sent, it appears that many individuals would benefit from a lower dietary fat intake, but care should be taken to achieve this by adjusting the relative proportions of low and high fat foods so as to maintain a well-balanced diet.

ACKNOWLEDGMENT

The National Cancer Institute of Canada supported this work.

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[Received June 20, 1984]

The Biochemistry of Selenoproteins

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ABSTRACT

There currently are 7 known bacterial selenoenzymes. All but thiolase contain selenocysteine (Se-Cys), presumably at the active site, and all but thiolase catalyze oxidation-reduction reactions. Selenide appears to be a central intermediate in selenium (Se) metabolism in animals, and it may be the precursor used for formation of the Se-Cys moiety in glutathione peroxidase (GSH-Px). The incorporation of Se into GSH-Px appears to occur via a post-translational mechanism, but the nature and extent of Se-Cys formation in higher animals has not been established. GSH-Px deficiency remains a logical explanation for a number of Se-deficiency signs, but other known selenoproteins and other functions may match up with defects apparently not prevented by GSH-Px.

INTRODUCTION

Many of the topics and the compounds discussed in this symposium in association with carcinogenesis reappear when considering the biochemistry of selenium. The discovery in the early 1970's that Se was an integral part of the enzyme GSH-Px (1) provided new support for the antioxidant theory of vitamin E function and, along with the discovery of superoxide dismutase (2), suggested that oxidative and/or free radical attack on cellular components may be responsible for the toxicity of a number of drugs. Some of these prooxidant or procancerous species are even more toxic when administered to Se-deficient animals, indicating that the biochemical functions of Se may be related to the processes involved in carcinogenesis.

Some of the anti-cancer activity of Se undoubtedly is related to the cytotoxicity of inorganic Se. The pharmacological effects of Se excess will not be the subject of this article. Instead, this article will review the biochemistry of Se and the effects of Se deficiency in animals as it relates to the biochemical functions of Se. The main sections of this review will discuss (1) bacterial selenoenzymes, (2) Se-Cys and GSH-Px, (3) Se metabolism, (4) GSH-Px function, (5) some of the selenoproteins and (6) functions of Se in animals apparently not related to GSH-Px. Recent reviews in related areas of Se biochemistry are available (3-6).

The story concerning Se essentiality in the US began in the late 1940's, when Klaus Schwarz came to the US from Germany. In Germany he had been studying dietary liver necrosis, a disease that could be prevented by either dietary vitamin E or the sulfur amino acids (7). After arriving in the US, he found that rats fed diets based on American brewer's yeast did not develop this disease. Rats fed torula yeast-based diets, however, developed liver necrosis in 3 to 4 weeks. Schwarz then isolated the organic factor present in American brewer's yeast that would protect against dietary liver necrosis, calling it factor-3 (8). In 1957 he identified Se as the crucial component in factor-3 (9). Factor-3 never has been fully characterized, but Schwarz and coworkers (10) have reported that several dialkyl diselenides have activities equivalent to factor-3 and were more active than

inorganic selenite.

Mills (11) discovered an enzyme in red blood cells (RBC) that would protect the cell against peroxidative damage. GSH-Px destroys hydrogen peroxidase and in the process oxidizes 2 molecules of glutathione (GSH) to GSSG. A protective sequence in the RBC and other cells uses glucose, glucose-6-phosphate dehydrogenase and glutathione reductase to maintain intracellular GSH concentrations (12). Adequate intracellular GSH is necessary, because the V_{max} of GSH-Px is first-order with respect to GSH (13). Little and O'Brien (14) showed that GSH-Px, unlike catalase, also would destroy hydroperoxides; this ability makes GSH-Px a unique peroxidase within animal cells. Scott et al. (15) showed that Se was essential for the growth of the chick but, as with the rat, only when the diet was deficient in vitamin E. The demonstration that Se was essential in the face of adequate dietary vitamin E came in 1969 when Thompson and Scott (16) reported that chicks, fed a diet deficient in Se but supplemented with vitamin E, developed a degeneration of the pancreas. Se alone was shown to be essential for that rat in 1969 (17).

Bacterial Selenoproteins

Pinsent (18) reported that selenite along with molybdate was necessary for the optimum development of formate dehydrogenase activity when *E. coli* was grown aerobically in nitrate-free media even though Se and molybdenum (Mo) had no effect on growth. In hindsight, this was the first suggestion of a Se-dependent enzyme. At latest count, 7 bacterial proteins have been identified as selenoenzymes.

Formate Dehydrogenase. Formate dehydrogenase of *E. coli* was shown in 1972 (19) to be a Se-containing enzyme. This enzyme catalyzes the oxidation of formate to CO_2 with the concomitant reduction of nitrate to nitrite. With a molecular weight of 600,000 daltons, formate dehydrogenase has four 110,000 dalton subunits each containing one atom of Se. The Se has been shown to be present as Se-Cys (20). In addition to 4 g-atom of Se, each mole of the enzyme also contains 4 g-atom of Mo, 56 g-atom of nonheme iron (Fe), 53 g-atom of acid-labile sulfur (S), and 4 moles of cytochrome b (21). A number of slightly different formate dehydrogenases have been identified from different bacteria, including some that do not require Se (5).

Glycine Reductase. The second bacterial enzyme identified as a selenoenzyme was glycine reductase from Clostridia (22). Glycine reductase has 3 types of subunits including a 12,000 dalton Se-containing subunit called protein A. The other 2 subunits are membrane-bound; Fe co-purifies with one of these subunits (23). Glycine reductase, as well as formate dehydrogenase, is inhibited by the thiol specific reagents iodoacetate and iodoacetamide (22,21). Cone et al. (24) used these reagents to alkylate Se, and demonstrated that the Se in protein A was present as Se-Cys. The enzyme catalyzes the reductive deamination of glycine to acetate and ammonia, and also involves a substrate-level phosphorylation of ADP (25). A variety of donor substrates can provide reducing equivalents for the reaction. The enzyme presumably goes through a selenophosphate intermediate (5), and thus the nuclear spin of ^{77}Se-labeled glycine reductase might help researchers to identify the intermediates in phosphorylation reactions.

After a gap of several years, a number of other bacterial enzymes were identified as Se-containing enzymes. In none of these cases, however, did bacteria grown under usual conditions produce sizable quantities of the selenoenzymes, nor did Se deficiency impair growth of these organisms in typical media. It is only when one critically selects the media for the bacteria that there is a strict requirement for Se. Most of the bacteria are grown anerobically so an electron sink, such as nitrate in the case of formate dehydrogenase, is necessary for growth.

Nicotinic Acid Hydroxylase. The third bacterial enzyme shown to be a selenoenzyme was nicotinic acid hydroxylase from *Clostridium barkeri* (26). The enzyme adds water across a double bond of nicotinic acid and then the ring is dehydrated to form the 6-oxo derivative. This reaction is the first step in the metabolism of nicotinic acid to ammonia, pyruvate, acetate and CO_2. The enzyme contains nonheme Fe, S and a flavin moiety in addition to Se.

Xanthine Dehydrogenase. Xanthine dehydrogenase, an enzyme with a molecular weight of 300,000 daltons, was the fourth bacterial selenoenzyme to be discovered (27). This Clostridial enzyme oxidizes xanthine, purines or aldehydes, with concomitant reduction of dyes, ferricyanide or oxygen. The enzyme also contains Mo, Fe, S and flavin.

Thiolase. The fifth bacterial enzyme shown to be a selenoenzyme was thiolase (28). The enzyme is 160,000 daltons in size with a 40,000 dalton Se-containing subunit. This is the enzyme involved in the cleavage of acetoacetyl CoA to 2 acetyl CoA molecules during β-oxidation of fatty acids. In *Clostridium kluyveri* (a fatty acid-producing bacteria), the enzyme apparently acts in a synthetic role to catalyze the condensation of acetyl CoA to form acetoacetyl CoA. The typical thiolase isolated from bacteria or from pigs contains a cysteine at the active site, and the amino acid sequence at the active site of these enzymes was shown to be Lys-Val-Cys-Ala-Ser (29). In the selenothiolase, however, the Se is present as selenomethionine (Se-Met) (30). Depending on the S/Se ratio in the media they found either that only a little Se-dependent thiolase was present or that 50-60% of the thiolase activity was due to the Se-form. In no case did Hartmanis and Stadtman find preparations with only Se-dependent thiolase activity.

The presence of Se-Met rather than Se-Cys at the active site of the Se-dependent thiolase is difficult to rationalize because of the difference in mRNA codons for methionine (Met) and cysteine (Cys), and thus presumably Se-Met and Se-Cys. A point mutation could not change a Cys to a Met but a frame shift toward the 5′-end of the mRNA would shift Val-Cys codons (GUA-UGU) to a Met (AUG) codon. It is interesting that this is the only selenoenzyme discovered to date not involved in an oxidation-reduction reaction, and the only selenoenzyme shown to contain Se-Met rather than Se-Cys; this exception strongly reinforces the idea that a Se-Cys moiety in an enzyme serves as an electron-carrying intermediate during redox reactions.

Se-Dependent Hydrogenase. In 1982 Yamazaki (31) discovered a Se-dependent hydrogenase from *Methanococcus vannielli.* This enzyme has a molecular weight of 340,000 daltons, and the Se is present in a 42,000 dalton subunit. There are a reported 3.8 g-atom Se per mole of enzyme, which apparently is one per subunit. Most interestingly, Yamazaki (32) recently has reported that 2 g-atom nickel (Ni) per mole of enzyme co-purify with the hydrogenase activity. The enzyme catalyzes the hydrogenation using H_2 gas of a variety of substrates such as methyl viologen. 8-hydroxy-5-deazaflavin is its natural acceptor substrate. The Se has been reported to be present as Se-Cys (31).

W-dependent Formate Dehydrogenase. The seventh enzyme on the list is a tungsten-dependent formate dehydrogenase from *Clostridium thermoaceticum* (33). The molecular weight of this formate dehydrogenase appears to be 340,000 daltons and thus about half of the usual 600,000 daltons, but it still is unclear whether tungsten (W) is simply substituting for the 2g-atom Mo per mole that would be present in a formate dehydrogenase of this size. Two Se-Cys are present per 340,000 dalton enzyme. Yamamoto et al. (34) recently have reported that this protein has a strict re-

quirement for W rather than Mo for full activity, and that it contains (on a g-atom basis) 2 W, 2 Se, 36 Fe and about 50 S per mole.

SELENOCYSTEINE/GLUTATHIONE PEROXIDE

Both Se-Cys and selenomethionine (Se-Met) are found in the tissues of plants grown on Se-containing soils. Those plants that tolerate high Se levels in the soil and that accumulate Se, such as several species of Astragalus (the milk vetch genus), sequester the Se in rare amino acids such as methyl selenocysteine and selenocystathionine (35). Monogastric animals, however, do not accumulate Se-Met in their tissues when fed diets supplemented with inorganic Se (36,37), and Olson and Palmer (38) found only small but nonetheless detectable quantities of Se-Cys (as 2,7 diamino-4-thio-5-selenaoctanedioic acid) in rats fed selenite. These trace amounts of Se-Cys were thought at first to be unimportant, but with the discovery that the bacterial selenoenzymes contained Se-Cys, the presence of Se-Cys in animal tissues has assumed new importance.

Se-Cys in GSH-Px

The form of Se in reduced GSH-Px was shown to be Se-Cys in 1978 by Forstrom et al. (39) and Wendel et al. (40). ^{75}Se-labeled GSH-Px was purified, reduced with GSH, alkylated with iodoacetate, hydrolyzed in 6 N HCl, and then subjected to automated amino acid analysis. The ^{75}Se was found to co-elute with authentic carboxymethylselenocysteine (CM-Se-Cys) when chromatographed on the long column of a Beckman 121 amino acid analyzer. CM-Se-Cys eluted approximately 7 min after CM-Cys and between aspartate and threonine. Edman degradation of peptides obtained by tryptic digestion of GSH-Px indicated that the Se-Cys was incorporated into the peptide backbone of the enzyme (41). Ladenstein et al. (42) crystallized the selenoenzyme and used X-ray crystallography to establish the structure of GSH-Px at 2.8 A resolution. The Se is present 35 residues from the N-terminal end of the bovine erythrocyte enzyme and at residue 41 of the rat liver enzyme (43). A refined structure at 2.0 A resolution has indicated that the dimer is the functional unit and that there are 2 active (GSH-binding) sites per tetramer (44). The mechanism used to form and incorporate Se into GSH-Px thus has become one of the important areas of Se research.

Se Incorporation

There are 2 postulated mechanisms—translational and post-translational—for inserting Se into GSH-Px. If Se-Cys is incorporated during translation by direct insertion into the peptide backbone of the enzyme facilitated by a Se-Cys-specific tRNA, then there should be no interference with this process by the other Se metabolites. If the insertion of Se is post-translational—after the peptide backbone has been synthesized beyond the 35th/ 41st residue—then other metabolites of Se would interfere with the incorporation of ^{75}Se from ^{75}Se-Cys into GSH-Px.

We set out to directly test these 2 hypotheses using the isolated, erythrocyte-free perfused liver (45). An erythrocyte-free perfusate was used because red blood cells rapidly take up selenite, metabolize it to selenide, and release $Se^{=}$ back into the plasma (46). Se-adequate livers were perfused for 4 hr with a perfusate that consisted of glucose, individual amino acids mirroring the composition of rat fibrinogen, insulin, cortisol, antibiotics and heparin in Krebs-Ringer bicarbonate buffer containing 3% bovine serum albumin. Liver viability was assessed by bile duct cannulation and measuring the rate of bile production.

Each liver was perfused with 6.8 ng Se/g liver. Under these conditions, approximately 30% of the cytosolic ^{75}Se was incorporated into GSH-Px within 4 hr. Sephadex G-150 chromatography of the liver supernatant yielded ^{75}Se and GSH-Px activity peaks that co-eluted at a molecular weight corresponding to 90,000 daltons. The other ^{75}Se peaks (void volume, 40,000-dalton and low molecular-weight peak) apparently were due to proteins with affinity for ^{75}Se, as these peaks were reduced substantially when the supernatant was dialized prior to chromatography. When the pooled GSH-Px-containing fractions were purified further with carboxymethy cellulose chromatography, the GSH-Px activity and ^{75}Se co-eluted, well ahead of the major protein peak, and with a specific activity (^{75}Se/EU) across the peak the same as across the Sephadex G-150 peak. This indicated that Sephadex G-150 chromatography alone was sufficient to separate ^{75}Se-labeled GSH-Px from other ^{75}Se-containing or ^{75}Se-binding proteins (45).

If an individual lobe of the perfused liver is ligated and removed at various times during the perfusion, the time-course of ^{75}Se incorporation into GSH-Px can be obtained. A linear rate of ^{75}Se incorporation was observed in liver from both Se-adequate and Se-deficient rats. The rate in the Se-deficient liver was 1/3 that observed in the Se-adequate liver, even though there was no detectable GSH-Px activity in the chromatograms of Se-deficient liver supernatant. In intact rats, ^{75}Se incorporation was delayed 2 to 3 hr in Se-deficient rats, whereas detectable ^{75}Se incorporation was observed within 30 min of ^{75}Se administration in Se-adequate rats (47). This delay is consistent with the time required for mRNA induction. Cycloheximide pretreatment of both Se-adequate and Se-deficient rats completely blocked ^{75}Se incorporation into GSH-Px, indicating that detectable quantities of a pre-GSH-Px protein, into which Se can be inserted, do not accumulate even in Se-deficient rat liver (47).

An isotope dilution technique was used next to differentiate between the ability of various Se compounds to provide Se for GSH-Px synthesis. A 100-fold excess of unlabeled selenite or selenide very effectively eliminated ^{75}Se incorporation from [^{75}Se] selenite, whereas unlabeled selenocystine (Se-Cys_2) was relatively ineffective (45). This experiment demonstrated that selenite and selenide were more readily metabolized than was Se-Cys_2 to the form used for incorporation into GSH-Px. When conditions were reversed and [^{75}Se] Se-Cys_2 was used, a 9-fold excess of unlabeled selenite or selenide was far more effective than unlabeled Se-Cys_2 in decreasing the incorporation of ^{75}Se into GSH-Px. These experiments thus demonstrate that inorganic forms of Se, such as selenite or selenide, are more readily metabolized than is Se-Cys_2 to the form of Se used for insertion into GSH-Px. The results further suggest that a post-translational modification of another amino acid residue is the mechanism for formation of the Se-Cys residue present in GSH-Px. For instance, nucleophilic attack of selenide on the β-carbon of serine, cysteine or dehydroalanine in the peptide backbone of the enzyme would result in formation of a Se-Cys residue in GSH-Px (46). The insertion of Se could be facilitated by a specific GSH-Px Se-Cys synthetase, or at the substrate level by a moiety such as a selenopersulfide of GSH (GS-SeH).

Evidence Supporting Translational Se Incorporation

In 1979 Hawkes et al. (48) reported preliminary results suggesting that Se-Cys was incorporated directly into GSH-Px using a Se-Cys-specific tRNA. Hawkes et al. (49) described the isolation of ^{75}Se-Cys-acylated tRNA that was prepared from ^{75}Se-Cys in a cell-free system or from [^{75}Se] selenite in a rat liver slice system. Only 15% of the

^{75}Se-Cys from the acylated tRNA prepared from selenite was shown to be authentic Se-Cys, and the authors stated that as much as 61% of the ^{75}Se in acylated tRNA prepared from liver slices incubated with ^{75}Se-Cys was in a form other than Se-Cys. ^{75}Se incorporation into GSH-Px from selenite was blocked by puromycin or cycloheximide in the liver slice system, and dilution of [^{75}Se] selenite by a 6-fold excess of Se as $Se\text{-}Cys_2$ reduced ^{75}Se incorporation by only 50%. These results obtained with the liver slice system are thus in agreement with those of Sunde and Hoekstra (45, 47). Using the cell-free system, however, ^{75}Se from Se-Cys-tRNA was more efficient (on a percentage basis) than were Se-Cys or selenite in providing Se for GSH-Px synthesis. This result suggests that Se-Cys is inserted into GSH-Px in a tRNA-mediated process. Recent reports from the same laboratory, however, have indicated that 82% of the ^{75}Se incorporation from ^{75}Se-Cys was not inhibited by cycloheximide in this rabbit reticulocyte system (50), and that Se-Cys and Cys in this system were in direct competition for incorporation into TCA-insoluble material (51).

Se-Cys apparently is a rare amino acid in both bacteria and in higher animals. Most rare amino acids are formed post-translationally (52), and all 64 codons have been unambiguously assigned to the common amino acids or to initiation/termination codons. The recent report of Wilhelmsen et al. (51) further shows that Se-Cys can be incorporated into general proteins (which normally do not contain Se-Cys) by the same mechanism that inserts Cys. Thus the post-translational hypothesis remains the more logical of the 2 postulated mechanisms explaining Se insertion into GSH-Px.

Post-translational Processing of GSH-Px

The distribution of Se and GSH-Px activity in rat liver indicated that a majority (60%) of liver GSH-Px was found in the cytosol and that 28% was localized in the mitochondria (53,54). Using antibodies prepared against rat liver GSH-Px, Yoshimura et al. (55) showed that GSH-Px protein was localized predominantly in the cytoplasm of the parenchymal cells—the endothelial cells and the Kuppfer cells were devoid of immunoreactive protein. Yoshida et al. (56) used immunochemical analysis to demonstrate the virtual absence of immunochemically recognizable GSH-Px in liver from Se-deficient rats. When Se-deficient rats were administered 50 μg Se IV, 5 hr were required before GSH-Px was detected immunochemically. These experiments, confirming the results of Sunde and Hoekstra (47), demonstrated that a pre-GSH-Px protein does not accumulate in the liver of Se-deficient rats. Recently Voigt and Autor (57) reported preliminary results indicating that the initial gene product from total rat liver RNA, translated in a cell-free rabbit reticulocyte system, was an immunoreactive protein of 28,000 daltons, as compared to 23,000 daltons for mature GSH-Px. These results clearly suggest that more than just Se-insertion occurs during maturation of GSH-Px.

SELENIUM METABOLISM

Se metabolism in higher animals parallels sulfur (S) metabolism as long as the Se is bound to carbon in an animo acid form (Fig. 1). Thus Se-Met is converted to Se-Cys which, in turn, is then degraded to release inorganic Se. As in S metabolism, the conversion of inorganic Se into an amino acid form does not occur readily in monogastric animals (38). In this diagram of Se metabolism, we have proposed that inorganic Se in the -2 valence state, as HSe^- or a similar compound, is the form inserted into a pre-GSH-Px protein to form the active enzyme (58).

Se Chemistry

Se compounds have higher redox potentials than the corresponding S compounds. Thus Se metabolism in animals

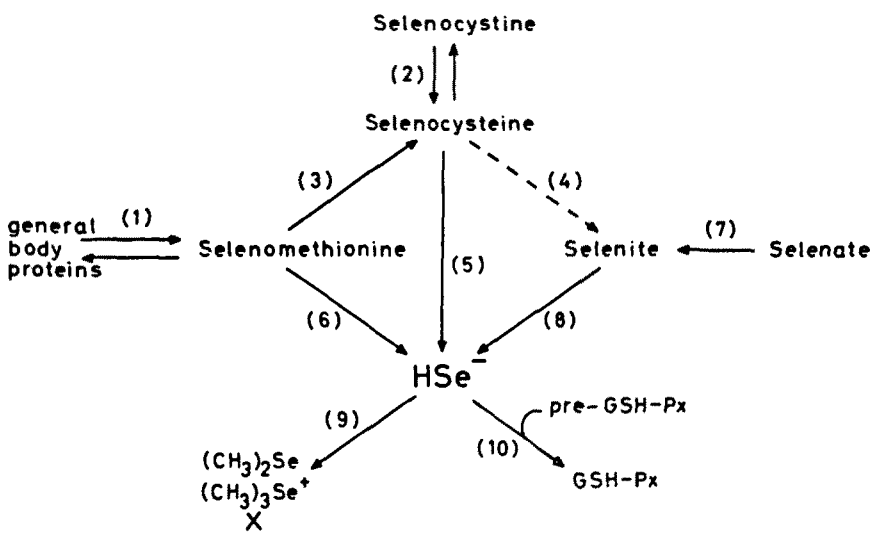

FIG. 1. Proposed Metabolism of Selenium. Reaction 1, incorporation of selenomethionine into general body proteins in place of methionine; Reaction 2, reduction of selenocystine to selenocysteine by GSH or by thioltransferase; Reaction 3, transsulfuration pathway analogous to sulfur metabolism; Reaction 4, selenite release from selenocysteine, analogous to sulfur metabolism, probably does not occur because of the high reduction potentials of selenium compounds; Reaction 5, selenocysteine lyase; Reaction 6, transamination pathway analogous to bacterial methionine-γ-lyase and to the methionine transamination pathway; Reaction 7, selenate reduction to selenite in animals may occur via APSe or PAPSe intermediates analogous to sulfur metabolism; Reaction 8, GSH/glutathione reductase catalyzed reduction of selenite to selenide; Reaction 9, methylation of selenide to produce the excretory compounds dimethyl selenide, trimethyl selenonium ion or unknown form(s) (X) which correspond to toxic, adequate or suboptimal levels of selenium intake; Reaction 10, hypothetical GSH-Px selenocysteine synthetase.

tends toward reduction, whereas S metabolism generally is oxidative (3). Selenate thus is reduced to selenite and then selenite is reduced to selenide in a series of GSH-dependent steps (59). The redox potentials further suggest that selenide rather than selenite would be the expected inorganic form released during Se-Cys degradation (45). Se is much more acidic than S; the pK of Se-Cys is 5.3, whereas Cys has a pK of 8.3 (60). This means that at physiological pH Se-Cys is mostly deprotonated whereas Cys is protonated. Enzymes that act on these substrates would be expected to act differentially on Se-Cys and Cys because of the charge on the side chain of Se-Cys. Se in Se-Met, in contrast, is covalently bound between 2 carbon atoms. The covalent radii of Se and S are approximately the same (1.17 versus 1.03 A). Thus, Se-Met would be expected to substitute for Met as a substrate for those enzymes that metabolize Met. Met and Se-Met are absorbed from the intestine by the same system (61,62), Se-Met readily acylates Met-tRNA with a K_m much the same as for Met (63), and is incorporated into proteins in place of Met in both *E. coli* and rat liver (64,65). Huber and Criddle (66) grew bacteria on selenate supplemented media, and replaced 70-75% of the Met residues in β-galactosidase with Se-Met without affecting the V_{max}. The only difference was that the Se-substituted enzyme was slightly more susceptible to heat denaturation.

Se-Met Biopotency

Met, unlike the other essential amino acids, still is catabolized to a relatively large extent even when it is the limiting amino acid in the diet (67), and as the level of Met in the diet increases, the percentage as well as total amount of Met catabolism increases. Because Se-Met apparently is an excellent analog for Met, Se-Met catabolism might be expected to be affected similarly.

To test whether the level of dietary Met would affect the proportion of Se from Se-Met that was available for GSH-Px synthesis, Se-deficient rats were repleted with various levels of dietary Se for 7 days while being fed different levels of dietary Met. Biopotency was quantitated by measuring the increase in tissue GSH-Px activity elicited by the 7 days of Se-Met or selenite repletion (68). We found that selenite biopotency was unaffected by the level of dietary Met, but that Se-Met biopotency for GSH-Px synthesis was reduced dramatically when the diet was suboptimal in Met. These results, indicating Se-Met incorporation into protein when the diet was suboptimal in Met, could explain the observations of Cary et al. (69), that muscle Se was raised in rats fed Se-Met as compared to rats fed selenite. These experiments show that Se-Met has 2 fates in the animal; it can be incorporated into general body proteins in place of Met or it can be catabolized to release Se which will then be available for GSH-Px synthesis.

It is important to note that what we are looking at is the biopotency of these Se compounds—that is, the ability of these forms of Se to raise tissue GSH-Px activity in a deficient rat. The level that gives maximal response in repleting a deficient rat (0.5 ppm Se as selenite) (68) is different from the dietary level necessary to maintain tissue GSH-Px levels (0.1 ppm Se as selenite) (70). Secondly, the plateau in liver GSH-Px activity above 0.5 ppm Se means that Se is no longer the rate-limiting factor for the synthesis of GSH-Px.

Nature of Tissue Se

During the conference there was some discussion about the proportion of Se that could be accounted for by GSH-Px. We conducted some experiments with radioactively labeled sheep that examined the nature of the Se in sheep liver and blood (71). Liver and erythrocyte GSH-Px was purified, and the specific radioactivity (dpm/EU) or the total Se specific activity (μg Se/Eu) of the purified GSH-Px was used to estimate the percentage of the liver or erythrocyte Se that could be accounted for by GSH-Px. For ovine liver only 10 to 15% of the total liver Se could be accounted for by GSH-Px, and 25% of the liver supernatant Se was present as GSH-Px. In contrast, 75% of the whole blood Se and 100% of the erythrocyte Se were accounted for by GSH-Px. These sheep were injected and dietarily supplemented with Se as selenite to induce maximal levels of GSH-Px. Animals with a lower Se status might be expected to have a greater proportion of the Se in liver present as GSH-Px, although the absolute amount of Se might be less. The form of the non-GSH-Px Se may have been Se-Met (formed by rumen bacteria from endogenous Se released into the rumen) or the non-GSH-Px Se may have been present in some of the other selenoproteins (discussed below). In a separate experiment, when rats were labeled with ^{75}Se, and the liver GSH-Px purified through several ion exchange steps, the constant ratio of ^{75}Se/EU across the GSH-Px peak was used to estimate Se distribution in rat liver. We found that 60% of rat liver Se was accounted for by GSH-Px and 70% of the liver supernatant Se was accounted for by GSH-Px. Levander et al. (54) earlier reported similar results for rat liver mitochondrial GSH-Px. It seems logical to assume that those tissues with high levels of GSH-Px, such as rat liver or sheep erythrocytes, might have a greater proportion of the Se accounted for by GSH-Px than tissues with lower GSH-Px activities, such as sheep liver.

Se-Cys Biopotency

The same biopotency technique was used to compare the metabolism of Se-Met, $Se\text{-}Cys_2$ and selenite (58). Yasumoto et al. (72) reported that vitamin B_6 deficiency would decrease the biopotency of Se-Met, but that selenite biopotency was unaffected. At first glance this result would make sense if the activities of the B_6-dependent enzymes involved in Met/Se-Met catabolism were so depressed that Se-Met catabolism was restricted. The transaminases and cystathionase involved in S/Se metabolism would be such B_6-dependent enzymes. In our experiments, however, we found that there was no difference in the biopotency of selenite, $Se\text{-}Cys_2$ or Se-Met at either 0.2 or 1.0 ppm Se, when control rats were restricted to the food intake of B_6-deficient rats and the diet was supplemented with Met. In our experiments, the B_6 deficiency was sufficient to impair growth by 37% and to depress cystathionase activity by more than 50%, whereas in the experiment of Yasumoto et al. (72) no growth depression was reported. In addition, Se was supplemented in those experiments at a level (2 ppm) where Se clearly was no longer rate limiting for GSH-Px synthesis in our previous experiments (68). The reason for the impaired Se-Met biopotency in the experiments of Yasumoto et al. (72) remains unclear.

Se-Met and Se-Cys Metabolism

In Figure 1 Se-Met has 2 fates, either incorporation into general body proteins in place of Met (reaction 1), or degradation. Se-Met degradation can follow the transsulfuration pathway (reaction 3) leading to Se-Cys (73). Alternatively, Se-Met may be transaminated and decarboxylated, with final release of methane selenol (reaction 6) in a manner similar to the Met transamination pathway described by Steele and Benevenga (74). In bacteria a methionine-γ-lyase activity has been shown to use Se-Met as a substrate and to release methane selenol (75). Methane selenol catabolism would yield methane and selenide. Se-Cys might be degraded with release of selenite (reaction 4) in a manner similar to the degradation of Cys, but the high reduction potential of Se compounds suggests that direct release of

selenide would occur instead. Soda and coworkers (76) found such an enzymatic activity in rat liver, Se-Cys lyase, that specifically catabolizes Se-Cys to alanine and selenide (reaction 5). As suggested by the lower pK's for selenols as compared to thiols, Cys was found to be a poor substrate for this enzyme. The normal cellular concentrations of free Se-Cys are most likely well below the K_m of Se-Cys lyase, so low concentrations of Se-Cys can be present inside cells (76). By analogy with Cys the adequate intracellular levels of GSH should readily reduce Se-Cys_2 to Se-Cys (reaction 2) either nonenzymatically (77) or catalyzed by the low molecular weight thioltransferases (78).

Inorganic Se Metabolism

Selenate is reduced to selenite (reaction 7) presumably via APSe or PAPSe intermediates similar to the APS and PAPS intermediate involved in sulfate reduction (79). The GSH/glutathione reductase/NADPH pathway involved in the reduction of selenite to selenide (reaction 8) has been well characterized by Hsieh and Ganther (59), as has the excretory methylation of selenide (reaction 9). When moderate levels of Se are ingested the selenide is methylated to form $(CH_3)_3Se^+$ (trimethyl selenonium ion) which is excreted in the urine (80). With acute Se toxicity, $(CH_3)_2Se$ (dimethyl selenide) is formed; this volatile compound is expired via the lung (81). Trimethyl selenonium ion may not be the major urinary form when the diet is deficient or marginally deficient in Se (82). The reductive pathway of Hsieh and Ganther (59) is important not only in terms of Se excretion, but also because it is the pathway used to metabolize selenite rapidly to selenide in erythrocytes and other tissues (46). This pathway also may be critically important in depleting lens GSH and thus causing selenite-induced cataract (83).

Selenide

Hydrogen selenide thus appears to be a central compound in Se metabolism (58). Selenide may be the chemical form that binds to a number of proteins, perhaps proteins with exposed thiols, to yield the various non-GSH-Px ^{75}Se peaks that are present in gel filtration profiles of liver supernatant. As postulated by Diplock (84), selenide also could be the chemical form that binds to microsomes. While selenide is highly toxic (85), the intracellular toxicity of this species may be lessened by its non-specific binding to proteins. Finally, HSe^- or a close metabolite may be the form inserted post-translationally into a pre-GSH-Px polypeptide to form the active GSH-Px subunits (reaction 10) (45). The small amount of Se-Cys usually detected in animal tissues (38) may be only from selenoenzyme synthesis.

GSH-Px FUNCTION

The discovery that GSH-Px was a Se-containing enzyme (1) and that GSH-Px destroyed hydroperoxides (14) as well as H_2O_2 provided strong evidence for the antioxidant theory of vitamin E function. The discovery of superoxide dismutase (2) also helped to demonstrate that superoxide and other activated oxygen species exist in vivo and that peroxidant damage could affect cells. It is unfortunate, perhaps, that in almost every case of Se deficiency, one can logically explain the signs of selenium deficiency in terms of an absence of GSH-Px which leads to peroxidative damage (86). The protective mechanisms of cells are compartmentalized within various subcellular components (6). A deficiency disease that is prevented by either vitamin E or Se, such as dietary liver necrosis in the rat, suggests that the origin of the toxic molecular species is in the cytosol where GSH-Px is localized, but that the target may be in the lipid (and vitamin E) soluble membrane. The metabolism of various drugs, for instance, leads to the production of superoxide either in the membranous or the aqueous compartments of the cell (87). Superoxide in turn could lead to the formation of lipid hydroperoxides, singlet oxygen or hydroxy radicals, all species capable of causing cell damage. In the membrane, vitamin E could quench lipid hydroperoxides or singlet oxygen. Superoxide and H_2O_2, in a modified Haber-Weiss-type reaction catalyzed by iron, can react to form hydroxy radical (88). Superoxide dismutase and GSH-Px thus can act in concert to minimize the formation of these aqueous activated oxygen species.

Compartmentalization

The chick provides an excellent illustration of the compartmentalization of protection in animal cells. A chick fed a diet deficient in the sulfur amino acids, very low in vitamin E and perhaps with an excess of unsaturated fatty acids, will develop muscular dystrophy (89). Met supplementation together with a small level of dietary ethoxyquin will prevent the development of this nutritional disease. Se supplementation will sometimes delay but will never prevent muscular dystrophy (90). With ethoxyquin and Met administration, chicks still develop another disease—exudative diathesis. Exudative diathesis is an increased permeability of the capillaries that results in the accumulation of a bluish-green fluid under the ventral skin of the chick. Either vitamin E or Se can prevent the development of this disease (91,15). If deficient chicks are supplemented with Se, they develop encephalomalacia, a degenerative brain condition (92). The brain is a tissue with a high lipid content, and it seems that the water soluble GSH-Px is unable to protect the lipids from peroxidation. If the chicks are supplemented with vitamin E but not Se, they will develop pancreatic atrophy (previously called fibrosis or degeneration) (93). This condition is not prevented by normal levels of vitamin E, but recent reports (94) indicate that pharmacological levels (550 ppm) of Vitamin E (as well as BHT, DPPD or ascorbate) will prevent the disease. Interestingly, Se-Met is reported to be 4 times as effective as selenite for the prevention of pancreatic atrophy (95). This may relate to the high anabolic rate of the pancreas, or to a non-GSH-Px role for Se. With each of the diseases prevented by Se, the signs of the disease can be explained logically by an absence of GSH-Px. That does not rule out the possibility of other important biological functions for Se.

GSH-S-Transferase

GSH-S-transferase may also have a protective role in the tissues of some species because of its hydroperoxidase activity (137,96,97). Burk et al. (98) perfused livers from Se-deficient rats with organic peroxides and demonstrated that GSH-S-transferase would destroy the peroxides and release GSSG into the perfusate. This work strongly suggests that GSH-S-transferase may have a biological role in tissues with low GSH-Px activity. These 2 enzymes, however, are not equivalent because GSH-Px also destroys H_2O_2 and GSH-S-transferase also conjugates compounds with GSH.

SELENOPROTEINS LOOKING FOR A FUNCTION

10K Muscle Selenoprotein

It would seem unusual if there existed only one function for Se in higher animals and yet a handful of selenoenzymes in bacteria. Researchers have been looking without success for alternative selenoproteins and for other functions for Se. In 1972 Pedersen et al. (99) reported a 10,000 dalton selenoprotein which they found to be lacking in lambs suf-

fering from muscular dystrophy. Subsequent purification and identification of this protein has not been successful (100,101), although it recently was reported that the Se is present as Se-Cys (102). The classification of this protein as a selenoprotein must therefore remain tentative until more results are obtained.

80K Selenoprotein

A second selenoprotein is the ^{75}Se binding protein first identified by Herrman (103) in the plasma of rats. Burk and Gregory (104) used DEAE-Sephadex chromatography to further purify GSH-Px-containing fractions from gel filtration chromatography and found a non-GSH-Px ^{75}Se binding protein that was retained by DEAE-Sephadex, whereas GSH-PX passed straight through the column. This protein, called ^{75}Se-P, had an apparent molecular weight of 79,000 and 83,000 daltons when isolated from rat plasma and rat liver, respectively. Elution with NaCl released the ^{75}Se in several peaks. Motsenbocker and Tappel (105-107) have published a series of papers in which they examined the appearance and fate of this protein. Initially, they reported a 45,000 dalton Se-containing subunit of an 80,000 dalton plasma protein, but they recently have reestimated these molecular weights as 53,000 and 85,000 (108). The Se is reported to be present as Se-Cys, and Motzenbocker and Tappel have suggested that it serves as a transport protein that carries Se from the liver to the kidney and other tissues. They also have reported a 75,000 dalton ^{75}Se-binding protein in kidney that may be the same protein as the plasma/liver protein (106). A two-subunit transport protein, carrying only one atom of Se per 80,000 dalton protein and with the Se attached covalently to a residue in the peptide backbone, would be thermodynamically very expensive, so the nature and role of this ^{75}Se-binding protein await further characterization.

Burk and Gregory (104) have found a greater percentage of liver ^{75}Se binds to ^{75}Se-P in liver from Se-deficient rats as compared to Se-adequate rats. Three hours after injection of a low (0.76 μg) dose of ^{75}Se, they found 8% of the liver ^{75}Se in GSH-Px in Se-adequate rats as compared to 1.5% in Se-deficient rats; 1.6% of the liver ^{75}Se was present in ^{75}Se-P in Se-adequate rats as compared to 3.5% in Se-deficient rats. At 72 hr, the labeling pattern of neither GSH-Px nor ^{75}Se-P had changed significantly in the Se-deficient rats whereas the percentage of the ^{75}Se in GSH-Px in the Se-adequate rats had increased 3-fold. The exact nature of this Se-binding peak remains unclear, but this approach seemingly will provide a better picture of Se metabolism in animals.

Sperm Selenoprotein

Se was reported in 1973 to be required for the production of normal sperm (109,110), and these defects now can be associated with a 17,000 dalton Se-protein in rat sperm discovered by Calvin (111) and with a 20,000 dalton Se-protein in bovine sperm discovered by Pallini and Bacci (112). The selenoprotein is localized in the midpiece region of the sperm in association with the mitochondrial helix. In Se-adequate mice the mitochondria exist as regular, rectangular organelles located next to a central lumen. Using electron microscopy, a few aberrations are observed in the regular mitochondrial structure in sperm midpiece from first-generation Se-deficient mice, and sperm from second generation Se-deficient mice have severely disrupted mitochondrial organization (113). The SDS-insoluble sheath surrounding the mitochondria contains a protein with a high Cys content, and the Se is present in this Cys-rich structural protein (114). The form of the Se in this protein has not been characterized, but a selenoamino acid moiety would be a likely possibility.

130K Plasma Protein

One of the more recent selenoproteins to be identified was a 130,000 dalton protein from rat plasma. The protein was identified by Gasiewicz and Smith (46), and it appears that both Cd and Se are necessary for the labeling of this protein. Importantly, this protein has served to identify the form of Se released from erythrocytes. When rat plasma is incubated with cadmium and selenite and without erythrocytes, this protein is not observed. If erythrocytes are placed in the media, however, the protein becomes labeled with cadmium and Se; if hydrogen selenite is bubbled through the media during the incubation, the protein also becomes labeled. This work thus finally has identified the product released from erythrocytes as selenide.

Se-Cys Proteins

Hawkes et al. (115) have separated a number of ^{75}Se-labeled selenoproteins by chromatography from blood, liver, kidney, testes, skeletal muscle, lung, heart and epididymus of rats. The rats were isotopically equilibrated with ^{75}Se by providing $^{75}SeO_3^=$ in the drinking water for 5 mo. They reported that over 80% of the Se was present as Se-Cys, and roughly 50% of the total body Se was present as GSH-Px Se. Using DEAE sephacel chromatography with a buffer containing 0.1% triton-X-100 and 7M urea, they have identified 8 different sizes of proteins that bind ^{75}Se—>89,000, 46,000, 36,000, 26,000 (=GSH-Px), 20,100, 15,000, 9800, and 6800 dalton proteins. Ion exchange chromatography has further identified 9 different charge forms. Because protein(s) of one molecular weight can have several charge forms, they have estimated that there may be as many as 19 to 23 different selenoproteins. Hawkes, Wilhelmsen and Tappel (116) find only a 36,000 dalton protein in the plasma, whereas Motsenbocker and Tappel (108) have reported that the major plasma selenoprotein is 85,000 daltons with a 53,000 dalton subunit. Because these results are not in agreement, the conclusions of these experiments must remain tentative until confirmed by more rigorous methods.

FUNCTIONS LOOKING FOR A SELENOPROTEIN

Heme Metabolism

The role of Se in heme metabolism was one of the first areas to be studied in an attempt to find a non-GSH-Px biological function for Se. Burk and Masters (117) reported that phenobarbitol injection did not increase the concentration of cytochrome P450 in Se-deficient rats. When they examined the various enzymes involved in heme metabolism they found that heme synthesis was relatively unaffected by Se deficiency but that heme oxygenase activity was increased 8-fold by Se deficiency in the rat (118). A number of other mineral imbalances also alter heme metabolism (119), so it still is not clear whether this is a specific effect of Se. The stimulation in heme catabolism apparently is not due to a direct effect of Se deficiency in heme catabolism but because Se-deficient liver has a defect in its ability to use heme for the assembly of heme proteins (118).

Drug Metabolism

The toxicity of paraquat and diquat are elevated with Se-deficiency. Burk et al. (120) found that diquat was more toxic than paraquat, and that both were far more toxic to Se-deficient rats than to Se-adequate rats, as assessed by survival time or ethane evolution. An acute lethal dose of

diquat or paraquat, however, did not cause elevated ethane evolution in Se-adequate rats, suggesting that peroxidation is an important aspect of the toxicity in Se-deficient rats. Both vitamin E and Se deficiency increased the toxicity of paraquat (87), but vitamin E deficiency in the rat did not increase the toxicity of diquat or paraquat as dramatically as did Se deficiency (120). A single 50 μg Se injection of selenite 10 hr prior to sacrifice did not substantially raise measured lung or liver homogenate GSH-Px activity, but 50 μg Se administration 6 or 10 hr prior to diquat administration substantially increased survival time and decreased ethane evolution. These authors have interpreted these results to indicate that GSH-Px is not responsible for the protective effect of Se.

In contrast to rats, dietary vitamin E (100 ppm) supplementation of E- and Se-deficient chicks did not decrease the toxicity of paraquat or nitrofurantoin (121,122). Dietary Se supplementation, however, at 0.04 ppm Se allowed 90% survival in 8-day old chicks administered 175 mg paraquat/kg without significantly elevating plasma GSH-Px. GSH-Px activity in other tissues was not measured. Thus, these drug toxicity studies in both the chick and the rat suggest that GSH-Px may not be the important protective factor, but these experiments have not conclusively eliminated the possibility that crucial increases in GSH-Px at the specific site of attack by the toxic species are the protecting biochemical mechanism.

Transsulfuration

Another metabolic role for Se may be in the conversion of Met to Cys. Bunk and Combs (123) reported that Se-deficient chicks fed a crystalline amino acid diet for 28 days had negligible weight gains and high mortality even though they were supplemented with 0.8 or 1.2% D,L-Met. Replacement of part of the dietary Met with Cys increased weight gain 4-fold and reduced mortality from 65 to 40% without altering the incidence of pancreatic atrophy. Se supplementation (0.05 ppm as selenite) increased weight gain 8-fold and completely eliminated the mortality and pancreatic atrophy, thus suggesting that there was an inability to convert Met to Cys in Se-deficient chicks. These experiments were conducted with a fast-growing strain of broilers; the apparent inability to convert Met to Cys with Se deficiency was not observed in a slower growing strain of leghorn chicks (124). The lack of Se response in a slower-growing strain of broilers suggests that this defect could be related to the protein (Met) requirement rather than directly to Se deficiency.

Immune Response

As with a number of other essential elements, animals with Se-deficiency have been reported to have an impaired immune response. Ducks deficient in vitamin E and Se were less able to resist malarial infection than were vitamin E and Se supplemented ducks (125). The neutrophils from Se-deficient rats and cattle were shown to phagocytize bacteria readily but were not able to kill the bacteria as readily as Se-adequate controls (126,127,128). Arthur et al. (129) have shown that toxic oxygen radical production was reduced in Se-deficient neutrophils. A lack of GSH-Px—either normally acting as a protective enzyme or as an integral part of the bacteriocidal mechanism—may be responsible for this Se effect.

Arachidonate Metabolism

Hydroperoxides are intermediates in the metabolism of arachidonic acid to prostaglandins, thromboxanes and leucotrienes. Because of the lack of specificity for the peroxide substrate, GSH-Px was suggested as an important enzyme in prostaglandin metabolism (130). Prostaglandin endoperoxide synthetases also have peroxidase activity, so GSH-Px would not be essential for prostaglandin metabolism (131). Bryant and Bailey (132) reported that the relative products of arachidonate metabolism were altered by Se deficiency, and they suggested that GSH-Px (and thus Se) may have a specific role in platelet metabolism of essential fatty acids.

White Nail Beds

A recent report (133) documenting Se-deficiency in a child receiving total parenteral nutrition adds white fingernail beds to the list of symptoms of Se deficiency in humans—the others are chronic muscle pain, elevated plasma enzymes indicative of tissue damage, and Keshan disease in China (134). Some 25 months after the initiation of parenteral nutrition, the entire fingernail bed of all fingers from both hands was observed to be white even though the nails were fully developed and normal. Addition of Se to the IV fluid (97 μg Se as selenite per day) restored the beds to their normal pink state. White nail beds have been reported in patients with cirrhosis (135), but not in all patients with Se deficiency concurrent with total parenteral nutrition (136), suggesting that this condition may be due to liver necrosis caused by an absence of GSH-Px, or it may be due to lack of another Se function.

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[Received June 20, 1984]

Growth Stimulatory Activity of Unsaturated Fatty Acids for Normal and Neoplastic Breast Epithelium

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ABSTRACT

Studies with experimental animals first showed that dietary lipids in excess have a very large stimulatory effect on the development of breast tumors either induced by carcinogens or occurring spontaneously. These observations took on added significance when epidemiologists found a strong positive correlation between breast cancer incidence and the level of dietary fat. Although not unequivocably established, the total observations concerning this phenomenon suggest a cause and effect relationship between high dietary lipids and breast cancer development. In an attempt to understand how the lipids might be acting, we have begun to assess the effects of various fatty acids on the growth and function of breast epithelium from both normal and neoplastic tissue. The results to date suggest that the unsaturated fatty acids are needed for mammary cell division and that they may play roles in this process by serving as substrates for prostaglandin synthesis, as membrane structural elements or possibly as activators of C kinase when they are in the form of diglycerides. Whatever the mechanism of growth stimulation, it appears that the fatty acids are rate limiting for growth and that physiologic mechanisms for recruiting fatty acids from proximal fat cells exist within the mammary gland. It thus appears that the fat cell serves as a physiologic buffer and that exceeding this buffer such as by consuming excessive lipids may override this buffering capacity and thus favor the division of normal or neoplastic breast cells.

INTRODUCTION

Although it has been more than 40 years since the demonstration that carcinogen induced or spontaneously developing mammary tumor incidence in experimental animals was modulatable by dietary fat (1), the numerous reports on other tumor model systems that have confirmed this observation have failed to elucidate the basis for this effect. Dietary lipid effects on hormone levels (2) or on carcinogen metabolism or clearance rates (3) have been suggested, but the results from other laboratories are not consistent with either of these possibilities (4,5). The type of lipids which promote the response of mammary cells to carcinogens also has proven variable. Original reports indicated that unsaturated fatty acids were more efficient than saturated fatty acids and suggested that these compounds might act as promoters (6). More recently it has been found that a minimal amount of unsaturated fatty acid (essential fatty acid) is needed and that over and above this minimal amount an increased tumorigenic response is obtainable with additional amounts of either a saturated or an unsaturated fatty acid (7). This latter result is consistent with recent epidemiological surveys suggesting that the strongest link between dietary fat consumption and human mammary cancer incidence relates most strongly to the total amount of fat in the diet rather than to the amount of unsaturated fatty acid in the diet (8). Unfortunately, retrospective estimates of consumption are notoriously unreliable because of subjects' faulty memories and lack of accuracy concerning the actual amount of fat consumed (for example, of the amount of dietary fat available for consumption, how much is discarded in the food preparation process?). In spite of these problems, there is a strong positive correlation between lipid intake and the incidence of mammary cancer in humans and in the efficiency with which carcinogen induction of mammary cancer takes place in experimental animals.

Because of the lack of a consistent demonstration that dietary lipids affect serum mammatrophic hormone levels such as prolactin, estrogens or progestins, we have considered the possibility that lipids may directly affect the growth of the mammary epithelium and thus increase the possibility of neoplastic conversion by increasing the size of the cell population at risk. What has emerged from these studies is the fact that the growth of both normal and neoplastic mammary epithelium is facilitated by unsaturated fatty acids. Experiments both in vivo and in vitro with cultures of mammary epithelium have led us to propose that there is an integration between the mammary epithelium and mammary fat cells that is linked through mammotrophic hormones and an intermediary cell type, the mast cell. The experiments that have led us to this postulate are reviewed in this report.

RESULTS AND DISCUSSION

The Model

First let us introduce the model we have formulated and then describe briefly the experimental evidence which supports the various aspects of the model. Then we wish to speculate on the implications of the model insofar as mammary cancer and dietary lipids are concerned.

The model is presented in Figure 1, which depicts a cross