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THE SIGNIFICANCE OF INDUCED FORESTOMACH TUMORS

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INTRODUCTION

Scientific interest in forestomach carcinogenesis has been greatly heightened by the observation that butylated hydroxyanisole (BHA) is carcinogenic to rats at this site (1). BHA is one of only three synthetic phenolic antioxidants that are permitted to be added to food in Canada. These chemicals inhibit oxidative spoilage of food (rancidity) during transportation and storage and are thus of importance to the functioning of the modern centralized food industry. The demonstration that high levels of BHA incorporated into the food of male and female rats resulted in papillomas and carcinomas of the forestomach led to an international research effort focused on the forestomach and designed to outline the way in which these tumors arose and determine whether they are relevant to humans (2).

This report addresses the development of cancerous lesions in the rodent forestomach, the nature of the chemicals that induce these lesions, and their interactions with the animal body. Presently available evidence indicates there are at least two different types of chemical carcinogen that act on the forestomach and that these may have very different degrees of relevance to the human population.

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SPECIES DIFFERENCES IN RODENT FORESTOMACH CONFIGURATION

In rodents, both the forestomach and the esophagus are lined with a squamous epithelium. This has led some investigators to the view that the esophagus and forestomach are a single entity. In rats, for example, the forestomach is a bag that holds food before it passes through the stomach into the intestinal tract. In consequence, the food that has passed rapidly through the esophagus in the form of a bolus is held in the forestomach, which therefore receives a much greater overall exposure to any contaminant in the ingested food. Grice (3) has suggested that the exposure of the forestomach by a foodborne agent greatly exceeds that of the esophagus.

Other rodent species may have a forestomach resembling that of the rat or may possess one of three other types (4). The house mouse (*Mus musculus*) or the wood mouse (*Apodemus sylvaticus*) combine the fore and glandular stomachs in a unilobular form. In the vole (*Microtus agrestes*), the two organs are separated by a narrow neck giving the appearance of two sequentially placed tissues, whereas in the grasshopper mouse (*Orychomys torridus*) there is a unilobular organ in which the glandular part is confined to a small pouch in the greater curvature. Nonrodent species generally lack a forestomach, although in the pig, for example, there is an extension of the esophageal epithelium, known as the pars esophagea, which may resemble the rodent forestomach. Species other than rats, mice, and hamsters have not been extensively used in testing for chemical carcinogenicity and therefore there is no substantive evidence whether these different forms of forestomach affect the development of tumors.

PATHOLOGY OF INDUCED FORESTOMACH LESIONS

Recent detailed studies of the effect of 2% BHA in rat forestomach (5) make an ideal system to demonstrate the types of lesions seen in this organ.

Anatomical Appearance

The rat stomach is the expanded portion of the gastrointestinal tract between the esophagus and the small intestine. It is divided by the limiting ridge into a proximal segment containing the forestomach, which is approximately 60% of the surface area of the total stomach and a distal segment, the glandular stomach, with about 40% of the stomach surface. The esophagus enters the stomach at about the middle of the lesser curvature close to the limiting ridge in the forestomach. Distally the glandular stomach connects with the small intestine. The main function of the forestomach appears to be the storage of food prior to its entry into the glandular stomach which is responsible for the initial digestion of the food.

The wall of the forestomach is composed of four layers known as: the mucosal (luminal) surface, submucosa, muscularis externa, and the serosa. The mucosa is further subdivided into three layers: epithelium, lamina propria, and muscularis mucosae. The epithelium of the mucosa is made up of 3 to 4 layers of stratified squamous cells resting on a layer of basal cells (Figure 1). The luminal surface is lined by a thin layer of keratin. In contrast, the luminal surface of the glandular stomach is lined by a simple columnar epithelium. The pores through which the gastric glands excrete the gastric juices are also lined by simple columnar epithelium. The lamina propria and the muscularis mucosae are similar in both the forestomach and the glandular stomach.

Pathology of Induced Lesions

Oral administration of 2% BHA induced lesions only in the forestomach of F344 rats. The initial, 9-day lesions consisted of an acute inflammatory reaction with necrosis and edema of the mucosa and submucosa (Figure 2). The areas of epithelial necrosis were small, superficial, and multiple and did not penetrate deeper than the muscularis mucosa. Polymorphonuclear leucocytes infiltrated the mucosa. However, these changes were confined to the lesser curvature close to the esophageal opening and the limiting ridge.

With continued oral administration up to 91 days, the early lesions were replaced by hyperplastic stratified squamous epithelium (Figure 3), which formed an accordian-like pleated pattern and thus resulted in the formation of papillae and rete pegs similar to those described in the skin. There was also hyperkeratosis and acanthosis. These secondary lesions arose in the lesser

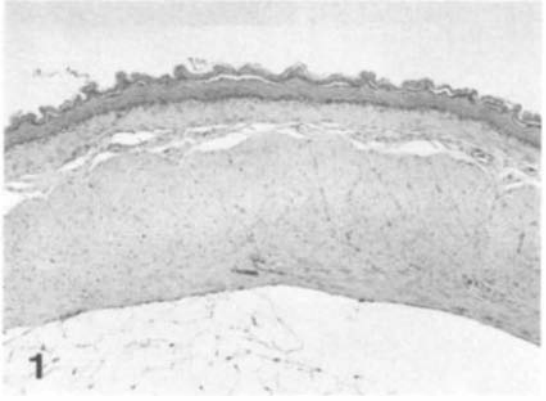


Figure 1 Forestomach from a control rat showing normal appearance of the mucosa, submucosa, and muscularis externa. The mucosa has a thin layer of keratin, and underneath are the layers of stratified squamous epithelium, lamina propria and the muscularis mucosae (H&E × 140).

Figure 2 Forestomach mucosa after 9 days of treatment with 2% BHA showing hyperplasia of the squamous epithelium with the formation of papillae (arrow) and rete pegs (arrow head) similar to those of the skin. Hyperkeratosis and acanthosis (bar) are also present. Inflammatory cells, composed mostly of polymorphonuclear leucocytes, are found in the lamina propria and submucosa (H&E × 140).

curvature of the forestomach and replaced the initial lesions, spreading to other regions of the forestomach with continued treatment. The actively proliferating basal cells pushed the maturing squamous cells into the lumen of the forestomach and, at the same time, developed infiltrating fingerlike projections that encroached toward the lamina propria. With time, the lamina propria and the muscularis mucosa were penetrated by the proliferating basal cells and the lesion extended to the submucosa where keratin-filled cysts were formed. Intraluminal epithelial protrusions became papillomatous and polyploid (Figure 4). These latter lesions affected all layers of the forestomach

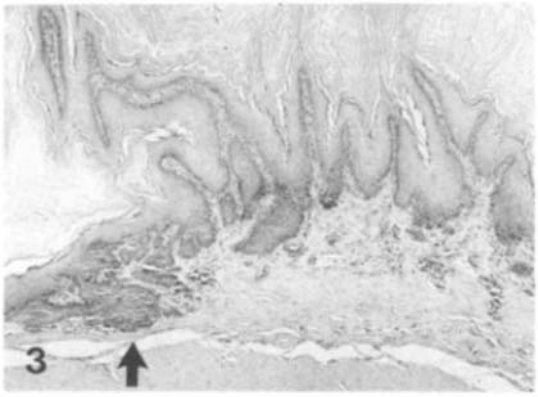


Figure 3 The hyperplastic squamous epithelium approaching the muscularis mucosae after infiltrating the layer of lamina propria (arrow) (H&E × 140).

wall when 2% BHA treatment was continued for 24 months. At 24 months, there was a markedly thickened forestomach wall due to multiple cauliflowerlike protusions into the lumen and to the variably sized keratin cysts and fibrosis.

When administration of 2% BHA was stopped after 6 months continuous treatment, the induced lesions gradually disappeared and the mucosa regained its normal appearance. This was first observed 3 months after 2% BHA treatment was stopped. There was also lesion reversal after 1 year of treatment, but this was not so complete as after 6 months treatment. One year after the cessation of 12 months BHA treatment, the submucosa was still thickened due to fibrosis and the presence of small islands of squamous cells. There were still small polyploid lesions along the lesser curvature.

The squamous cells found in the submucosa were usually well differentiated except in a few cases in which malignant degeneration had occurred (Figure 5). After 12 months treatment with 2% BHA, 5 of 40 rats had poorly differentiated squamous cell carcinomas and 3 of these had well differentiated metastases to the lymph nodes (Figure 6). Others had invaded the muscularis externa and/or the serosa. After 24 months of continuous treatment, 2 of 40 rats developed squamous cell carcinoma, one with metastasis to a lymph node located behind the stomach. These lesions were both poorly differentiated.

CHEMICAL CARCINOGENS ACTIVE IN THE RODENT FORESTOMACH

In a review of the scientific literature, Kroes & Wester (6) identified 60 chemicals that induced forestomach tumors (squamous cell carcinomas and/or

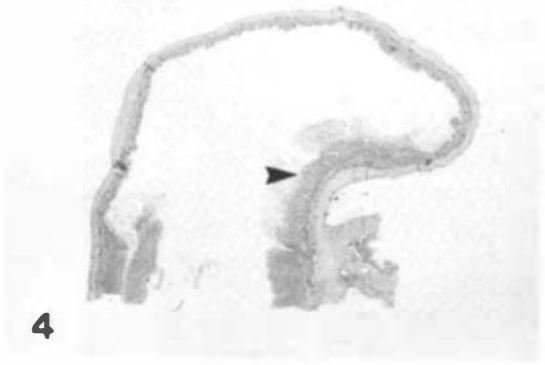


Figure 4 A papillomatous lesion on the lesser curvature extending from the limiting ridge to the dome of the forestomach (arrow head) (H&E × 6).

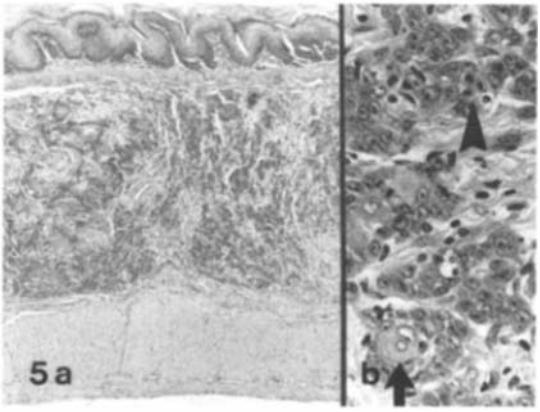


Figure 5 (a) A poorly differentiated squamous carcinoma found in the submucosa of the forestomach (H&E × 70). (b) A higher magnification of the same lesion showing abnormal mitotic figures (arrow head) and single cell keratinization (arrow) (H&E × 560).

papillomas). The majority of these agents were genotoxic, that is they directly, or after metabolic activation, possessed the ability in vitro or in vivo to alter DNA sequences such as those in oncogenes (7–9), and thus lead to populations of cancer precursor cells. These agents included several nitrosamides (for example, N-methylnitrosourea, N-butylnitrosurea), polycyclic aromatic hydrocarbons (7,12-dimethylbenz(a)anthracene, benzo(a)pyrene), aromatic nitro compounds (4-nitroquinoline N-oxide, aristocholic acid) and halogenated hydrocarbons (ethylene dibromide, bis(chloromethyl)ether). In contrast, nitrosamines (N-nitrosomorpholine, N-nitrosoephedrine) and aromatic amines (2-acetylaminofluorene) were infrequent in this series despite the fact that large numbers of such chemicals have been tested for carcinogenicity (10, 11). The apparent paucity of nitrosamines in this series may have been due to a failure to identify all of the active chemicals. There are several reports of other forestomach carcinogenic nitrosamines in the literature including, for example, Clapp's (12) demonstration that diethylnitrosamine led to forestomach carcinomas in RF mice. As discussed in the next section, the forestomach epithelium may be deficient in the ability to activate, metabolically, carcinogens such as certain nitrosamines and aromatic amines and thus is refractory to the induction of tumors by these agents. In hamsters, for example, 2-acetylaminofluorene administration leads to only a low yield of benign forestomach squamous cell papillomas but its proximate carcinogenic form, N-hydroxy-2-acetylaminofluorene, gives both carcinomas and papillomas (13). Few other aromatic amines have been recorded as inducing tumors in this tissue (11).

Kroes & Wester's (6) series also contains a number of chemicals that

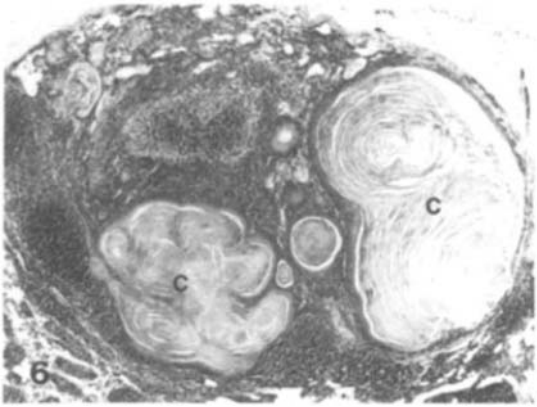


Figure 6 A metastatic focus in a lymph node showing keratin filled cysts lined by stratified squamous epithelium (c) (H&E × 70).

appear to lack genotoxicity in the broad sense of intracting with the DNA in tests such as the *Salmonella* microsome assay (14, 15). These agents include butylated hydroxyanisole (BHA), sodium saccharin, diallyl phthalate, and propionic acid. In a careful comparison of the overall tumorigcnicity of ethylene dibromide and BHA, Moch (16) stressed that the latter chemical appeared to induce cancer only in the forestomach, whereas the genotoxic ethylene dibromide affected a much wider range of tissues.

More forestomach carcinogens have been demonstrated in rats than in mice or hamsters (6). This probably reflects the greater number of chemicals tested in rats compared to the other species. There is insufficient evidence at present to make judgments on species susceptibility.

The forestomach, like other epithelia, develops tumors as a result of the use of an initiation/promotion type protocol. For example, 7,12-dimethylbenz-(a)anthracene-initiated forestomach epithclium was promoted by the well-recognized skin tumor promoter, 12-0-tetradecanoylphorbol-13-acetate (17). It is not surprising that, in initiation-promotion studies, BHA, which itself induces forestomach tumors, has been shown to "promote" forestomach carcinogenesis initiated by methylnitrosourea or MNNG (18). Although, as will be discussed, the overall promotional effect of BHA may be taken to be the major causative factor in inducing forestomach tumors (5), there is as yet no adequate evidence to support the concept that other nongenotoxic forestomach carcinogens act in this manner.

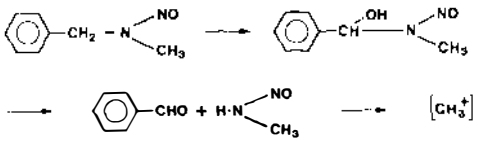
Alterations in the dietary environment of the test animal may also affect the induction of forestomach tumors. Newberne and his associates, for example, demonstrated that feeding a zinc-deficient diet (<2ppm compared to 7ppm in the normal diet) enhanced the incidence of squamous cell papillomas (but not

carcinomas) of the forestomach in rats initiated by N-methyl-N-nitrosobenzylamine or diethylnitrosamine (19, 20).

METABOLISM OF XENOBIOTICS BY THE FORESTOMACH EPITHELIUM

The small amount of forestomach epithelium has precluded much attention being given to its ability to metabolize xenobiotics. It now appears to be of greater interest since, as discussed in the previous section, there may be a deficiency in the expression of some enzymes concerned in the activation of certain carcinogens. This suggests that elements of the P450 system, or specific enzymes therein, are not adequately expressed in the forestomach epithelium. There is a need for knowledge in this area. In the authors' laboratory, one investigator (C. Rogers, unpublished observations) attempted to determine the ability of the forestomach epithelium to activate procarcinogens by cocultivating rat forestomach cells with genetically deficient V79 cells which are mutable in the presence of some activated carcinogens. The experiment had to be abandoned when it was found that the forestomach cells killed the indicator V79 cells in the culture.

Mehta and her colleagues (21) and others (22) used forestomach microsomes in a study of the metabolism of N-methyl-N-nitrosobenzylamine in different organs and species. With small tissues from rats and mice, it was necessary to pool the tissue from several animals for each single observation. The metabolism of this potent esophageal and forestomach carcinogen was investigated by measuring the production of benzaldehyde as a marker for the production of the active methyl species believed to be concerned in the carcinogenicity of this nitrosamine:



Benzaldehyde was produced readily with microsomes from Syrian hamster and BALB/C mouse liver, lung, and kidney. The esophageal microsomes from mice and hamsters, in contrast to those from rats, failed to produce detectable levels of benzaldehyde. Microsomes derived from Sprague-Dawley rat or BALB/C mouse forestomach epithelium failed to produce benzaldehyde, although those from the Syrian hamster gave low levels. Overall, these results did not correspond to the species and tissue differences in tumorigenicity of this nitrosamine. The authors suggested that factors other than metabolic activation might be of critical importance in the genesis of tumors.

Carcinogen-DNA adduct formation is a direct indication of the ability of a tissue to activate metabolically a carcinogen to an unstable intermediate (electrophile or free radical) capable of interacting with DNA. After making allowances for possible strain and species differences, the results of adduct studies with N-methyl-N-nitrosobenzylamine (23, 24) were in agreement with the microsome studies (21).

CELL-PROLIFERATION STUDIES

Increased cellular proliferation (cell replication) is well known to provide an environment in a tissue that is favorable to the expression of cancer (25). The rate of cellular proliferation in a particular tissue or cell type is usually measured by counting the proportion of cells in mitosis, in which case the nuclei of the cells demonstrate a series of recognizable configurations; by tritiated thymidine radioautography; or by incorporation of bromodeoxyuridine (a thymidine analog) followed by labeling with a specific antibody. The thymidine-labeling approach depends on the fact that thymidine is a specific DNA precursor and that if thymidine carrying a radioactive label (3H or 14C) is injected into an animal, the DNA of every cell that is synthesizing DNA will contain the radiolabel. The proportion of cells in DNA synthesis can then be measured either by extracting the DNA and counting the amount of radioactivity that it carries, or, better, by exposing a photographic emulsion to a thin slice of the tissue and counting the number of nuclei that because they contain the radiolabel, have exposed the film. The radioautographic technique is preferable to biochemical extraction because it identifies the actual cells that have incorporated thymidine and thus allows the investigator to identify which cell types are affected and where they are located in the tissue, using multiple sections if needed.

The induction of hyperplasia should not be regarded as a quantitative indicator of the level of cell proliferation. Hyperplasia, the increased number of cells in a tissue, is a histopathologically identifiable lesion, which is the consequence of the difference between cell gain by replication and cell loss due to a variety of possible factors. Hyperplasia may therefore reflect either a large increase in cellular replication accompanied by large losses of cells, or minimal cell replication accompanied by even less cell loss. Active cell proliferation, rather than hyperplasia, is likely to be a significant event in some forms of carcinogenesis.

Application of cell-proliferation techniques to the rat forestomach has revealed important features about the tissue's behavior. First, the squamous cells of the epithelium react differently to chemical stimuli according to the part of the forestomach from which they arise (26–28). Untreated cells in the lesser curvature, that is cells proximate to the epithelium of the glandular stomach, exhibit a very slightly higher rate of cell turnover than do the cells in

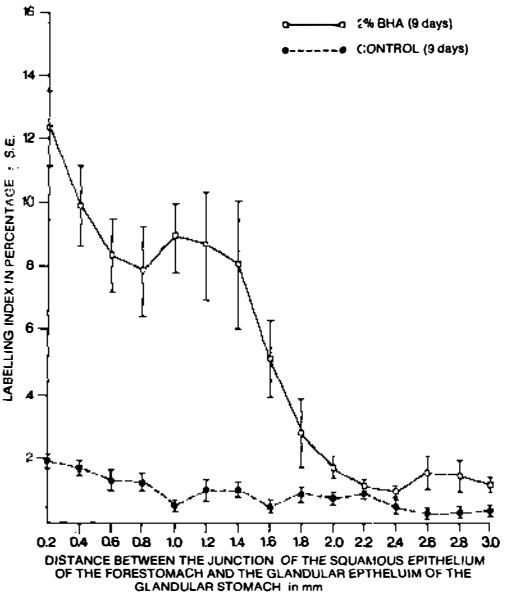


Figure 7 The [methyl-3H]-thymidine labeling index of groups of 125 forestomach cells measured from the junction of the forestomach and fundus in untreated Fischer 344 rats (•) and in rats treated with 2% BHA for 9 days (□). Reproduced with permission from [26].

the main viscus of the forestomach. This difference is markedly exaggerated when 2% BHA, or certain other chemicals were fed in the diet (Figure 7: 26). Such chemicals include: ethyl, n-propyl, and n-butylparaben although others such as 4-methoxyphenol led to proliferation in the main body of the viscus rather than in the lesser curvature (28).

A further observation on the untreated rat forestomach relates to the change in the (3 H)-thymidine labeling index with the age of the rats (27, 29). Cells in the lesser curvature of untreated rats demonstrate a progressive increase in labeling from a value of about 2% in 100g Fischer 344 rats to about 4% at 12 months and 8% at 24 months. Cells in the main body of the viscus do not show a detectable increase of this magnitude (29).

The differences in the lesser curvature and the main viscus of the forestomach tissue are of interest for two reasons: they are not readily apparent by direct histopathological observation; and Ito et al (1) reported that the majority of tumors induced by 2% BHA arose in the lesser curvature, an observation confirmed by Nera et al (29). The progressive increase with time in cellular proliferation in the lesser curvature is of interest because such an increase is not often found in aging tissues. It could indicate an instability in the control mechanisms of the tissue that may be concerned in carcinogenesis.

CASE REPORTS

It is becoming clear that carcinogenic chemicals may be divided into two broad categories: (*a*) those that give consistent positive results in a series of genotoxicity tests and/or form adducts or cause damage in DNA in vivo, and (*b*) those that fail to react with DNA, and may be referred to as nongenotoxic carcinogens. The former generally act on a wide range of tissues and have been extensively studied (30, 31). The broad outline of their interaction in humans and animals with DNA sequences, such as oncogenes, to form precursor tumor cells, is at least partially understood. There has been much less progress with the second group, the nongenotoxic or non-DNA interactive carcinogens, despite their being found with increasing frequency in standard carcinogenesis bioassays. Ashby & Tennant (32) reported that 57 (42%) of 134 carcinogens identified in the US National Cancer Institute/ National Toxicology Program bioassay series were nongenotoxic insofar as they failed to give positive results in the Ames *Salmonella* microsome assay (14, 15). The possible human relevance of these nongenotoxic carcinogens must be considered on a case-by-case basis [33, 34].

Butylated Hydroxyanisole (BHA)

Carcinogenesis BHA has been adequately tested for carcinogenicity in rats, hamsters and mice. In each case the forestomach epithelium was the only tissue to be significantly affected. Ito et al (1, 35) reported that 2% BHA led to carcinomas, papillomas, and hyperplasia in the Fischer 344 rat forestomach epithelium; 1% BHA to papilloma and hyperplasia; and, 0.5% BHA only to hyperplasia. Levels below 0.25% BHA in the diet have not led to significant changes including hyperplasia. Untreated rats rarely exhibited lesions more severe than occasional hyperplasia after 24 months (36). Only one carcinoma was reported in one experiment in hamsters fed 1% and 2% BHA in the diet; but others have reported more tumors (37). These concentrations of BHA enhanced the background level of forestomach papillomas and hyperplasia found in control hamsters (35). In mice, 1.0% and 0.5% dietary BHA induced forestomach hyperplasia but only a single papilloma (35). A recent report (38) noted the induction of 1 carcinoma and 2 papillomas in 20 mice fed 2% BHA, a yield that was enhanced by coadministration of retinyl acetate. Additionally, high levels of BHA promoted the formation of tumors initiated by genotoxic carcinogens in the Fischer 344 rat forestomach and urinary bladder. In contrast, coadministration of lower levels of BHA and certain genotoxic carcinogens inhibited tumor formation through the induction of detoxifying metabolizing enzymes by the BHA (39, 40). Sato et al (41) claimed that BHA initiated tumors in the mouse skin. The evidence for this is by no means statistically sound in view of the few tumors reported [4], the small size of the

groups used, and the failure to make allowance for the fact that multiple groups were being compared with a single, equal-sized, control.

Genotoxicity BHA does not exhibit a genotoxic effect in the Ames *Salmonella* microsome assay, in the DNA-repair assay (42), nor in certain mammalian cell systems (43). The only system in which BHA has been shown to be genotoxic is highly artificial. Phillips et al (45) stripped the medium and the metabolic activating system (S-9 fraction) of catalase and other enzymes that degrade hydrogen peroxide and thereby permitted the hydrogen peroxide formed from the antioxidant to be transformed into oxygen radicals, which have the ability to damage DNA. Such a test system is only relevant if the test agent, in vivo, produces high enough levels of hydrogen peroxide to swamp the normal systems responsible for its degradation. That is, the BHA-induced lesions, if any, would be expected to show a threshold at relatively high dosage levels.

Metabolism On present evidence, BHA does not appear to be metabolized to a genotoxic agent in the rat (Figure 8). BHA is an effective antioxidant because it is able to surrender a hydrogen atom to prevent formation of lipid peroxides. Two of the resultant free radicals combine to form the dimer—DiBHA. Dimerization may also result from peroxidase activity. DiBHA has been found in the intestines and plasma after oral administration of BHA (45) and as a product of in vitro incubation of BHA with horseradish peroxidase (46) or rat liver microsomes (47). Formation of DiBHA appears to be a minor metabolic pathway. No DiBHA was found after incubation of BHA with rat forestomach preparations [48].

O-Demethylation of BHA is part of a major metabolic pathway that depends on P450 activity. The resulting tertiary butyl hydroquinone (TBHQ) also possesses antioxidant activity. The hydroquinone may cycle by way of a redox reaction to yield the quinone. The volatility of the quinone has hindered positive identification, but Cummings & Prough (49) and Armstrong & Wattenberg (47) have successfully isolated this metabolite. BHA is a known inhibitor of P450 activity (50). The quinone binds to the flavoprotein reductase and increases the NADPH oxidase activity of the enzyme resulting in hydrogen peroxide production and a decrease in normal cytochrome P450-dependent monooxygenase activity (49). O-Demethylation activity has been documented in rat liver but not in the forestomach. We have used forestomach preparations and find that a small amount of formaldehyde is formed, consistent with some O-demethylation of BHA (F. Iverson, L. Hierlihey, C. Armstrong, unpublished results).

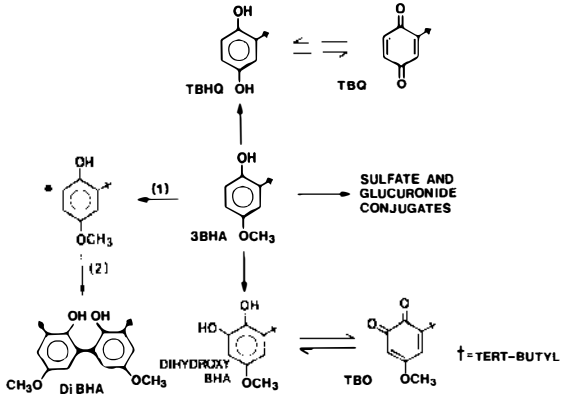


Figure 8 The metabolism of 3-BHA.

An analog of BHA, 4-hydroxyanisole, has been studied as an antineoplastic agent. Its activity is believed to result from conversion to the ortho-quinone. The BHA equivalent (TBOQ) has also been identified (47) and in conjunction with the para-quinone metabolite (TBQ) has been shown to account for the majority of the binding of BHA to protein in both forestomach and liver (48, 51, 52). Other studies (53) have shown that the major BHA isomer (3BHA) is converted to the para-quinone, whereas the minor form of BHA (2BHA) is transformed to the ortho-quinone. The reactive nature of quinones suggests that they may be important in the carcinogenic activity of BHA. DeStafney et al (51) have suggested that the depletion of sulfydryl groups by quinones could establish a threshold for the carcinogenic activity of BHA. Cummings et al (53) have shown that the binding of the quinone to cytochrome P450 is blocked by formation of a glutathione conjugate. Any contribution of the quinone to the development of cancer must be viewed in the light of the finding by Hirose et al (48, 52) of no binding of BHA-derived radioactivity to DNA in rat forestomach preparations and no indication of quinone or other metabolite formation. Moreover the same workers have shown that depletion of GSH inhibits the proliferative response to BHA (54). This suggests that GSH is required in the carcinogenic process. GSH conjugates of BHA have been identified after in vitro (54) but not in vivo studies. The sulfate and glucuronide conjugate of BHA have been shown to be the major metabolites in rats (53).

Acetylsalicylic acid (ASA) administration also inhibits cellular proliferation induced by BHA in rat forestomach (28) and suggests a role for prostaglandin synthetase. Indomethecin, however, is a specific inhibitor of the

cyclo-oxygenase portion of the bifunctional enzyme but has no effect on the BHA-induced response (54); this finding implies that ASA affects proliferation by other means. In vitro BHA and TBHQ effectively inhibit prostaglandin synthetasc activity, again obviating the direct role of this enzyme in the forestomach response to BHA.

Cytotoxicity Sparagli et al (55) noted that BHA interacted with cell membranes to produce solubilization of proteins. Thompson & Moldeus (56) extended this work to show that BHA became inserted easily into the cell membrane and disrupted mitochondrial function and produced a decrease in ATP, an influx of calcium, and subsequently cell death. Thus, the lipophilic and structural properties of BHA rather than reactive metabolite formation appear to be responsible for BHA's cytotoxicity at high concentrations. In vivo, large doses (1g/kg) BHA produce an early inflammatory reaction in the lesser curvature of the rat forestomach. This early reaction appears to regress but some impetus to cell replacement may occur by this pathway, which is only effective at very high doses of BHA.

Reactive oxygen species As already mentioned, BHA-derived quinones can inhibit the P450 portion of the hepatic monooxygenase and thus lead to hydrogen peroxide formation. The BHA-induced acute inflammatory reaction of the forestomach with infiltration of polymorphonuclear cells (PMN) may support oxidative bursts. Finally, the redox cycling of the quinone–semiquinone metabolites may produce oxygen radicals.

The contributions, if any, of these possible pathways to formation of forestomach tumors is unknown. Because the forestomach is low in P450 activity, substantial and continuing peroxide production by enhanced flavoprotein oxidase activity would not appear possible. BHA is also known to suppress the formation of superoxide anions by PMNs, thus rendering the pathway an unlikely candidate as a cancer-inducing mechanism (57). TBHQ, the hydroquinone metabolite of BHA, does not induce a hyperplastic response in the rat forestomach (26, 58, 59) suggesting that redox cycling of the hydroquinone is not a necessary prerequisite for neoplasia in this tissue. Moreover, the dramatic BHA-induced increases in NADPH oxido-reductase (DT diaphorase) should prevent redox cycling and provide protection from chemically induced carcinogenesis (59, 60).

GSH production is increased in the forestomach in response to BHA administration. Normally, this would be considered a protective situation because GSH would scavenge any reactive metabolites. The observation that GSH depletion inhibits the carcinogenic response to BHA in the rat forestomach (55) should be investigated further to determine if the depletion of GSH produces a specific effect on cell proliferation.

Cell proliferation Histopathological and radioautographical studies have demonstrated threshold effects in the forestomach in response to BHA. Feeding 2% BHA in the diet for only 9 days led to a grossly apparent thickening of the lesser curvature of the forestomach, to histopathological evidence of hyperplasia, to a marked increase in the (3H)-thymidine labeling index, and to less marked alterations in the main body of the viscus. At this time and at 91 days, the dose-response curve for BHA-induced proliferation indicated a no apparent observed effect level at about 0.25% BHA in the diet (27, 28). A similar threshold was observed in a 2-year study in which levels of BHA below 0.25% failed to induce significant numbers of lesions such as hyperplasia (36).

Perhaps the most important observation to come from the 91-day study of BHA was that if basal diet was substituted for the BHA-containing diet at this time, the (3H)-thymidine labeling index fell to control levels within 7 days, the shortest period investigated (27). Induced histopathological lesions were much slower to regress and remnants of these lesions were still apparent even 63 days after BHA treatment was stopped. Other short-term reversibility studies conducted after relatively short periods of 2% BHA exposure have confirmed the slow disappearance of established histopathological changes (61).

Lesion reversibility In some cases, but not so far in the forestomach, the withdrawal of the inciting agent that leads to the disappearance of the originally induced lesions, is later followed by the reappearance of tumors in the tissue of interest. Ito et al (62) demonstrated such an occurrence with alpha-hexachlorocyclohexane in mouse liver. To ensure that this was not the case with BHA in the rat forestomach, two reversibility studies were established (29, 36). In the second of these studies, Fischer 344 rats were treated with 2% BHA for 0, 3, 6, 12, and 24 months and these rats were then placed on the basal diet for the remainder of the 24-month experimental period. In addition, serial sacrifices of experimental and control animals were made each time the BHA diet was discontinued and at 15 months. These animals and a subset of those killed at the termination of the experiment at 24 months were injected with (3H)-thymidine so that the labeling index could be measured. The serial sacrifice studies showed that normal or "quasi" normal forestomach epithelium remained dependent on the presence of 2% BHA for its continued excessive proliferation well into the experiment. Carcinomas, some papillomas and some downgrowths had lost their dependence on the continued presence of BHA. Rats fed BHA for 3 months and then returned to the basal diet for the remaining 21 months had forestomach epithelia that were indistinguishable from controls. Those given 2% BHA for 6 months and the basal diet for the succeeding 18 months exhibited a few downgrowths in the

lesser curvature, especially near the esophageal opening. Feeding 2% BHA for 12 months led to hyperplasia, downgrowths, papilloma and the occasional carcinoma of the forestomach as demonstrated in the serial sacrifice part of the study and in a few animals dying at about this time. With the exception of the carcinomas and a few papillomas and downgrowths, most of these lesions regressed when basal diet was fed for another 12 months. Masui et al (36) opined that their evidence was in agreement with the hypothesis that most of the BHA-induced tumors arose from the downgrowths, Nera et al's (29) experiment does not necessarily support this. The important observation from Nera's study is that up to 12 months continuous exposure to BHA appears to be necessary to induce tumors.

BHA overview BHA, from all the evidence, appears to be a forestomach-specific carcinogen. To ensure that it is not likely to induce tumors by a similar mechanism in other organs or tissues of species without a forestomach, short-to medium-term studies of the administration of BHA to dogs [63, 64], pigs [65] and cynomolgus monkeys (66) were established. In no case was any biologically significant lesion observed due to BHA administration. This series of experiments confirms the idea that BHA is specific to the forestomach and together with the concept put forward by Grice (3) that the time of residence in the rat forestomach is critical to its action, confirms that BHA may exert its effect through a toxic rather than a genotoxic action.

In summary, BHA appears most unlikely to induce tumors in humans and other species without a forestomach. It is virtually nongenotoxic, the only exception to this statement being in extreme conditions in which the enzymatic environment has been manipulated to avert the detoxication of hydrogen peroxide. Its ability to induce forestomach cell proliferation, hyperplastic downgrowths, and tumors reaches a threshold at a level between 0.125% and 0.25% in the diet, a level considerably in excess of that to which humans are exposed by way of the food supply. The dose of BHA used in experiments in which tumors were produced, is considerably above the normally defined Maximum Tolerated Dose for BHA in Fischer 344 rats (27, 29). BHA does not appear to have any lasting effect in short- to medium-term dosing periods in species without a forestomach. On the basis of this extensive evidence there is nothing of statistical or biological significance that points to BHA having an adverse effect in humans exposed to the currently used levels in the food supply.

Ethyl Acrylate

Ethyl acrylate (EA) is an industrial chemical noted for its irritant properties, particularly to the gastrointestinal and respiratory tracts of exposed workers (67). Experiments in which EA was given by gavage at a concentration

equivalent to 100 or 200 mg/kg body weight 5×/week in corn oil as a solvent to Fischer 344 rats, or at concentrations up to 100 mg/kg to B6C3F1 mice, demonstrated that the chemical was a forestomach carcinogen in both sexes of both species (68). Growing concern that the slides from this experiment might have been over-read pathologically led to their being reviewed by a panel of consultants, an exercise that reduced, but did not eliminate, the excess tumor incidence in the forestomach of both species (69). Miller et al (70) failed to induce significantly excessive numbers of any tumor in an inhalational study of EA in Fischer 344 rats or B6C3F1 mice. Similarly, a 400-day skin painting study also failed to give evidence that EA was carcinogenic (71). No initiation/promotion type studies were reported (67). These results are consistent with the concept that a high concentration of EA in the forestomach is needed for tumors to be induced and that the gavage route can attain such a concentration.

Examination of the shorter term effects of EA showed that the effects of 20, 200, and 400 mg EA/kg body weight given in corn oil by gavage to Fischer 344 rats affected only the forestomach; 200 mg/kg injected intraperitoneally or subcutaneously did not induce forestomach lesions; the toxicity of EA induced in the forestomach by one or a few doses of EA were severe. The lesions induced by 14-day treatment with 100 mg/kg regressed completely within 2 weeks, although lesions induced by twice this level of dosing still presented hyperplastic lesions after 2 weeks (72–74). Smith (69), in further, more extensive studies succeeded in inducing forestomach toxicity. Tests showed that EA given by gavage for 12 weeks was far more effective in inducing forestomach toxicity than a similar dose given in the drinking water. This again stresses the importance of the local concentration of EA attained at the forestomach. However, even 20 mg/kg given by gavage for 12 weeks led to diffuse hyperplasia.

It is difficult to assess the importance of this information to species such as humans who do not possess a forestomach. It is difficult to descry any evidence of a threshold in the data presented by Smith (69). EA is negative in many tests for genotoxicity but is positive in one recent test (75). The metabolic pattern presented by Smith (69) is consistent with EA being converted, like vinyl chloride, to an epoxide that could be postulated to be a proximate carcinogenic form. Further, there is no long- or short-term information on the effects of EA on species without a forestomach.

Propionic Acid

Von Greim (76) reported that feeding 4% propionic acid (PA) in the diet to Wistar rats led in time to pronounced hyperplasia, papillomas, and severe inflammatory lesions in the forestomach, whereas 0.4% PA led only to hyperplasia. Abnormal cell proliferation of the forestomach epithelium was

induced by 4% PA after a lag phase of about 30 days (28). It appears very unlikely that lower concentrations of PA will induce relevant genotoxic effects. This example serves to indicate that even the simplest chemicals will induce unexpected adverse toxic effects if applied at excessively high doses.

Ethylene Dibromide

Ethylene dibromide (EDB) was introduced into commerce as a lead scavenger in tetra-alkyl lead-treated gasoline and as a soil and grain fumigant (77). It differs from BHA, EA, and PA insofar as it induces tumors in tissues other than the forestomach. In a review of two bioassays conducted by the US National Cancer Institute/National Toxicology Program (78, 79), Moch (16) indicated that besides metastatic forestomach tumors, which are the most prevalent response to EDB when given by gavage in corn oil, EDB also induced lower numbers of hepatocellular carcinomas in female rats and hemangiosarcoma in male rats. In mice, both sexes developed alveolar/bronchiolar lung adenomas. Inhalation studies with EDB led to high yields of nasal tumors in both sexes of both species, as well as lung carcinomas and adenomas, mesothelioma of the tunica, and fibroadenomas of the mammary gland. Skin tumors have also been reported after topical application of EDB in mice (80). The induction of this wide range of neoplasms in different sites is in complete contrast to tumor induction by BHA, which appears to be completely confined to the rodent forestomach.

Unlike BHA, EDB is a potent genotoxic agent inducing mutations in prokaryocytes, fungi, insects, and cultured mammalian cells and chromosomal aberrations also in cultured mammalian cells (77). Structurally, EDB would not be predicted to be a particularly strong genotoxic agent, but it is activated by interaction with the thiol group on glutathione to a one-armed sulfur mustard derivative (81):-

$$R\text{-}SH + Br\text{-}CH_2.CH_2\text{-}Br \rightarrow R\text{-}SCH_2CH_2\text{-}Br.$$

The fact that EDB is potently genotoxic whereas the other agents considered here (BHA, PA, and EA) are either nongenotoxic or less clearly genotoxic is a strong pointer to a different mechanism of tumor induction. Such presumptive evidence is not adequate to establish that the nongenotoxic chemicals are unlikely to be effective in humans. Decisions on relevance to humans require a much broader review of all the relevant toxicity data (33).

CONCLUSIONS

The present spate of work on forestomach carcinogenesis is the result of the observation that an important food additive, the phenolic antioxidant BHA,

induced tumors in this tissue when administered in the diet at high dosages that were well in excess of the Maximum Tolerated Dose (18, 27, 29). Internationally based studies (2) have produced evidence strongly supporting the view that BHA is unlikely to induce tumors in humans under the presently allowed levels of exposure via the food supply. This opinion is predicated on the scientifically based facts that: (*a*) BHA (in contrast to, for example, EDB) is nongenotoxic under normal test conditions, (*b*) at high doses, BHA induces a considerable level of cellular proliferation, mainly in the lesser curvature of the rat forestomach which is the area where most of the tumors and other lesions arise, (*c*) this induced proliferation demonstrates a no-apparent-effect level well above the levels to which humans are exposed to BHA via the food supply, (*d*) the proliferation remains dependent on the continued presence of BHA until relatively late in the experiment, (*e*) levels of BHA up to the maximum tolerated dose do not appear to induce malignant lesions in the rat forestomach, and (*f*) similar effects are not demonstrable in short- or medium-term studies in species without a forestomach.

Perhaps the most important consequence of these studies on the relevance of exposure of BHA to the rat forestomach epithelium is a recent series of studies suggesting that other nongenotoxic carcinogens may not, in some cases, necessarily be effective carcinogens in humans (34, 35). This is an extremely important development since the number of apparently nongenotoxic carcinogens is rapidly increasing; Ashby & Tennant (32), for example, found that 42% of the carcinogens in their series were ineffective in the Ames *Salmonella* test. Important progress is being made, for example, in the interpretation of the renal adenoma in male rats (82, 83), some rodent urinary bladder tumors (34, 35), and thyroid follicular cell tumors (84). The most important tumor of all those induced by nongenotoxic carcinogens, the rodent hepatocellular carcinoma, still needs critical attention.

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