNA for cytokeratins and the desmosomal protein, desmoplakin, in response to TPA. In a bovine mammary cell line, he found that synthesis of a specific 45 kD acidic cytokeratin, found only in dense cultures, was inhibited by TPA. Ben-Ze'ev postulated that TPA produced a coordinated down-regulation of proteins involved in cell-cell contact. The permeability of gap junctions is also decreased by tumor promoters.[19] The effect may be due to loss of the gap junction protein, connexin, from the junctional complex.

Since these early studies, the molecular mechanisms involved have become partially clarified. In particular, the major mode of action of TPA has been found to be activation of protein kinase C (PKC).[20] Kaiser et al.[21] found that within 2 hours of treatment with 26 nM TPA, adducin, a calmodulin-binding protein that promotes binding of spectrin to actin in adherent junctions of cell-cell contacts of both keratinocytes and MDCK cells, was completely removed from the contacts. The protein remaining within the cells was diffusely distributed and showed increased phosphorylation. Mullin et al.[22] found that other tumor promoters, including teleocidin and aplysiatoxin, increased the junctional permeability of the LLC-PK1 renal cell line by 40 fold. These observations are consistent with the hypothesis that TPA acts by stimulating protein kinase C, which alters the phosphorylation state of adducin and other cytoskeletal proteins and leads to dissociation of the protein complexes that maintain cell-cell contacts and cell morphology *(Figure 2).*

The effects of tumor promoters are not confined to cell junctions, however. They have protean effects on gene expression[20,23] in many cell types and even enhance differentiation in some cells. When PKC was overexpressed in cultured fibroblasts, for example, TPA conferred multiple growth abnormalities, including anchorage independence.[24] While most of these actions are beyond the scope of this review, in mammary cells TPA decreased expression of the membrane neutral metalloendopeptidase and increased production of secreted proteases and their inhibitors.[25] From the scanty information available, in the mammary gland tumor promoters might be expected to set in motion a whole range of cellular activities that tend to alter the cell from a differentiated to a proliferative state *(Figure 2).*

Other agents operating through the PKC pathway. Because activation of PKC is an important element in the alteration of epithelial morphology away from the differentiated state, any agent that alters its activity will likely have an effect on mammary morphology. The hormone, epidermal growth factor (EGF), stimulates phospholipase C releasing diacylglycerol (DAG). DAG in turn stimulates PKC in many cell types.[26] EGF has effects similar to TPA on milk secretion by primary mammary cultures.[14] Dioxins up-regulate the EGF receptor and activate both PKC and phospholipase C,[27] which suggests they may have effects on the cytoskeleton. It has been hypothesized that the most widespread human

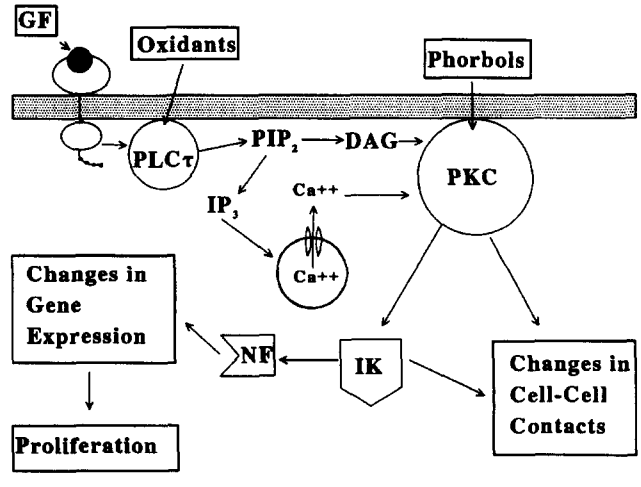

Figure 2 Possible routes of signal transduction in the mammary cell. Growth factors or oxidants, acting through phospholipase C (PLCτ), stimulate the breakdown of phosphatidylinositol (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 stimulates calcium release from intracellular compartments. Ca^{++} and DAG activate protein kinase C (PKC), which in turn activates other intracellular messengers (IK) that alter cell-cell contacts or activate nuclear factors, leading to changes in gene expression and cell proliferation.

symptom of dioxin toxicity, chloracne, an epithelial disorder, may result from these EGF-like effects of dioxins.[27]

Oxidants rapidly and reversibly decrease the paracellular resistance of MDCK cells.[28,29] This effect appears to be specific because other compounds that deplete cellular ATP like 2-deoxyglucose did not have this effect.[28] The mechanism probably involves activation of phospholipase C to break down phosphatidylinositol to inositol triphosphate and DAG. Analogs of DAG have been shown to have effects on paracellular resistance similar to those of phorbol esters.[29] In cultured amnion cells,[30] oxytocin activated the PLC-PKC pathway, increasing PGE_2 production, with the possibility of setting in motion a number of secondary effects. Although various observations suggest that prostaglandins are involved in the regulation of milk production,[31,32] their role and the possible role of inhibitors of the cyclooxygenase pathway on mammary morphology are not clear at present.

Other effects on cell morphology. Many other toxins have been reported to alter cell morphology. However, it is important to distinguish between primary effects of these substances on cell morphology and secondary effects resulting from disruption of cell metabolism. For example, hydrogen peroxide (5 mM) caused membrane blebbing, a change in the state of actin, and profound morphological disruption within 3 hours in the $P388D_1$ cell line.[33] However, in this case, the primary effect was thought to be inhibition of ATP synthesis. The resulting loss of metabolic intermediates may lead to an increase in intracellular free calcium and many changes associated with cell death.

There are compounds other than tumor promoters that act directly on cytoskeletal elements. The microtubule destabilizing element, colchicine, for example, produces marked changes in liver cell morphology and junctional permeability[34] after administration of 1 mg in the mouse. It decreases casein secretion in rabbit mammary explants.[35] Heavy metals such as Cd^{++}[36] or Ni^{++}[37] may alter microtubular integrity. Cytochalasin D, a compound that disrupts microfilaments, alters intestinal tight junctional permeability at concentrations below 10 μg/mL.[38,39] Early effects (<12 hours) of the chemical carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA) studied at 4 μg/mL in cultured lingual epithelium included disruption of gap junctions and desmosomes accompanied by profound morphologic alterations in the cytoskeleton.[40] Retinoic acid decreased gap junction communication in cultured hepatocytes.[41] Phalloidin, a mushroom poison, given at 500 μg/kg body weight for 7 days, altered the microfilament structure and bile secretion in rat livers by an unknown mechanism, suggesting that toxins of biologic origin may also act through alterations in morphology.[42] Finally, chronic administration of the potent dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in nonhuman primates induced hypertrophy and hyperplasia of most epithelial cells, although the mammary epithelium was apparently not studied.[43]

The above observations make it clear that primary and specific alterations in the cytoskeleton are produced by a variety of xenobiotics at concentrations less than 10 ppm (often much less). In most cases, there is neither information on their specific toxicity in the mammary gland nor on their potential for altering the composition and volume of milk. There is, however, one instance, namely mastitis, when a change in milk composition clearly signals the presence of inflammatory agents. It is well known in both animals[44] and humans[45] that mastitis is accompanied by a dramatic increase in the sodium, chloride, and protein concentration of milk, accompanied by a decrease in the lactose concentration *(Figure 3)*. These changes are thought to reflect opening of the tight junctions allowing the influx of plasma constituents into the milk space. It is tempting to speculate that oxidants released by macrophages or other cells of the immune system set in motion the train of events illustrated in *Figure 2* to create this response.

Model systems for further study. Fortunately, effects of environmental agents on mammary morphology are readily amenable to studies in tissue culture using some of the in vitro systems currently available. Because one of the most sensitive indicators of disruption of cytoskeletal contacts with cell-cell junctions is the transepithelial resistance,[38] mammary cell lines with tight intercellular junctions grown on filter supports are probably most suitable for such studies. Certain strains of the Comma 1D line have been shown to have these properties,[46] as well as derivatives of the IM2 mammary cell line.[47] Because some of these cells also secrete milk proteins under the appropriate conditions,[48,49] they might serve as a very appropriate model for future studies. However, derivation of a more homogeneous cell line that forms tight junctions and is stimulated by lactogenic hormones to secrete both milk proteins and milk lipids is a highly desirable goal. Ultimately, in vivo studies on milk secretion must be carried out in species in which both milk composition and volume can be easily measured. Goats are most amenable to such research. In humans, the concentration of sodium ion in the milk is a sensitive indicator of tight junctional permeability.[50]

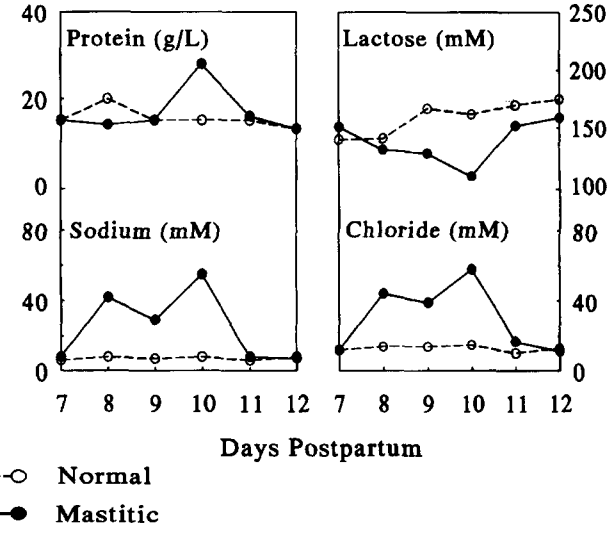

Figure 3 Changes in milk composition in a mastitic breast. Daily milk samples from the mastitic right and normal left breast of a lactating woman 7 to 12 days postpartum were analyzed. Data replotted from reference 45.

Potential effects of xenobiotics on milk lipid synthesis and secretion

Triacylglycerols make up 98%[51] of the lipids and provide about 40% of the calories present in human milk. There are several sources of substrates for mammary lipid synthesis. Free fatty acids may be derived from dietary lipids carried to the mammary gland in the form of chylomicra. Ultrastructural studies have demonstrated that chylomicra become embedded in the capillary wall of the mammary gland and undergo lipolysis.[52] Lipids may also be transferred from adipose tissue and possibly liver stores as free fatty acids bound to serum albumin or as triglyceride contained in very low density lipid particles (VLDL). The regulation of this transfer is poorly understood but appears to involve, at the very least, inhibition of lipoprotein lipase in adipose tissue and up-regulation of the enzyme in the mammary gland during lactation.[53] It has been established from tracer and arteriovenous difference studies across the mammary gland that serum lipoproteins are important contributors to milk lipids;[54] further definition of the role of lipoprotein receptors in lipid uptake is needed.

The enzyme lipoprotein lipase (LPL) is crucial to triglyceride uptake from the plasma. This enzyme is bound to heparin sulfate proteoglycans in the capillary endothelium. There it hydrolyses triacylglycerols in chylomicra and VLDL particles to free fatty acids and glycerol.[55] The fatty acids cross both the capillary endothelium and the plasma membrane of the mammary alveolar cells by mechanisms that remain controversial.[56] There is evidence in some cell types for a specific plasma membrane transporter for free fatty acids. This transporter has not yet been identified in mammary cells.

Once across the plasma, membrane free fatty acids bind to intracellular fatty acyl-binding proteins that are present in high quantity in the mammary cell.[57] These proteins may act to ferry highly toxic free fatty acids from one cellular site to another. Whether they serve as carriers for the transfer of lipid soluble xenobiotics across the cytoplasm to the growing milk fat globule is not yet known. The mammary fatty acid binding protein or a related protein has, however, been postulated to act as a mammary growth inhibitor.[57]

Triacylglycerols are synthesized in the mammary gland by esterification of fatty acids derived from both the plasma and de novo fatty acid synthesis from glucose within the mammary cell itself.[6] As in adipocytes, the enzyme fatty acid synthetase is responsible for de novo fatty acid synthesis, using glucose or acetate as substrate. Unlike adipocytes and other somatic cells, in mammary cells, fatty acids are terminated at chain lengths of 10 to 14 carbons by a mammary specific enzyme, thioesterase II.[58] The source of fatty acids in milk can be determined from their chain length because the majority of fatty acids derived from the plasma have a chain length of 16 carbons or greater, while those synthesized within the mammary gland have a chain length of less than 16 carbons.

Once synthesized, the triacylglycerols form droplets that move apically through the cytoplasm until they butt up against the apical membrane of the cell. This highly specialized membrane, containing a number of unique proteins including butyrophilin, xanthine oxidase, and two or more forms of mucin,[59] gradually envelopes the milk fat globule *(Figure 1)*. Eventually the globule breaks off into the milk space, where it exists as a membrane-coated lipid droplet, often including a small crescent of cytoplasm, that provides both phospholipids and triglycerides to the infant.

Although there are potentially many points at which xenobiotics might interfere with this pathway, two possibilities deserve emphasis. The activities of both LPL[60] and fatty acid synthetase[61] are tightly regulated by the metabolic status of the individual and might, for this reason, be particularly sensitive to toxic effects of xenobiotics. Because thioesterase II is specific to the mammary gland, inhibitors of this enzyme, if they exist, might be expected to have specific effects on the fatty acid composition of human milk.

Potential effects of xenobiotics on mammary lipoprotein lipase (LPL) activity. Feeding and fasting have been shown to have profound effects on the tissue activity of LPL in humans and animal models.[60,62,63] The effects differ from tissue to tissue; for example, fasting decreases adipose tissue LPL activity but increases the activity of the muscle enzyme.[60] In the mammary gland, fasting appears to have effects similar to those observed in adipose tissue.[63] The hormonal mechanisms involved in these responses are not completely understood, but insulin increases LPL activity in white adipose tissue[64] and β-adrenergic agonists decrease it.[65] These responses are sensitive to nutritional status; for example, the responsiveness of adipose tissue to insulin was shown to be altered by fat feeding.[66] Cytokines released from macrophages may also regulate LPL activity, as shown by the observations that both tumor necrosis factor and interleukin-1 reduce LPL activity in 3T3-L1 cells, a tissue culture model for adipocytes,[67] and adipose tissue LPL activity is decreased by tumor necrosis factor.[68] The complexity of regulation of this enzyme suggests that xenobiotics could act at many points to alter mammary LPL activity, in turn leading to alterations of milk lipid synthesis and secretion.

Although no reports of the effects of polychlorinated-biphenyls (PCBs) on mammary LPL are available, the potent dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) at low concentrations has been observed to inhibit adipose tissue LPL in a variety of species[69] and produce hypertriglyceridemia. For example, Brewster et al.[70] studied rabbits and found that 1 μg/kg body weight of TCDD given as a single dose decreased adipose tissue LPL by 56% and doubled plasma triglycerides and cholesterol. At this dose, there were few signs of systemic toxicity. These effects appear to be species dependent; similar changes have been observed in guinea pigs and hamsters but not in rats.[69] Hypertriglyceridemia has been observed in humans after TCDD exposure.[71–73] The effects of TCDD on LPL and serum triglycerides are thought to be independent of the dioxin receptor[27] and may represent an acute response to the toxin.

Based on the effects of adrenergic agonists on tissue LPL activity, β-adrenergic blockers might be expected to alter the mammary enzyme. In fact, the antihypertensive agent, propranolol, and other β-receptor antagonists directly inhibit the in vitro activity of LPL,[74,75] a nonreceptor-mediated effect. The bovine enzyme purified from milk is much more sensitive to inhibition than LPL activity in human-postheparin plasma, where 3 mM propranolol is required for 50%

inhibition of enzyme activity. Although the high concentration necessary suggests that the effect is not pharmacologically relevant, Hostetler[74] has proposed that chronic use of β-blockers may augment membrane concentrations of these agents and thus inhibit LPL activity. On the other hand, the receptor-mediated regulatory effects of adrenergic agents on tissue LPL activity are likely to have more significance. Very little information exists on this point, although an early study[76] showed that reserpine and α-methyldopa increased cardiac LPL in dogs and rats.

Chronic ethanol treatment given as 25% of the drinking water increased the LPL activity of extracts of the mammary gland from lactating rats.[77] However, milk production and total mammary weight were decreased more than 50% in this model, suggesting that ethanol produced mammary involution, perhaps secondary to decreased oxytocin release. The relevance of the increased LPL activity under these circumstances is open to question.

Thioesterase. The mammary glands of most species contain a mammary-specific enzyme, thioesterase II, that releases medium-chain fatty acids from the 4′-phosphopantetheine thiol of the fatty acid synthetase.[78] Sequence analysis shows the enzyme to be a serine protease with an active site that differs in location from other serine active-site esterases.[79] Presumably the enzyme is inhibited by inhibitors of serine proteases like phenylmethylsulfonyl fluoride because the thioesterase inherent in fatty acid synthetase is inhibited by this substance. However, we were unable to find any data on specific inhibitors of this enzyme.

Other potential effects of xenobiotics on milk lipid synthesis and secretion. At a dose of 1 μg/kg in rats, TCDD was observed to inhibit de novo fatty acid synthesis in liver and adipose tissue by 45%.[80] This observation suggests that dioxins could interfere not only with LPL-mediated uptake of fatty acids but also with their synthesis within the mammary gland, severely compromising lipid secretion. Higher doses of some agents have been observed to increase the lipid content of rat milk including the plasticizer, di(2-ethylhexyl) phthalate, and chronic ethanol ingestion.[77] In a well-designed study of the effects of phthalate, Dostal and coworkers[81] gave 1 g/kg/day orally for 3 to 5 days. Comparison with pair-fed controls indicated that the increase in milk fat was not due to decreased food consumption. The pups grew poorly, suggesting that milk volume was decreased and that the increased fat content could have resulted from an imbalance between synthesis of the components of the aqueous fraction of milk and milk lipid, rather than an increase in milk lipid synthesis per se.

Recommended further investigations. The available observations suggest that dioxins and related compounds could alter the amount of milk lipid or its composition. Further studies should include careful measurement of milk fat content, volume, and fatty acid composition in women with high body burdens of dioxins. Careful monitoring of the milk composition of lactating women taking adrenergic antihypertensive drugs may reveal whether alterations in adrenergic responsiveness should be of concern. Animal studies that include measurements of mammary LPL activity and fatty acid synthesis would also be useful both for screening of potentially toxic agents and determination of their mechanisms of action.

In the design of future studies, some caution is necessary. Because milk fat content varies with the degree of emptying of the mammary gland,[50] accurate measurement of milk fat content in animals other than humans and dairy species in which the total milk content of the mammary gland can be extracted at each milking is almost impossible. This fact plus the unavailability of a good in vitro model system for milk fat synthesis severely limits the scope of feasible studies. In humans, measurement of the fat content of milk samples that are obtained casually is not useful. If the fat content of milk is to be measured, careful attention to obtaining a representative milk sample is essential as described below.

Synthesis and secretion of lactose

The major sugar in human milk is lactose. It is significant because of its caloric value to the infant and also as the major determinant of the volume of secreted milk. The mechanism and regulation of lactose synthesis have been reviewed.[6] Briefly, lactose is synthesized within the Golgi apparatus and, due to impermeability of the membranes, generates the principle osmotic driving force for fluid secretion. The synthesis occurs via coupling of glucose and UDP-galactose (UDP = uridine diphosphate glucose), which is catalyzed by lactose synthetase. This enzyme complex consists of galactosyltransferase, activated by metal ion binding at high and low affinity sites (I: Mn; II: Ca), and α-lactalbumin. Under physiological conditions, the critical regulatory factors appear to be glucose, manganese, and α-lactalbumin concentrations.[82] The kinetics of galactosyltransferase activity, both for the solubilized and vesicle-bound form of the enzyme, have been studied in detail.[83,84]

Mechanistic studies of galactosyltransferase suggest that several classes of chemical compounds may potentially inhibit lactose production and secretion by the mammary gland. Interactions of metals with manganese at its high affinity binding site could theoretically reduce the maximum activity of the enzyme. Witsell et al.[84] have shown that zinc ion produced a concentration-dependent (0.1 to 10.0 mM) inhibition of lactose synthesis by mammary gland Golgi vesicles in the presence of 10 μM manganese. Uridine derivatives can inhibit interactions of the substrate UDP-galactose with galactosyltransferase. The cancer chemotherapeutic agent, 5-fluorouracil, has been shown to inhibit this enzyme purified from human serum and ovarian tumor tissue;[85] uridine, uridine monophosphate (UMP), and UDP-glucose competitively inhibit the enzyme isolated from milk.[86] Inhibitors of polyamine biosynthesis such as the combination of α-difluoromethylornithine and ethylglyoxal bis(guanylhydrazone) also reduce activity of galactosyltransferase.[87] The mechanism of this effect is probably the decreased levels of polyamines such as spermine and spermidine, believed to be the cationic activators of the low affinity manganese site on galactosyltransferase. Perturbations of Golgi membranes are also likely to impair lactose synthesis and secretion. Mitranic et al.[88] have shown that colchicine inhibits vesicular membrane-bound galactosyltransferase but not the

soluble form of the enzyme. They present evidence suggesting that colchicine alters the biophysical characteristics of the Golgi membranes (increased fluidity). Phlorrhizin and phloretin, inhibitors of monosaccharide carriers in membranes, also inhibit lactose synthesis in Golgi membrane vesicles from mammary glands of lactating rats; the mechanism of this effect, however, is more complex than simple inhibition of glucose influx into the Golgi compartment.[89]

Clinical studies indicate that the concentration of lactose in milk is the least variable of all the macronutrients when examined at different times over a 24-hr period or across a population.[90] Maternal dietary factors reportedly do not influence lactose secretion. It has been difficult to establish mammary model systems that secrete lactose for detailed in vitro study.

Milk protein synthesis and secretion

Transcriptional regulation. One of the most important control points for the production of milk proteins during lactation is at the level of gene transcription. Rates of mRNA synthesis from casein genes start to increase during late pregnancy and remain high through lactation. Induction of β-casein or whey acidic protein (WAP) messages is dependent on the presence of prolactin, insulin, and glucocorticoids.[91] Cell-cell and cell-substratum interactions are also important.[92,93] In contrast, lactoferrin, another milk protein, seems to be regulated by an entirely independent form of control.[94] The cloning of many of these genes from a variety of species has allowed the sequences of promoters and other flanking regions to be analyzed and compared.[95] These studies have revealed potential binding sites for known transcription factors, such as the NF1 site in both the ovine and bovine β-casein gene, as well as at least two novel sequences that are conserved both in different milk protein genes and in the same gene from different species *(Figure 4).* Interestingly, one of these sequences (called the "milk box", −140 to −110 of the rat β-casein gene) is also found in the long terminal repeat of the mouse mammary tumor virus with a high degree of homology. The other (found between −95 and −80 of the rat β-casein gene) is found both in other casein genes and in the sheep β-lactoglobulin gene.

Conserved Promoter Sequences in Rat ß-Casein, MMTV-LTR and Sheep ß-Lactoglobulin

Figure 4 Conserved promoter sequences in rat β-casein, MMTV-LTR, and sheep β-lactoglobulin.

Doppler et al.[96] made a series of constructs using the rat β-casein promoter to drive expression of the chloramphenicol acetyltransferase (CAT) gene. The constructs were transfected into HC11 cells, a clonal line of mammary epithelial cells. CAT activity from the largest construct, containing 2.3 kb of upstream sequence, the noncoding exon 1 and about 450 bp of the first intron, could be induced 22 fold by preincubating the cells with prolactin and hydrocortisone. As the upstream sequences were progressively deleted, there was little effect on the basal activity, but hormonal induction of CAT activity decreased, particularly apparent as the sequences from −285 to −265 and from −185 to −165 were deleted. The significance of these findings is not clear because neither of these sequences correspond to the conserved regions mentioned above, neither is very well conserved in the β-caseins of other species, nor are they found in the promoters of other milk protein genes.

Yoshimura and Oka[97] made similar constructs using 5.3 kb of the mouse β-casein promoter together with 1.6 kb of 3′ sequence and transfected them into primary mouse mammary epithelial cultures. Removing upstream sequence from this construct had little effect on the degree of hormonal induction but did affect the basal activity, suggesting that there may be a transcriptional repressor far upstream in the β-casein promoter.

Rosen et al.[98] have shown that only 500 bp of promoter sequence from the rat β-casein gene (−524 to −8) was sufficient for hormone responsiveness and tissue specificity. When this construct was introduced into transgenic mice, inconsistencies between in vitro and in vivo systems became apparent. The −524 to −8 construct failed to function. The longer −524 to +480 sequence, which again contained exon 1 and part of the first intron, was required to give correct tissue specificity and appropriate hormonal control of CAT activity. Furthermore, even when the entire rat β-casein gene with 3.5 kb of 5′ sequence and a further 3.0 kb of 3′ sequence was introduced into transgenic mice, expression of the transgene was only 0.01 to 1.0% of the endogenous β-casein gene. In contrast, constructs with the rat WAP gene introduced into mice were expressed at 10 to 100% of the endogenous WAP gene. It may be relevant that the globin genes, which like the casein genes are arranged in a multigene locus, have remote locus control regions that exert coordinated control of the entire gene cluster. However, no such sequences have yet been found for the casein gene locus.

While the transfection and transgene experiments have yielded slightly contradictory results, they do indicate the minimal region important for efficient transcriptional control. Another approach has been to identify nuclear factors interacting with sequences in these regions. Electrophoretic mobility shift (band shift) assays using sequences corresponding to the rat β-casein milk box showed apparently identical shifts with nuclear extracts from a range of cultured cells, both mammary and nonmammary in origin. Nuclear extracts from Comma-D (a mammary epithelial cell line) and NIH-3T3 cells also gave identical footprints.[98] However, when an oligonucleotide corresponding to the other conserved sequence (−95 to −80 of the rat β-casein gene)

was used, a mammary-specific band could be obtained with rat mammary tissue.[98]

More recently, Schmitt-Ney et al.[99] have detected a total of four nuclear factors in HC11 cells. When the cells are exposed to lactogenic hormones, two of these factors seem to increase in concentration and may be positive regulators of β-casein expression, and the other two factors decrease and may be suppressors. This interpretation is supported by transfection studies in which promoters mutated in this region gave rise to much higher basal CAT activity than the wild type promoter.[99] One of the positive regulators may be binding to the milk box. However, nuclear extracts from mouse mammary gland produced only one shifted band,[99] binding to the same region shown by Rosen et al.,[98] to have mammary-specific activity.

Although none of the relevant transcription factors has been purified, the identification of specific DNA sequences that bind nuclear proteins makes their isolation likely in the near future. Such information is critical as a basis for understanding potential effects of xenobiotics on mammary gene expression.

Although little is known about the precise mechanisms by which hormones and cell-substratum regulate milk protein gene expression, a general hypothesis is that they may act through coupled membrane receptor-tyrosine kinase interactions. The prolactin receptor is a member of the hematopoietin receptor superfamily, which includes growth hormone and cytokines.[100] Although these receptors have not been demonstrated to contain intrinsic tyrosine kinase activity, ligand binding has been shown in several cases to increase tyrosine phosphorylation of specific substrates via activation of an associated tyrosine kinase.[101-103] Analogous studies have been performed recently for the prolactin receptor (P. Kelly, personal communication). The involvement of tyrosine kinases in the signal transduction pathway for milk protein gene expression has also been inferred from recent studies in which expression of the polyoma middle T or activation of the erbB-2 tyrosine kinase by a newly described ligand induced casein expression in mammary tumors and cell lines.[104,105] Presumably, multiple kinases are involved downstream from the tyrosine kinases to generate a kinase cascade capable of transducing the hormone signal from the cell surface to the nucleus.[106] The resulting phosphorylation or dephosphorylation of specific transcription factors may influence their activities in regulating milk protein gene expression. While the details of these signal transduction pathways remain to be elucidated in the mammary gland, they provide likely targets by which xenobiotics may affect lactation.

Posttranslational modification. The synthesis and secretion of milk protein depends on the formation of a unique complex of protein and inorganic calcium and phosphate termed the casein micelle. This complex is a true biocolloid and facilitates the transport of calcium to the neonate.[107] A requisite for this capability is the posttranslation phosphorylation of casein. In this respect, the human milk system is quite unique because the degree of phosphorylation, while not complete, is relatively constant in human β-casein; the protein contains five possible phosphorylation sites (5P), and the 2P and 4P forms predominate. In most other species, the caseins are usually completely phosphorylated.[108] While the reasons for this characteristic of human milk are unknown, this mixture represents the minimum degree of phosphorylation necessary for colloid formation. The totally unphosphorylated caseins will not form the necessary colloidal complexes for calcium binding and transport.[107] Thus, it appears as though colloid formation in human milk occurs on the very edge of colloid stability. Minor changes in the degree of posttranslational modification could easily upset this balance. In addition to transport out of the mammary gland, a number of digestion studies have shown that phosphopeptides derived from casein may facilitate calcium transport and uptake in the intestine.[109] Thus, changes in the degree of phosphorylation could jeopardize this function.

Another unique feature of the human casein system occurs in κ-casein, the fraction responsible for overall colloid stability. Human κ-casein is very highly glycosylated relative to most other species. The carbohydrate content contains a high percentage of amino sugars.[110] As in the case of phosphorylation, glycosylation may also play a role in the intestinal tract. It has long been recognized that breast-fed infants have *Bifidobacterium bifidus* as the major constituent of their intestinal flora, which is not the case for formula-fed infants. Studies on *B. bifidus* growth factors have constantly pointed to amino sugars as important factors.[111,112] It has been speculated that κ-casein may provide requisite growth factors for this bacterium.

A final posttranslational modification, proteolysis of β-casein by plasmin, occurs in milk within the lumen or ductal system. This cleavage results in a change in aggregation state of the casein micelles. In the older literature, this cleaved protein, now known as γ-casein, was termed galactothermin because of its increased degree of aggregation at 37°C.[113] It has been speculated that this proteolysis could be involved in the beginning of the digestive process.

There is mounting evidence that a regulatory step in casein secretion is at the level of intracellular degradation.[114] Explants of mammary glands taken from pregnant animals degrade synthesized casein intracellularly, whereas cultured lactating tissue shows little intracellular degradation unless prolactin is omitted from the medium. A secreted milk constituent appears to stimulate casein degradation and has been postulated to be a major regulator of milk volume secretion.[115] In addition, in vitro studies have shown that a human milk factor identified putatively as α-lactalbumin has the potential to inhibit cell division and protein synthesis in selected primary cultures and cell lines.[116] This would again argue for a type of autocrine regulation of mammary protein secretion by a secretory product. The fact that "back secretion" of α-lactalbumin into serum occurs in human lactation focuses attention on the role of milk proteins in the general circulation.

Certain proteins are degraded in the endoplasmic reticulum.[117] This process appears to require calcium and was inhibited in cultured Chinese hamster ovary (CHO) cells when the endoplasmic reticulum was depleted of calcium with the calcium ionophore, A23187, or the CaATPase inhibitor, thapsigargin.[117,118]

Potential effects of xenobiotics on ion transport

Like most eukaryotic cells, mammary cells have low intracellular sodium and high intracellular potassium ion concen-

trations,[119,120] which are maintained by an Na/K ATPase in the basolateral membrane.[121] Secretion of the aqueous phase of milk is driven by lactose synthesis in the Golgi. The synthesized lactose serves to draw water osmotically into this compartment. Mechanisms must be present in the Golgi membrane and the apical membrane of the alveolar cells to limit entry of monovalent cations with the water.[6] In this respect, the mammary epithelium acts like an absorptive epithelium. It is known that the apical membrane of the mammary cell is permeable to monovalent ions,[122] but the mechanisms that maintain the steady state ion distribution across this membrane are unknown, largely because of the lack of an in vitro model system that would permit study of its ion transport properties. The transport of calcium, present in high concentration in milk (8 mM in human milk; 33 mM in bovine milk), is better understood. The cytosolic concentration of this ion is in the submicromolar range, and the apical membrane is not permeable to this ion.[123] The ionized calcium concentration in human milk is about 3 mM.[50] The remainder is bound to casein or citrate. A calcium ATPase in the Golgi membrane is postulated to pump calcium from the cytosol into the Golgi compartment. There it participates in formation of the casein micelle.[124]

Low concentrations of ouabain, a specific inhibitor of Na/K ATPase, prevented lactogenesis in explants of rabbit mammary gland treated with prolactin.[125] Further, prolactin appears to increase the activity of this enzyme as measured by the uptake of ^{86}Rb into slices of lactating mammary gland.[126] This finding suggests that xenobiotics that inhibit Na/K ATPase might interfere with milk secretion. Many insecticides interfere with Na/K ATPase including chlordecone,[127] DDT,[128,129] and toxaphene.[128] Arochlor, a mixture of various PCBs, inhibited the ATPase activity of rat liver, brain, and kidney, but only 20 to 30% at a concentration of 25 mg/kg given daily for 7 days. However, some components of the mixture appeared to be more toxic.[129] Other substances that inhibit Na/K ATPase under some conditions include aluminum fluoride,[130] vanadate,[131] and lead.[132] The relevance of these observations to the in vivo toxicity of these agents is brought into question by a study in rats where 1 to 50 μM chlordecone, triethyltin, tributyltin, and mercuric chloride were all found to inhibit Na/K ATPase activity in brain cells and erythrocytes in vitro.[133] However, doses of these compounds that produced rapid and gross symptoms of neurotoxicity in vivo had no effect on Na/K ATPase activity of the brains or erythrocytes examined 5 to 9 hours postdose.

The calcium ATPases of both the plasma membrane and the endoplasmic reticulum are inhibited by vanadate ions at micromolar concentrations.[134] Thapsigargin and 2,5-di(tert-butyl)-1,4-benzohydroquinone (tBuBHQ) are specific inhibitors of the endoplasmic reticulum Ca ATPase that do not affect plasma membrane calcium transport.[135] Studies of the effects of these inhibitors should provide additional insight into mechanisms of calcium transport in the mammary alveolar cell and the potential role of toxins in this process.

Potential effects of xenobiotics on other secretory pathways

Transport of trace elements. The concentrations of most of the essential trace elements in human milk appear to be highly regulated. The levels of iron, zinc, copper, and manganese in milk from women who are deficient in these nutrients are similar to those in controls or women who take daily supplements.[136] These observations suggest that there are homeostatic mechanisms within the mammary gland that control the uptake of trace elements from plasma or possibly regulate the secretion of these elements into milk.

Little is known about the mechanisms of trace element transport into the mammary gland. However, because most trace elements are delivered in blood bound to carrier proteins, it is likely that the uptake is mediated by receptors for such proteins. It has been shown, for example, that there are transferrin receptors on the plasma membrane of mammary epithelial cells, and that the number of receptors is affected by maternal iron status.[137] This phenomenon is likely to play a key role in the regulation of influx of iron bound to transferrin in the plasma. Plasma carrier proteins for other trace elements include ceruloplasmin for copper, α_2-macroglobulin and albumin for zinc and transferrin for manganese.

Smaller fractions of trace elements are transported in "free" form or loosely coordinated to low molecular weight chelators or anions.[138] It is possible that this pool of trace elements is transported via passive or facilitated diffusion. The fraction of trace elements transported via this pathway, however, is probably quite limited as only a small percentage of the total trace element concentration is found in this pool. In early lactation, transport may also occur via the paracellular route. However, it should be noted that the temporal patterns for concentrations in milk and the plasma to milk ratios vary considerably among the trace elements, which suggests that the paracellular pathway is not of quantitative significance.

In contrast to the elements mentioned above, the level of selenium in human milk seems to be strongly affected by maternal selenium status.[139] It has been shown that women in low selenium areas have low milk selenium levels, and that women taking selenium supplements have increased milk levels. Thus, transport of selenium into milk is dependent on plasma selenium levels and much less, if at all, regulated by cellular homeostatic mechanisms.

There is a paucity of information on the effects of environmental agents on milk trace element levels. There is a high likelihood that nonessential metals such as aluminum, cadmium, mercury, lead, and nickel may interfere with transport mechanisms because interactions have been shown in other tissues. Because some nonessential toxic metals have similar coordination chemistry to trace elements, they can compete for binding sites on transport proteins or membrane sites. However, few studies have examined interactions in mammary gland cells or plasma-to-milk transfer. Other nonmetal environmental agents may possibly interfere with these processes as well. Greater understanding of normal mechanisms of trace element transport into milk is needed to predict the potential perturbation by xenobiotic agents. Research priorities include characterization of transport mechanisms of essential and toxic metals into milk and determination of effects of toxic metals and other xenobiotics on transport of essential trace elements into milk.

Production of host defense systems in milk. Human milk is characterized by an array of defense factors that are biochemically heterogeneous and are adapted to persist and act at muco-

sal sites.[140] The defense system consists of antimicrobial factors, antiinflammatory agents, and modulators of immunity.

Antimicrobial factors. The major immunoglobulin of human milk is secretory IgA. This immune molecule is made by plasma cells within the interstitium of the mammary gland. It is secreted by a transcytotic pathway that involves binding to a specific receptor on the basolateral surface of the mammary epithelial cells, endocytosis, transport across the cytoplasm, and exocytosis into the milk after proteolytic cleavage of the receptor, resulting in the secretion of the IgA with bound secretory component.[7] Toxins potentially could interfere with both the production of IgA and the transcytotic pathway. There is substantial evidence for effects of dioxins on the immune system,[141] with suppression of a number of immunologic parameters at dosages less than 1 μg/kg/week in mice and rabbits.

Other major antimicrobial agents in human milk include lactoferrin, lysozyme, oligosaccharides, glycoconjugates, antiviral lipids, and activated leukocytes.[140] The mechanisms for the production of these components are poorly understood.

Anti-inflammatory agents. Human milk also contains a host of anti-inflammatory agents that include the antioxidants urea, vitamin E, and β-carotene.[140] These factors are of interest because they may potentially counteract effects of oxidative xenobiotics excreted in milk.

Immune modulators. A number of immunologic modulators including vitamin E, tumor necrosis factor-α, interleukin-6, and interleukin-1 have been discovered in human milk.[140] The cytokines are of particular interest because they are major orchestrators of the immune system but may produce an excessive inflammatory response with perturbations of nutritive secretory pathways if present in very high concentrations.

Bile salt stimulated lipase. The major lipase in milk, bile salt-stimulated lipase, shares substantial structural homology with other esterases such as acetylcholinesterase and human pancreatic carboxyl-ester hydrolase.[142] The enzyme is completely inhibited by diisopropylfluorophosphate (DFP, 0.1 mM), a cholinesterase inhibitor that binds covalently to a serine in a site common to these enzymes.[142] Other anticholinesterases, such as certain insecticides, would be expected to affect the activity of this enzyme.

Transport of vitamins. The secretion of vitamins into milk in humans has been reviewed by Bates and Prentice,[143] and in the recent text from the National Academy of Sciences.[90] The following points are directed to those vitamins for which xenobiotic-induced inhibition of transport into milk is most likely to cause deficiency syndromes in the offspring. Bates and Prentice in their review[143] refer to deficiency syndromes documented in breast-fed infants only for vitamin B_{12}, vitamin D, and vitamin K. Reduced levels of riboflavin (B_2) have been shown in breast-fed infants of deficient mothers, although clinical symptomatology was not found. Other limited studies suggest that long-term high dose oral contraceptive use may decrease vitamin B_6 in milk and produce a deficiency syndrome in infants.[90] Xenobiotics that reduce plasma levels of these vitamins in the mother may well produce deficiencies in the infant. Examples of this type of interaction in the literature are minimal. Possibilities that warrant exploration include:

Vitamin A: Levels of retinol in the liver are influenced by exposure to dioxin, but whether secretion of retinol into milk is affected has not been examined. Retinol is secreted primarily in esterified form and is absorbed by the infant after deesterification by bile salt-stimulated lipase; whether inhibition of this enzyme by esterase inhibitors secreted in milk can induce deficiency in infants is unknown.

Vitamin B_{12}: Low maternal dietary intake of B_{12}, as occurs with consumption of a strict vegetarian diet, increases the risk of B_{12} deficiency in breast-fed infants.

Vitamin D: The concentration of vitamin D in milk has been shown to correlate with maternal dietary intake; more significant changes in milk levels occur in response to dietary change than is observed for vitamin A or water-soluble vitamins.[143] Maternal exposure to UV light also increases vitamin D levels in milk. Human milk alone provides a marginal level of vitamin D to breast-fed infants.[144]

Studies were not found of xenobiotic-induced reduction of vitamin D secretion into milk. Of special concern might be agents which impair the 25- and 1-hydroxylation reactions, mediated by mixed function oxidases, that activate cholecalciferol.[145] For example, prolonged therapy with the anticonvulsants, phenytoin and phenobarbital, reduces plasma levels of 25-OH D_3, the major form in plasma also present in milk. It should be noted that 1-hydroxlation is increased by prolactin and estrogens. Studies were not found that analyzed the extent or regulation of these reactions within the mammary gland.

Vitamin E: Sows deficient in Vitamin E are reported to have a higher incidence of agalactia at birth[146]; the method for assessing this effect was not detailed in the paper. However, preliminary observation suggests that xenobiotics that impair vitamin E supply might interfere with lactogenesis or milk secretion or both. Piglets seem quite sensitive to alterations in lactation and may be a good in vivo model for the effect of toxins.

Vitamin K: Levels of vitamin K in milk are low relative to needs of the infant and insufficient to meet recommended intake for infants less than 6 months of age.[147] This fat soluble vitamin is localized in milk within fat globules and is not membrane bound. The mechanism of transport from plasma to milk is reportedly not known.[148]

Although documentation in the literature was not found, it would be expected that xenobiotics that decrease maternal plasma levels of vitamin K might induce or exacerbate infant deficiency. Mechanisms by which chemical agents cause insufficiency of vitamin K include inhibition of intestinal bacterial growth (antibiotics), impairment of bile salt function, and inhibition of reduction of the oxidized form (warfarin-like anticoagulants).[149] Whether mammary gland transport of vitamin K from plasma to milk can be altered by xenobiotics has not been studied.

Folate: Recent reviews suggest that concentrations of folate in milk are not closely related to dietary intake.[90,143] Deficiency syndromes in breast-fed infants were not found in the literature. An important example of a dietary interaction affecting folate in milk has recently been described by Picciano and coworkers.[150–152] Their studies demonstrate impairment of folate transport into the milk of moderately or severely iron-

deficient lactating rats fed normal levels of folate;[150] a smaller percent of total folate in milk was in the form of long-chain folylpolyglutamates.[151] In iron-deficient dams, unlike controls, an increase in dietary folate levels (above normal) did not increase milk folate concentrations. The defect induced by iron deficiency could not be explained by a change in folate-binding protein in milk. Folate is known to accumulate in the mammary gland (producing higher levels in milk than in plasma) as a result of the polyglutamation reactions mediated by methionine synthase and folyglutamate synthetase. In vitro studies with addition of substrate have not revealed reduction in the activity of these enzymes in mammary gland homogenates from iron-deficient rats.[152] The mechanism of this interaction is therefore unknown. Xenobiotics that induce an iron-deficient condition may therefore impair folate availability in breast-fed infants.

Secretion of hormones and growth factors. Milk (human, bovine, and other) contains many hormones and hormone-like substances of a peptide character: namely, gastrointestinal regulatory peptides, hypothal-hypophyseal hormones, thyro-parathyroid hormones, and growth factors.[153–155] Some of the peptide hormones, such as EGF, are produced by the mammary gland; others, like prolactin, are taken up by the mammary gland from the peripheral circulation. Many of them "survive" and are absorbed in a biologically and/or immunologically active form in the gastrointestinal tract[156] because of the immaturity of the gastrointestinal barrier function, low level of proteolytic enzymes,[157] and presence of protease inhibitors in milk.[158]

Studies with EGF and other hormones have shown the profound dependency of the content of the gastrointestinal tract on their orogastric intake, thus suggesting both a long-term and short-term regulatory role. Several hormones from milk have been demonstrated to reach the circulation of the newborn and exert profound effects on (neuro)endocrine regulation.[159,160] For example, prolactin in milk has been demonstrated to be a major source of the hormone in the neonatal circulation. This conclusion is based in part on analysis of the molecular forms of prolactin in milk and in neonatal serum, based on assays for bioactivity (Nb_2 bioassay) and immunoactivity (RIA).[156] Prolactin in milk, unlike that secreted from the pituitary, is substantially glycosylated, a form not readily detectable by RIA. The physiological significance of this milk-derived prolactin has been demonstrated in that its reduction during a critical postnatal period in female rats has been shown to alter the structural and functional characteristics of prolactin-secreting cells of the pituitary.[161,162]

The production and secretion by the mammary gland, as well as gastrointestinal processing of peptide milk-borne hormones, may be influenced by various endogenous and exogenous factors. Because of the possible importance of these peptide factors in milk, a serious need exists to explore these processes under normal conditions as well as under the influence of environmental xenobiotics.

Xenobiotic-induced oxidative injury

One mechanism of xenobiotic-induced toxicity is generation of "oxidative stress"; with formation of reactive species such as superoxide, hydrogen peroxide, and hydroxyl radicals capable of oxidizing cellular lipids and macromolecules; and postulated to lead to damage to membranes, DNA, and sulfhydryl-containing enzymes.[163] Reactive oxygen species are produced as intermediates in a large number of cellular enzymatic reactions including cytochrome P-450 catalytic cycles, prostaglandin synthesis, and xanthine formation from hypoxanthine. Cells possess a variety of enzymes that protect them from oxidative damage, including superoxide dismutase, catalase, and glutathione peroxidase.

Lipid peroxidation is a cellular response to a variety of agents and may take place in both hepatic and extrahepatic tissues.[164,165] Goel and colleagues[166] gave rats one half the LD_{50} of carbon tetrachloride, TCDD, hexachlorocyclohexane, or dieldrin in a single dose. Lipid peroxidation, assessed by measuring hepatic malondialdehyde, was increased fourfold to fivefold in response to all these compounds. Carbon tetrachloride and TCDD also decreased the concentration of glutathione peroxidase by about 25%. The number of hepatic peroxisomes has been shown to be increased by hypolipidemic agents such as clofibrate, the plasticizer di(2-ethylhexyl)phthalate, the herbicide lactofen, and the organic solvent 1,1,2-trichloroethylene.[167] The magnitude of the response may depend on the sex of the animal. For example, high doses of TCDD in rats produced an increase in malondialdehyde content of the liver that was accentuated in female rats. An increase in glutathione S-transferase often accompanies increased lipid peroxidation.[168] Increased lipid peroxidation apparently does not depend on activation of the gene for arylhydrocarbon hydroxylase (see below) because the response was seen in mice deficient in responses to activation of this gene.[169] Hepatic glutathione peroxidase is a selenium-dependent enzyme whose depletion was postulated to be responsible for increased lipid peroxidation. However, increased malondialdehyde production was observed in selenium-deficient rats in response to TCDD,[170] suggesting that depletion of glutathione peroxidase is not a necessary part of the mechanism of increased lipid peroxidation in response to xenobiotics.

The capacity of mammary cells for lipid peroxidation has received little study, although in recent experiments antioxidants and low oxygen tension enhanced the growth potential of rat mammary cells when used during the cell dissociation phase prior to primary culture.[171] These agents were shown to decrease lipid peroxidation during this phase. At least two oxidative enzymes have been shown to be present in both mammary gland and milk. Xanthine oxidase, the enzyme that catalyzes xanthine formation with the generation of hydrogen peroxide, is a prominent component of the milk fat globule membrane,[59] comprising 13% of the protein in this membrane. However, the actual activity of this enzyme in humans is quite variable; on the average the highest values found by Zikakis et al.[172] in mature milk (1.2 mU/mL) are substantially lower than these found in cows' milk (110 mU/mL) by Cerbulis and Farrell.[173] Xanthine oxidase is induced to high levels in the mammary alveolar cells from the lactating gland of the rat,[174] where it is found on the cytoplasmic surface of the apical membrane.[175] Sulfhydryl oxidase, an enzyme that catalyzes the formation of disulfide bonds with the

production of hydrogen peroxide, is present in the mammary gland and milk of most species apparently associated with membranes.[176,177]

There is currently uncertainty about the concentrations and nature of enzymes that potentially protect against oxidative injury in the mammary gland and milk. Multiple electrophoretic variants of superoxide dismutase have been reported in normal rat mammary gland[178]; the concentration increased with age and previous pregnancy.[179] The enzyme has been reported to be present in human milk.[180] Peroxidases have been reported both to increase[181] and to disappear[182] in the lactating rat mammary gland. Lactoperoxidase is present in bovine milk, but apparently not in human milk,[183] which only contains peroxidase derived from leukocytes. Glutathione peroxidase, a selenium containing enzyme, is present in the lactating mammary gland[184] as well as human milk.[139,185] A positive relationship exists between the concentration of selenium and glutathione peroxidase activity in milk of humans[186] and rats.[187] Milk levels are lower than plasma levels by a factor of at least 10. Glutathione peroxidase accounts for only about 25% of the total peroxidase activity of human milk with glutathione S-transferase possibly accounting for the remaining activity.[188] The source of human milk glutathione peroxidase is unknown. Cellular glutathione peroxidase appears to differ from the plasma enzyme,[189] and the possibility exists that the milk enzyme(s) may be present in various forms and from more than one source.

While the physiological significance of human milk glutathione peroxidase is unknown, it is known that selenium deficiency, which impairs the function of this enzyme in other tissues, exacerbates the toxicity of some xenobiotics, i.e., drugs, insecticides, and halogenated hydrocarbons that produce toxic oxygen derivatives. Conversely, selenium supplementation can alleviate the toxic effects of these agents.[190]

The potential of environmental toxins to produce oxidative injury in the mammary gland and the functional consequences of such injury require systematic investigation, particularly in the lactating animal. Important to these investigations will be an understanding of the role of antioxidative enzymes such as superoxide dismutase and glutathione peroxidase in the differentiated function of the mammary gland. The recent suggestion that oxygen radicals may serve as second messengers[191] renders such studies all the more urgent. A survey of the concentrations of oxidative and protective enzymes in the milk of women with high body burdens of PCBs and other toxins may help determine whether mammary oxidation-reduction mechanisms are affected by xenobiotics.

Potential effects of xenobiotics on the hormonal milieu

Milk ejection

In response to suckling, oxytocin is released from neurons with cell bodies in the hypothalamus and terminals in the posterior pituitary. This neuroendocrine reflex is subject to conditioning so that an infant's cry or even a picture of an infant may stimulate oxytocin release. Release is inhibited by emotional states. The hormone brings about milk ejection by stimulating the myoepithelial cells of the breast to contract.[6] Without this reflex, milk cannot be removed from the gland; the milk products build up and eventually inhibit milk synthesis and secretion. Alcohol is a well-known inhibitor of this reflex.[192] In rats, morphine has also been shown to inhibit the let-down reflex.[193]

Prolactin secretion

Prolactin is essential to milk secretion.[194] If high levels of prolactin are not present after parturition, lactogenesis (the onset of copious milk secretion) does not take place.[6] Under the stimulation of estrogen, prolactin levels increase gradually during pregnancy to concentrations 10 times those of the nonpregnant nonlactating woman. The number of mammotropes in the pituitary increases. After parturition, prolactin levels fall gradually. In breast-feeding women, suckling stimulates prolactin release so that the postpartum fall in plasma prolactin concentrations requires months rather than weeks. Prolactin secretion is under hypothalamic control; dopamine inhibits its release, and TRH and prolactin-releasing hormone stimulate it. The rise in estrogen during pregnancy appears to be essential for the increasing prolactin secretion and for lactogenesis. Women who lacked placental steroid sulfatase had severe estrogen deficiency during pregnancy, their prolactin levels failed to rise, and they were unable to establish lactogenesis.[195]

Because dopamine is a physiologic inhibitor of prolactin release, its congeners have the same effect.[196] Dopamine agonists such as bromocriptine are used clinically to suppress lactation[197] and hyperprolactinemia.[198] Dopamine antagonists such as sulpiride and metoclopramide stimulate prolactin release and have been shown to increase milk production.[199,200] More recently, intranasal infusion of TRH has been shown to increase plasma prolactin and milk production in women with inadequate lactation.[201]

Steroid hormones

Glucocorticoids, estrogens, and progesterone all play a role in mammary development and lactation. However, the exact mechanisms involved are not well understood.

Glucocorticoids. Glucocorticoids must be replaced in adrenalectomized or hypophysectomized animals if lactation is to take place.[196] In mammary tissue and organ culture, glucocorticoids, along with insulin and prolactin are necessary for casein secretion.[202] Although glucocorticoid receptors are present in the mammary gland,[203] glucocorticoid response elements have not been shown to be present in the promoter regions of milk protein genes,[204] so that direct effects on gene expression are probably not involved in their mechanism of action. They may act by enhancing the cellular response to prolactin.[194]

Estrogens. Estrogens are important in mammary growth, stimulating ductal development, and increasing the number of progesterone receptors.[205] Estrogens also increase stromal growth and prolactin secretion,[196] suggesting that part of their action is indirect. The mechanism of action of estrogens in the normal mammary gland is not clear, although they clearly promote growth of many mammary tumors.[205] Lactating mammary cells are refractory to the effects of estrogen

on progesterone receptors.[206] Estrogens have an inhibitory action on lactogenesis[197] and estrogen-containing contraceptives tend to inhibit milk production.[198] The mechanisms of these rather paradoxical inhibitory effects are entirely unknown. TCDD down-regulates hepatic and uterine estrogen receptors in rodents.[207] The antiestrogen, tamoxifen, increased TCDD toxicity in rats.[208] A positive relationship has been demonstrated between the estrogen receptor content of a number of human cancer cell lines and TCDD-inducible AHH activity (arylhydrocarbon hydroxylase).[209]

Progesterone. Progesterone inhibits milk secretion during pregnancy,[210] presumably acting through the progesterone receptors that have been induced by estrogens. There is no effect of progesterone in the lactating mammary gland,[196] and for this reason, progesterone-containing contraceptives do not affect milk secretion.[198]

Thyroid hormone

Adequate levels of thyroid hormone have long been known to be important for lactation[211,212]; thyroid replacement is essential for lactation in hypophysectomized goats and rats. However, the action is considered to be permissive rather than regulatory,[196] and, in general, thyroid hormone is not used in tissue culture model systems for milk secretion. Thyroid hormone increased milk secretion in cows, increasing the fat and lactose concentration but not the protein content of the milk.[213] Women who are clinically hypothyroid in the puerperium may have difficulty initiating lactation (N. Powell, personal communication). However, this effect has not received systematic study.

One effect of TCDD is to produce hypothyroidism.[214] For example, a single dose of 100 μg of TCDD to a 140 to 160 g rat reduced plasma T_4 as much as thyroidectomy in 7 days, although there was no effect or even a slight increase in plasma T_3. If this is true, and free thyroid hormone levels as well as the plasma TSH concentration must be measured to confirm it, dioxins may decrease milk production through decreasing circulating thyroid hormone.

Potential interactions of xenobiotics with nutrient delivery

Mammary blood flow

For substances readily extracted from blood by mammary gland cells, the delivery rate to the organ may be a limiting factor in secretory rate. In a review of mammary blood flow and lactation, Mepham[215] emphasized the strong positive correlation observed between mammary blood flow and milk volume in studies of lactating goats. The increase in flow during lactation results from vasodilation in the mammary vasculature and an increase in cardiac output. In rats, the increase in cardiac output may be mediated by prolactin or growth hormone. In cows, administration of either thyroxine or growth hormone increases cardiac output, mammary blood flow, and milk yield, but not the ratio of blood flow to milk yield.[216] Recent studies in goats suggest that effects of growth hormone on the mammary gland are mediated in part by insulin-like growth factor-I.[217] It is not clear whether the increase in mammary blood flow in response to this peptide is a direct effect on the vasculature or an indirect one in response to the increased metabolic activity in the mammary gland cells; the authors note that type-1 IGF receptors have been identified on bovine capillary cells.[217]

Key factors that regulate tone of the mammary vasculature under physiological conditions have not been established. Mepham refers to the summary by Linzell[218] of known endogenous vasoconstrictors (E, NE, 5-HT, $PgF_{2\alpha}$) and vasodilators (ACH, histamine, adenosine, bradykinin) and the correlation between flow and oxygen consumption, implicating a local role of pCO_2. A decrease in local levels of $PgF_{2\alpha}$ may contribute to the marked increase in mammary blood flow and change in milk composition that occurs in lactogenesis. Recent studies of prostacyclin suggest that its local release is not a critical determinant of variations in flow during lactation in goats.[219] Administration of growth hormone that increased milk yield also increased levels of prostacyclin in milk, but blood flow was not measured nor the effects of prostacyclin antagonists tested.[220] Literature was not found on the possible vasodilatory role of nitric oxide synthesis in the vascular endothelium of the mammary gland, of known importance in other tissues.[221] Recent studies have shown that neurogenic (mammary nerve stimulation) and hormonal (oxytocin) stimulation contribute to the marked increase in plasma levels of vasoactive intestinal peptide that occurs following initiation of suckling in lactating rats.[222] Whether this increase is sufficient to have a vasoactive effect was not tested.

Few studies were found that reported a decrease in milk volume or change in composition following administration of a xenobiotic with vasoactive properties. Polymyxin B, a relatively toxic antibiotic, has recently been reported to decrease blood flow to the mammary gland of starved-refed lactating rats and inhibit lipogenesis in this tissue.[223] However, the in vitro conversion of glucose to lipid was impaired in mammary gland acini from treated rats, so the contribution to the in vivo effect of reduced blood flow versus altered metabolic activity is not clear.

Malnutrition

The second critical factor determining delivery of nutrients to the mammary gland is the concentration in the blood supply to the tissue. For nutrient substances whose transport into milk is by passive diffusion without homeostatic regulation in the mammary gland, reduction in plasma concentration is likely to decrease secretion into milk. Xenobiotics may induce a relative state of malnutrition in which plasma levels of one or more critical nutrient substances are reduced. Mechanisms include anorexia with decreased maternal nutrient intake; impaired gastrointestinal absorption of nutrients; and altered systemic metabolism, including increased nutrient catabolism or impaired synthesis.

The extent to which decreased food intake impacts on lactation is species dependent. In lactating animals secreting milk at maximal capacity, a decrease in food consumption can more readily affect milk volume than has been observed in lactating women. A study in lactating rats whose diet was restricted to 40, 60, or 80% of normal for 1 week beginning on day 7 of lactation, indicated that milk production was

reduced based on lower weight gain of pups. However, milk concentrations of total lipid, total protein, and lactose were unaltered.[224]

As indicated previously, because of homeostatic regulatory mechanisms, the concentrations of many macronutrients and micronutrients in human milk are not substantially affected by a decline in their intake. Lactose, total protein, and total lipid in human milk are reported to be resistant to reductions in dietary content.[90] However, the form of the macronutrient, e.g., fatty acid composition of total lipid, is influenced by the dietary composition as is its source, e.g., diet versus adipose stores versus mammary gland synthesis. Furthermore, a recent comparison of the concentrations of nutrients in human milk samples from five studies carried out in developed countries to that from four studies in areas with higher incidences of malnutrition indicates that lipid levels are about 20% lower in malnourished women and lactose levels are about 10% higher.[50] Other studies in marginally nourished women have suggested that lipid levels in human milk correlate with total body fat.

Summary of xenobiotic effects by toxin category

This extensive review of the literature revealed only five studies in which possible effects of xenobiotics on milk secretion in animals have been properly evaluated. Of these studies, two showed that toxins at high doses, e.g., DDE[225] and lead,[132] have little effect on milk secretion. Three others, alcohol,[77] phthalates,[81] and TPA[16] had clear effects, although the mechanisms were unclear. There appear to be no studies in which the effects of environmental toxins on human milk composition have been usefully evaluated.

Alcohol

Ethyl alcohol exemplifies an agent potentially able to alter milk composition and volume by a multiplicity of mechanisms, including a decrease in release of oxytoxin from the pituitary gland, alteration of lipid metabolism in the mother, and decreased suckling through a depressant effect in the infant. Studies in lactating women have demonstrated that the milk-ejecting reflex, induced by oxytocin in response to the suckling stimulus, is depressed in women given ethyl alcohol (1 to 2 gm/kg).[192] This conclusion was reached by measurement of intraductal pressure, which exhibits phasic increases with let-down. Ethyl alcohol reportedly did not alter the increased pressure in response to injection of oxytocin, consistent with the hypothesis that the myoepithelial cells that contract in response to oxytocin were not altered. Studies in rats have demonstrated that milk production was substantially decreased in dams given ethyl alcohol via drinking water (25%) during lactation.[77] (Solid food intake was decreased in treated animals, but caloric intake was similar.) The composition of milk was changed, with an increase in triacylglycerol concentration and decrease in lactose concentration. A marked decrease in mammary gland weight was observed by day 15 after parturition and an increase in lipoprotein lipase activity per gram of tissue weight.

Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls are very stable compounds that found extensive use in the 1960s and 1970s until their persistent environmental effects become apparent. TCDD is the most potent of these compounds, and its mechanism of action has received extensive study.[27,226] Serious toxic effects of TCDD can be seen in vivo at intakes as low as 1 to 4 μg/kg/day. Reproductive toxicity in the form of decreased fertility was observed in rhesus monkeys at a dose of about 100 μg/kg/day. Cattle exposed to a polybrominated biphenyl-contaminated protein concentrate actually dried up.[227] Rogan and coworkers[228] calculated that the breast-fed infant of a moderately contaminated woman receives about 6 μg/kg of PCBs each day—substantially above any safe level. However, they state that "breast-fed infants thrive and there are no case reports of illness in breast-fed children at population levels." However, studies to examine breast milk volume and composition in instances of high PCB exposure have not been carried out, although some toxic effects in infants have been reported due apparently to intrauterine exposure, e.g., low birth weight[4] and decreased psychomotor development at 12 months.[229] Women with high body burdens of PCBs due to industrial accidents transferred large amounts of PCBs to their infants in breast milk.[4] However, according to Yakushiji,[4] there was no discernable effect on growth rate of the infants, even though they showed some of the signs of PCB toxicity.

More detailed studies on human milk composition are called for in the dioxin field. In particular, changes in cell morphology might be revealed by increases in the sodium content of milk of women with high exposures. The arylhydrocarbon hydroxylase (AHH) activity of breast milk epithelial cells would be a useful index of the functional consequences of PCB exposure.

Phthalate plasticizers

The exemplary study of Dostal and coworkers[81] on the effects of di(2-ethyhexyl) phthalate on rat milk composition and secretion has been discussed above. It is important to note that a dose of 2 g/kg for 3 days was given in this study. This dose probably exceeds the expected exposure in any imaginable natural situation. However, phthalate esters are widely used and widely distributed, with a concentration of 1 to 10 μg/L generally found in fresh water.[230] There is evidence that low chronic doses of these compounds are carcinogenic and may have reproductive effects as well.

Pharmacologic Agents

The effects of drugs on milk production has recently been reviewed by J. Philip and a World Health Organization Working Group.[198] Highlights reiterated here refer to pharmacologic agents notable for their ability to impair milk yield.

Oral contraceptive agents. Estrogens suppress lactation, probably by inhibiting the effect of prolactin on the mammary gland, and have been used alone and with androgens for this purpose. The effect on lactation of estrogens and progesterone, as used in oral contraceptive preparations, is not definitively established. Philip et al. (1988) summarized

results of 22 studies published between 1966 and 1984. They concluded that low-dose combination preparations, but not progesterone alone, decrease milk volume and the duration of breast feeding. In only a few of these studies was milk composition examined.

Dopamine agonists. Agents with dopamine agonist activity suppress lactation by inhibiting prolactin release from the anterior pituitary. Agonists activate dopamine by one or a combination of mechanisms: (1) direct interaction with the dopamine binding site on the D-2 receptor; (2) indirect effect via release of dopamine; and (3) conversion to dopamine. Examples of agonists whose primary effect is elicited by these mechanisms include: (1) bromocriptine and other ergot derivatives; (2) dextroamphetamine and nomifensine; and (3) levodopa.

Other drugs. Serotonin antagonists have been shown to decrease lactation, possibly by inhibiting prolactin release. Endogenous opioids may influence prolactin release, and under certain conditions opioid antagonists may inhibit prolactin release.[231]

Carcinogens and tumor promoters

Carcinogens act through alternations in DNA that interfere with growth control.[232] Because small numbers of molecules may be involved, very low concentrations of carcinogens may be effective in carcinogenesis. These concentrations are unlikely to produce the types of changes in large numbers of mammary cells necessary to interfere with milk secretion. On the other hand, tumor promoters alter the metabolism of large numbers of cells, establishing a milieu in which the actions of a carcinogen may lead to development of a tumor. We have already reviewed the evidence that tumor promoters produce changes in cell morphology that could interfere with milk secretion.

Insecticides

The primary mode of action of insecticides is as neurotoxins, and thus, in general, their toxicity is expected to arise from effects on the nervous system. However, because some of them, particularly the organochlorine insecticides, are very stable and persist a long time in the environment and in fat tissues of species that ingest them, they have other effects as well. Insecticides fall into three chemical categories: organochloride compounds, anticholinesterases, and pyrethroid insecticides.[233]

Organochlorine insecticides (DDT, aldrin, dieldrin, endrin, chlordane benzahexachloride). This group of compounds has the properties of low volatility, chemical stability, lipid solubility, and a slow rate of biotransformation and degradation. They are highly effective and have relatively low acute toxicity, but environmentally they are devastating because they build up in the lipid stores of animals high on the food chain, causing multiple long-term anomalies. Their major mechanism of action involves alteration of membrane channels, changes in activity of membrane ATPases, and possible alteration of calmodulin.[233]

The major metabolite of DDT is *bis*(p-chlorophenyl)acetic acid (DDA). This compound is bound to plasma proteins and excreted by the kidney. However, it is reabsorbed via the organic anion pathway (showing inhibitory effects at concentrations as low as 1 μM with maximal effects at 1 mM) without affecting the metabolism of the renal cell at concentrations up to 0.1 mM. Higher concentrations alter tissue respiration, intracellular electrolyte distribution, and Mg-ATPase activity.[234] In humans, the long-lived metabolite of DDT is 1,1-dichloro-2,2-bis[p-chlorophenyl]ethylene (DDE), a compound concentrated in the milk of exposed mothers. Because Rogan and Gladen[235] observed that mothers with high concentrations of DDE in their milk terminated breast-feeding earlier than control mothers, Schwetz and colleagues[225] initiated a study of the effect of long-term administration of DDE on pregnancy and lactation in rats. They used dosages (10 mg/kg/day for at least 9 weeks) that produced a concentration of DDE in the rat milk about 100 times that ever observed in human milk. There was no effect on milk secretion, pup growth, or the macronutrient composition of the rat milk, suggesting that DDT probably does not affect milk secretion. This study is notable as a model of how animal studies of lactation should be carried out.

Anticholinesterase insecticides. These compounds fall into two chemical categories, organophosphorus esters and carbamic acid esters; they act by reacting with the active site of cholinesterase, inhibiting acetylcholine hydrolysis, and prolonging its action at cholinergic synapses. Some agents, such as the prototypic DFP, bind irreversibly to the active site of the enzyme. Most are metabolized to a variety of compounds that vary widely from species to species and have varying toxicities. In the mammary gland, anticholinesterases might be expected to inhibit analogous serine proteases such as bile salt-stimulated lipase[142] and possibly thioesterase II.[79] Their acute neurotoxicity is, however, likely to be a more serious problem than inhibition of mammary enzymes.

Pyrethroid insecticides. The mechanism of action of these compounds is similar to that of the organochlorine insecticides. They appear to be detoxified efficiently in mammalian tissues, limiting their toxicity.

Heavy metals

Lead, fed to suckling rat dams in the drinking water (0.2% lead acetate), produced specific degeneration of the developing retina of their pups, which may result from inhibition of Na/K ATPase activity in this tissue. No inhibition of Na/K ATPase was seen in kidneys of these same animals.[132] Interestingly, in these studies, the relatively high dose of lead had no effect on growth rate, age of eye opening, brain weight, or hematocrit, suggesting that fairly high levels of lead do not interfere with milk secretion or alter milk composition in a nutritionally significant way.

A review of studies on the absorption and distribution of cadmium administered to lactating mice and rats[236] indicates increased oral absorption, increased renal accumulation, and a marked increase in mammary gland content compared with control animals. Transfer in milk to the neonate was also

examined. Data on pup growth rate or milk composition were not found.

Vitamins

The potential problem of xenobiotic-induced decreases in the milk concentration of certain vitamins has been described. Some vitamins, if consumed in marked excess, are excreted in milk in levels that may be potentially toxic to the breast-fed infant; this possibility has been documented for vitamin D.[237] Vitamin A is also potentially toxic to neonates, but Bates and Prentice in their review indicate that toxic levels are unlikely to be achieved in human milk.[143]

Research approaches

Our review of the available information shows the paucity of evidence regarding the effects of xenobiotics on breast milk secretion and composition. There are hints that some classes of toxins and drugs may have deleterious effects, and some of them may help in understanding the regulation of mammary gland function. More study is clearly necessary. Where body burdens of toxins are high in certain populations, an epidemiologic approach may be possible. For other compounds, an animal or in vitro model system may be more appropriate.

Epidemiological

Because of the sporadic nature of exposure to high levels of many xenobiotics, an epidemiologic approach may be useful for the study of certain toxins in breast milk in exposed populations. In the design of such studies it is important to keep in mind the time dependence of milk volume and composition.[50,238] While a complete review is not possible here, a few examples will suffice to illustrate the importance of the problem. *Figure 5* shows the time dependence of the concentration of zinc throughout lactation, as observed in a representative population of women.[239] In the inset, the marked time-dependent changes in milk volume, zinc, and protein concentrations are shown. *Figure 6* shows the change in free choline in human milk in the early postpartum period.[240] Lipophilic xenobiotics such as PCBs may accumulate during gestation and appear in much higher levels in colostrum and transition milk than in milk of later lactation.[241] Many processes are changing during the first 4 to 5 days postpartum and could be reflected in changes in the mechanisms by which xenobiotics are managed.

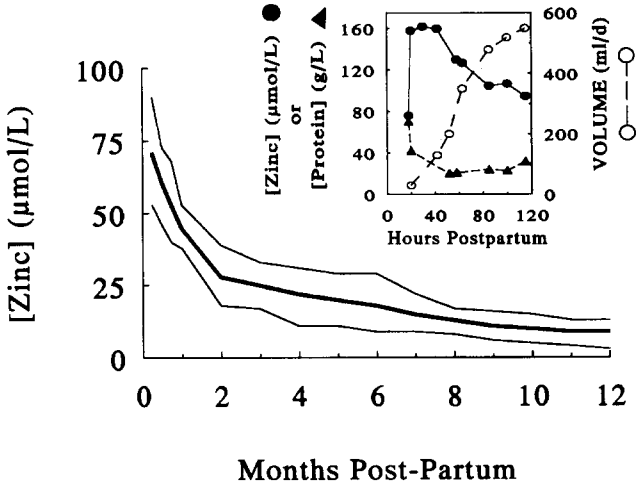

Figure 5 The time dependence of zinc concentration in human milk. Mean data from a representative sample of 13 lactating women studied throughout lactation (heavy line) are bracketed by light lines representing the standard deviation. In the inset, the changes in the zinc concentration (μmol/L) in a single subject during the first 5 days of lactation are shown along with the total protein concentration (g/L) and the milk volume secretion. Data replotted from reference 239.

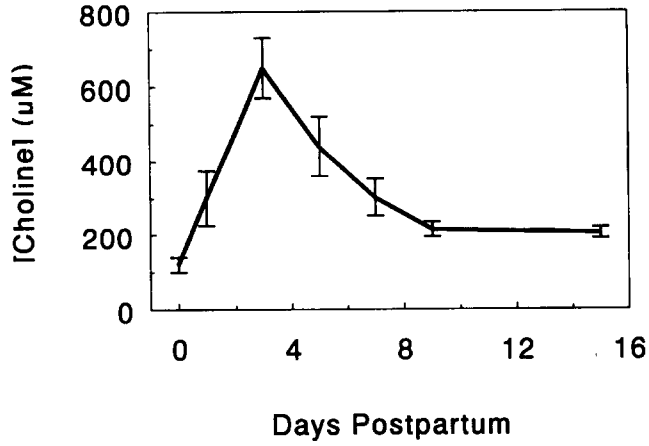

Figure 6 The time dependence of the choline concentration in human milk. Mean values from five or six well-nourished mothers at each time point. Data replotted from reference 240.

Lipid soluble xenobiotics. Because of the propensity for lipid-soluble toxins such as PCBs and DDT to accumulate in breast milk, the exposure of the breast at any given time to these compounds is amenable to measurement. For a full evaluation of their effects, a 24-hour breast milk sample from a fully breast-feeding woman at 1 to 3 months postpartum, collected by the Butte[242,243] procedure,* should be examined for total fat, protein, lactose, glucose, sodium, and chloride concentrations. The activities of enzymes involved in oxidative reactions such as glutathione peroxidase and superoxide dismutase, as well as glutathione-S-transferase should be measured and correlated with toxin concentration. Infant weight and length and developmental indices should be measured. If possible, a 3-to 4-day series of test weights to determine milk intake should be a part of the study. However, the growth rate of the infant, especially during the first 2 months postpartum, when normal growth is rapid, is a sensitive indicator of the adequacy of milk production. An attempt

*The Butte procedure probably gives the most reliable milk sample for determining the 24-hour secretion of any substance. It is most important for the measurement of fat-soluble substances because using this procedure the lipid content of the total milk sample probably consists of a representative proportion of fore and hind milk. In this procedure, the infant is fed at one breast each feeding for a 24-hour period while milk is extracted with a breast pump from the other breast. The pumped breast is alternated for each feed. The 24-hour milk sample is pooled for analysis.

should be made to study women with low, medium, and high body burdens of the toxins in question. These measurements should be made a part of every epidemiological study of effects of toxins on the breast-feeding infant to rule out effects due to altered breast-milk composition or volume.

In examining the fat content of human milk, the importance of obtaining a representative milk sample cannot be overemphasized. Although the use of automated fat assays in conjunction with assays for levels of toxins may be useful,[244] any conclusions about the secretion of either milk fat or lipid-soluble toxins will be invalid unless special attention is paid to methods of milk collection and storage prior to lipid analysis.[51]

Water-soluble xenobiotics. The same measurements of milk composition and infant growth should be made. It may not be possible to determine the toxicant burden from the milk, however, because these substances are usually excreted by other pathways.

It is becoming clear that breast milk volume is regulated by infant intake.[90] Toxins transmitted in the milk that alter infant appetite will appear to have an effect on milk volume. Such effects must be ruled out if toxic effects on milk volume are observed.

Experimental

Animal models. Animal models are essential for the prospective study of environmental toxins and for study of water-soluble xenobiotics. However, the application of results from such studies to humans should be made with caution. Toxins vary widely among species in their effects. This principle was made clear by studies of the dioxin receptor[27] whose apparent concentration shows considerable variation. Rats may not be a good model for humans for substances that decrease food intake because milk secretion and composition are very sensitive to food deprivation in this species.[245] In contrast, short-term starvation has little effect on the macronutrient content or volume of human milk.[238] Finally, the work of Schwetz and colleagues[81] on the effects of phthalates on milk secretion illustrates the importance of including pair-fed controls.

We found only one study in which appropriate techniques to measure milk volume and composition were applied.[225] Even in this study, however, the sodium concentration of the milk was not measured. This can be done if low oxytocin doses are used to obtain milk ejection. High doses will open the junctional complexes between the cells, increasing the milk sodium and chloride.

Tissue culture models. An ideal in vitro model for the study of milk secretion would consist of a homogeneous mammary epithelial cell line that forms an electrically tight monolayer when grown on a filter support, secretes milk proteins and lipids vectorially under control of lactogenic hormones, and can be transfected with foreign DNA.[246] Such a model would provide an in vitro system to evaluate both the passage of drugs and toxins into milk and their effects on milk secretion.

The first demonstration of milk protein secretion in cultured mammary epithelium occurred when Emerman and Pitelka[10] released primary cultures growing on collagen gels to float. Contraction of the gels and the corresponding shape change of the epithelial cells was accompanied by synthesis and secretion of casein. Other investigators were able to obtain secretory activity in nonreleased mammary cultures by plating cell clusters on extracellular matrix material derived from mammary epithelium[247] or a basal lamina-secreting tumor,[248,249] as well as by coculture with certain strains of 3T3 preadipocytes.[250,251] In all cases, the minimal hormonal requirements for milk protein synthesis were appropriate concentrations of prolactin, insulin, and cortisol.[251,252] All of these systems have substantial disadvantages. They are all primary cultures derived directly from partially differentiated mammary epithelium from pregnant mice or rats. Proliferative activity is minimal and the cells are not amenable to subculture on plastic. Further, the nature of the substratum, collagen gel, extracellular matrix, or other cells makes these models unsuitable for many desirable in vitro studies of milk secretion. For example, collagen gels impede access of nutrients, drugs, and hormones to the basal surface of the cells, precluding accurately timed measurement of isotope incorporation into protein, hormonal effects, or transcytosis of milk components and foreign substances such as toxins. Extracellular matrix materials such as Matrigel (Collaborative Research, Boston, MA, USA), as well as 3T3-L1 cells, tend to promote the formation of "mammospheres," hollow spherical structures that secrete into an inaccessible lumen.[248,249,251] Primary cells have the additional disadvantage that they are difficult to transfect with foreign genes and tend to vary from preparation to preparation.

A large number of mammary cell lines, derived from both human and rodent tissue, is available.[253] However, most are derived from mammary tumors that have lost their secretory function. Two cell lines have been shown to secrete milk proteins, the Comma 1D line[48,254] and the IM-2 line.[49] Both are heterogeneous and generally do not form a tight epithelium when grown on an appropriate filter support (Neville, unpublished; Parry, personal communication). A clonal derivative of Comma 1D cells, HC11 cells, make casein under the control of prolactin but have not been shown to secrete it.[96] Further, this cell line does not form a "tight" epithelium (Neville, unpublished). Another derivative line, CID-9, derived from Comma 1D cells by selective trypsinization, contains only 37% milk secreting cells by immunocytochemistry and was developed for growth on Matrigel.[252] Clearly, a cell line adapted for functional secretion on filters is needed. Such a line would be able to propagate rapidly in plastic tissue culture flasks, but would show all the characteristics of the lactating mammary epithelium when grown on flexible filter supports that allowed access of nutrients to the basolateral surface as well as the development of correct cell-cell and cell-matrix interactions. If such an ideal line does not become available, a number of "normal" mammary cell lines, each of which has at least one useful characteristic of the lactating gland might be of considerable utility.

Cells from human milk. Although many mammary cell lines derived from mammary tumors are available.[253,255] most of these have characteristics that differ significantly from the lactating mammary gland. A possible source of normal human mammary cells for in vitro study are the epithelial cells found in human milk. Thompson and Smith used such

cells to study the contribution of endogenous fatty acid synthesis to the human milk lipid.[256] Human milk cells may be particularly useful for the study of xenobiotic metabolism in the lactating glands of women who have body burdens of fat soluble toxins.

Summary and conclusions

Although we have presented evidence that there are several points at which xenobiotics might act to alter the composition and volume of milk secretion, there has been little systematic study of this area. The existing data make it clear that we do not yet know enough about the molecular and cellular mechanisms that regulate the rate of milk secretion to design a rational approach to elucidation of the effects of xenobiotics on lactation. For this reason, our recommendation for future studies targets areas of the regulation of milk secretion that remain poorly understood and model systems that may be useful in advancing our knowledge of these areas. Such model systems may eventually also serve as in vitro systems for the screening of xenobiotics for their effects on milk secretion. Because exposures of human populations to possibly toxic levels of xenobiotics are likely to antedate development of these models, it is also necessary to design an approach to epidemiologic studies that will allow the efficient collection of meaningful data in relevant populations.

A major area where further investigations are needed is characterization of the role played by transmembrane signalling and intracellular transduction of the signals thus generated in the regulation and maintenance of lactation. Protein kinase-mediated signal transduction has been well described in many other cell types and regulates multiple critical functions. However, the role of these signal transduction pathways has not been adequately investigated in mammary cells. Preliminary evidence, some of which has been outlined here, suggests that the phospholipase C-protein kinase C pathway is likely to be of some importance in understanding both normal mammary regulation and xenobiotic effects. A currently active research area of paramount importance is the molecular mechanism of prolactin signal transduction in the mammary gland. Signalling mechanisms such as those mediated by cAMP and eicosanoids have received extensive investigation in the past in the mammary gland but have provided no clear elucidation of the regulation of secretory mechanisms.

Further research is needed to characterize the molecular regulation of lipid secretion by mammary cells in a number of areas. We do not understand how lipid is transferred from the capillary into the mammary alveolar cell. The role of fatty acid-binding proteins in mediating intracellular lipid transport and partition of lipids and the mechanism by which the milk fat globule fuses with the plasma membrane to be secreted require elucidation. The enzymes involved in lipid metabolism and packaging are membrane associated and may be susceptible to perturbation by lipophilic xenobiotics as may the nontriglyceride components of the extensive membranes of the mammary cell and milk. Also of potential concern may be alterations of the enzymes secreted in milk that may play a role in infant digestion such as bile salt-stimulated lipase.

Studies should be encouraged to enhance understanding of the synthesis and secretion of milk proteins. The emphasis in this area should be in the regulation of the expression of genes for various proteins and their posttranslational modifications by processes such as phosphorylation and glycosylation.

Knowledge of the mechanisms of micronutrient uptake, metabolism, and secretion into milk is almost nonexistent. Transport mechanisms for trace metals have not been characterized; they may well be altered by heavy metals. Uptake of vitamins is better understood in tissues other than the mammary gland. Further characterization of the mechanisms of vitamin secretion into milk may allow us to better predict and understand potential xenobiotic effects.

The importance of nonnutrient components of milk such as cytokines, growth factors, free radical scavengers, and cells is not well defined but may potentially be significant to understanding the effects of xenobiotics as well as the possible protective effects of the mammary gland and milk against their actions.

Research on the regulation of mammary blood flow should improve our understanding of how vasoactive xenobiotics may influence milk composition and volume. Studies should clarify whether uptake into the mammary gland of certain substrates including xenobiotics can become flow limited under conditions of possible xenobiotic-induced vasoconstriction.

Certain areas of research methodology need rapid attention. Existing experimental model systems are inadequate for the types of research needed. New techniques should be applied to develop mammary cell lines with secretory functions. Creation of temperature-sensitive mutants that proliferate under certain conditions in culture and differentiate and express mature function under others may prove fruitful. The in situ perfused goat mammary gland may offer a model in which blood flow can be controlled, venous effluent collected, and milk secretions recovered. This technique would provide one approach for studies of disposition of xenobiotics in mammary tissue and their effects on transport of nutrients into milk. A primate model such as the Rhesus monkey is also likely to be useful for studying xenobiotics and mammary function. However, normal milk composition and volume in this animal must be characterized before it can become a practical model.

Finally, it appears essential that a battery of tests for assessment of milk composition and volume be developed for use in future large epidemiologic studies in human populations exposed to xenobiotics. These tests should be carefully chosen to be performed on small samples of milk collected under field conditions and to provide data on milk components most likely to be affected by these agents. They should be accompanied by measures of overall lactational performance and infant growth and development.

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