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An Evaluation of the Human Carcinogenic Potential of Ethylene Glycol Butyl Ether

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Summaries of the external peer reviewers' comments and the disposition of their recommendations are in Attachment 4.

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An Evaluation of the Human Carcinogenic Potential of Ethylene Glycol Butyl Ether
EXECUTIVE SUMMARY

Since the publication of NTP's draft report (NTP, 1998) on their 2-year inhalation bioassay of ethylene glycol butyl ether (EGBE; 2-butoxyethanol), there has been continued discussion among scientists from government, industry, and academia concerning the human carcinogenic potential of EGBE. NTP (1998; 2000) reported that their study results indicate *no evidence of carcinogenic activity* in male F344/N rats, *equivocal evidence of carcinogenic activity* in female F344/N rats based on increased combined incidence of benign and malignant pheochromocytomas, *some evidence of carcinogenic activity* in male B6C3F1 mice based on increased incidence of hemangiosarcomas of the liver, and *some evidence of carcinogenic activity* in female B6C3F1 mice based on increased incidence of forestomach squamous cell papillomas or carcinomas. The U.S. Environmental Protection Agency (EPA) IRIS (Integrated Risk Information System) assessment (U.S. EPA, 1999a) concluded that, in accordance with the proposed Guidelines for Carcinogen Risk Assessment (U.S. EPA, 1996), the human carcinogenicity of EGBE *"cannot be determined* at this time, but suggestive evidence exists from rodent studies." Under the pre-existing EPA guidelines (U.S. EPA, 1986), EGBE was judged to be a *possible human carcinogen*." These findings by EPA and NTP prompted investigators, largely supported by the Glycol Ethers Panel of the American Chemistry Council, to design research projects aimed at determining the mode of action for the formation of the forestomach and liver tumors observed in mice. In this paper, recent findings reported in scientific publications and meetings and EPA interim (U.S. EPA, 1999b) and draft final (U.S. EPA, 2003) cancer guidelines are used to provide an up-to-date evaluation of the mode of action involved in the origin of these tumors[1] in mice and their human relevance.

Establishing the mode of action is critical for determining relevance to humans and for choosing the approach most appropriate for dose-response modeling (i.e., whether to use a linear or nonlinear approach). As is extensively discussed in the Agency's interim and draft cancer guidelines (U.S. EPA, 1999b; 2003), in order to determine a chemical's mode of action, one must consider the full range of key influences a chemical or its metabolites might have as an initiator or promoter of the complex carcinogenic process. With this in mind, EGBE's role in the formation of female mouse forestomach (Attachment 1) and male mouse liver (Attachment 2) tumors observed following two-years of inhalation exposure (National Toxicology Program, 2000) were evaluated. These assessments are summarized below.

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[1]The increased incidence of pheochromocytomas reported by NTP is not addressed here because these tumors were reported to have been difficult to distinguish, were not statistically increased over chamber controls, and were not a contributing factor in the IRIS assessment of human carcinogenic risk from EGBE exposure (U.S. EPA, 1999a).

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Forestomach papillomas and carcinoma in female mice

Table 1 summarizes the dose-response data for key tumor types observed in female mice in the NTP (2000) inhalation study of EGBE. At the 250 ppm exposure level, the 10% incidence of squamous cell papillomas and 12% combined incidence of squamous cell papillomas or carcinomas were significantly increased over study controls and exceeded the ranges for historical controls of 0-2% and 0-3%, respectively. NTP (2000) reports that 8% is the highest incidence of forestomach neoplasms that has been observed in contemporary historical controls. NTP (2000) did not observe significant increases in forestomach papillomas and carcinomas at other exposure levels in female mice, nor at any exposure level in male mice or either sex of rats.

Recent reviews of available in vitro and in vivo genotoxicity assays are in agreement that EGBE is not likely to be genotoxic (Commonwealth of Australia, 1996; Elliot and Ashby, 1997; U.S. EPA, 1999a; NTP, 2000). NTP (2000) suggested that EGBE caused chronic irritation leading to forestomach injury including penetrating ulcers and that the observed "neoplasia [papillomas and one carcinoma] was associated with a continuation of the injury/degeneration process." Table 2 provides a summary of the strength of the evidence and the relevance to humans of this nongenotoxic and nonlinear mode of action for EGBE's role in the formation of these forestomach papillomas and carcinomas (Cantox, 2000; NTP, 2000; Green et al., 2002; Poet et. al., 2003).

The Agency believes that a nonlinear mode of action similar to that which is represented in Table 2 and described further in Attachment 1, is principally responsible for the increased incidence of female mouse forestomach tumors reported by NTP (2000). While the steps involved in the true mechanism of action may differ somewhat from those described, recent pharmacokinetic and genotoxicity investigations indicate that a linear mode of action requiring direct interaction of EGBE or an EGBE metabolite with cellular DNA is unlikely (see discussion in Attachment 1 under "*Other Possible Modes of Action for Forestomach Tumor Development in Female Mice*").

Liver Tumors in Male Mice

Table 3 summarizes the data for tumor types which were significantly increased in male mice exposed to EGBE by NTP (2000). Particular focus has been placed on hemangiosarcomas of the liver because this was the only tumor type that was increased over both concurrent and historical controls and because a mode of action involving EGBE has been proposed for this tumor (Siesky et al., 2002). Though the incidence of hepatocellular carcinomas was within the range of historical controls for male mice, consideration was given to this tumor because the dose-response trend is significant and because a similar mode of action has been suggested for this tumor.

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Table 4 summarizes the strength of the evidence and the relevance to humans of the mode of action described in Attachment 2 for EGBE's potential role in the formation of hemangiosarcomas and hepatocellular carcinomas in the livers of male mice. A metabolite of EGBE, butoxyacetic acid (BAA), has long been known to cause hemolysis in rodents (Carpenter et al, 1956). This hemolysis leads to the accumulation of hemosiderin (iron) in phagocytic Kupffer cells of the liver of both rats and mice (NTP, 2000). Recent research in mice and rats indicates that the increased iron levels associated with EGBE-induced hemolysis produces liver oxidative damage that is more severe in mice and increased DNA synthesis in both endothelial cells and hepatocytes that is unique to mice (Siesky et al., 2002). It is hypothesized that these events can contribute to the transformation of the endothelial cells to hemangiosarcomas and hepatocytes to hepatocellular carcinomas in male mice. Two recent analyses of carcinogenicity studies of B6C3F1 mice at NTP found a highly significant ($p<0.001$) association between liver hemangiosarcoma and Kupffer cell hemosiderin pigmentation, particularly when pigmentation is observed subchronically, that is limited to male mice (Nyska et al., 2004; Gift, 2005). Given the high background rate of these two tumor types in male mice (2.9% and 24%) relative to female mice (0.9% and 14%) and rats (0% and 0.4%; combined male and female) (NTP, 2002), it is reasonable to hypothesize that the endothelial cells and hepatocytes in the livers of male mice are more susceptible to oxidative stress resulting from iron buildup in local Kupffer cells. While additional research would be informative with respect to mechanistic issues such as the relative susceptibility of endothelial cells and hepatocytes to oxidative stress caused by the hemolytic effects of EGBE and the apparent resistance of female mice to the development of hemangiosarcomas despite experiencing similar hemolytic effects, there is enough evidence at this time to support an EPA determination that events associated with hemolysis contributed to the increased incidence of these tumors in male mice exposed to EGBE.

The Agency believes that a nonlinear mode of action similar to that which is represented in Table 4 and described further in Attachment 2, is principally responsible for the increased incidence of male mouse liver tumors reported by NTP (2000). While the steps involved in the true mechanism of action may differ somewhat from those described, recent pharmacokinetic and genotoxicity investigations indicate that a linear mode of action requiring direct interaction of EGBE or an EGBE metabolite with cellular DNA is unlikely (see discussion in Attachment 2 under "*Other Possible Modes of Action for Liver Tumor Development in Male Mice*").

Risk to Humans

Forestomach tumors in female mice - Available data establish a plausible nonlinear, nongenotoxic mode of action for the moderate increase observed by NTP (2000) in the incidence of forestomach tumors in female mice following chronic inhalation exposure to EGBE. EGBE appears to be one of a group of non-genotoxic compounds that can indirectly cause forestomach tumors through the sustained cytotoxicity and cell regeneration brought about by irritation and breakdown of the forestomach's gastric mucosal barrier. While this mode of action may be of

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qualitative relevance to humans, the exposure concentrations that would be necessary to cause hyperplastic effects and tumors in humans, if attainable, are likely to be much higher than the concentrations necessary to cause forestomach effects in mice, primarily because humans lack a comparable organ for storage and long term retention of EGBE. However, even if this fact is ignored, the analysis in Attachment 3 indicates that the exposure concentrations necessary to cause hyperplastic effects in humans would be much higher than the existing RfD and RfC for EGBE. Given these considerations, it appears reasonable to assume that the RfC and RfD developed for EGBE (EPA, 1999a) are sufficient for the prevention of hyperplasia and associate tumors in humans, including potentially sensitive subpopulations such as children.[2]

Liver tumors in male mice - Available data establish a plausible nonlinear, nongenotoxic mode of action for the moderate increase observed by NTP (2000) in the incidence of liver tumors in male mice following chronic inhalation exposure to EGBE. The proposed mode of action suggests that the endothelial cells and hepatocytes of male mice are sensitive to the formation of the subject neoplasms (as evidenced by the relatively high background rate of these tumors in male mice) and that excess iron from EGBE-induced hemolysis can result in sufficient iron-induced oxidative stress to cause the observed, marginal increase in the incidence of liver hemangiosarcomas and hepatocellular carcinomas in these animals (NTP, 2000). Given the relatively low sensitivity of humans, including subpopulations such as children, to the hemolytic effects of EGBE, it appears reasonable to assume that the EGBE RfC and RfD (EPA, 1999a) are sufficient for the prevention of hemolysis and associate tumors in humans.[3]

Conclusion Concerning EGBE's Cancer Risk - Information available to the Agency at this time indicate that nonlinear modes of action are likely responsible for the increased incidence of tumors observed by NTP (2000) in mice following chronic EGBE exposure. Application of nonlinear quantitative assessment methods indicate that the noncancer RfD (0.5 mg/kg/day) and RfC (13 mg/m^3) values developed for EGBE (EPA, 1999a) are adequately protective of these carcinogenic effects.

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[2]These analyses are consistent with the nonlinear assessment approach described in existing interim (U.S. EPA, 1999a) and draft (2003) cancer guidelines.

[3]These analyses are consistent with the nonlinear assessment approach described in existing interim (U.S. EPA, 1999a) and draft (2003) cancer guidelines.

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Table 1 - Key Tumors Observed in Female Mice Exposed to EGBE (NTP, 2000)

	Control	62.5 ppm	125 ppm	250 ppm
Squamous Cell Papilloma - Forestomach	0/50	1/50	2/50	5/50
Overall rate	0%	2%	4%	10%
Rate adjusted for intercurrent mortality[4]	0%	2.4%	4.8%	11.2%
First incidence (days)	NA	731	731	582
Squamous Cell Papilloma or Carcinoma - Forestomach	0/50	1/50	2/50	6/50
Overall rate	0%	2%	4%	12%
Rate adjusted for intercurrent mortality	0%	2.4%	4.8%	13.4%
First incidence (days)	NA	731	731	582

[4]Poly-3 test estimate taken from NTP, 2000 technical report series 484

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Table 2 - Concordance Table Showing the Relationship of Proposed Mode of Action for Formation of Forestomach Tumors in Female Mice to Humans

Event	Relation to Animal Tumors			Overall Weight of Evidence[5]	Qualitative Relation to Humans	Quantitative Relation to Humans
	Specificity[6]	Dose/ Temporal Relation	Biological plausibility			
1. **Deposition of EGBE/BAA in stomach and forestomach** via consumption or reingestion of EGBE laden mucous, salivary excretions and fur material	Moderate[7]	Lower/ Earlier	Moderate	Strong	Moderate	Moderate
2. **Retention of EGBE/BAA in food particles of the forestomach** long after being cleared from other organs	Moderate[4]	Lower/ Earlier	Moderate	Moderate	Low	Not Likely
3. **Metabolism of EGBE to BAA systemically and in forestomach**	High	Lower/ Earlier	High	Strong	??	??
4. **Irritation of target cells** leading to hyperplasia and ulceration	High	Lower/ Earlier	High	Moderate	Not Likely	Not Likely
5. **Continued injury and degeneration** leading to high cell proliferation and turnover	High	??	High	Weak	Not Likely	Not Likely
6. **High cell proliferation and turnover** leads to clonal growth of initiated forestomach cells	Moderate	??	High	Moderate	Not Likely	Not Likely

[5]*Weight of Evidence* is either strong, moderate or weak; a strong weight of evidence is defined as having several studies which support the proposed mode of action, preferably from multiple laboratories with limited evidence of contradiction. Weak evidence is normally defined as a single study from a single laboratory or with a significant amount of contradiction in the literature between reports.

[6]*Specificity* to the proposed mode of action. Specificity is high if an event is unique to this particular mode of action. Specificity is low if the event may be seen in many other modes of action.

[7]This step is confirmed by recent research in mice (Green et al., 2002; Poet et al., 2003) but has not been investigated in rats, which do not develop forestomach tumors following EGBE exposure.

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Table 3 - Key Tumors Observed in Male Mice Exposed to EGBE (NTP, 2000)

	Control	62.5 ppm	125 ppm	250 ppm
Hemangiosarcomas - All organs	1/50	1/50	2/50	5/50
Overall rate	2%	2%	4%	10%
Rate adjusted for intercurrent mortality[8]	2.2%	2.1%	5.0%	12.4%
First Incidence (days)	729	670	704	454
Hemangiosarcomas - Liver only	0/50	1/50	2/49	4/49
Overall rate	0%	2%	4%	8%
Rate adjusted for intercurrent mortality	0%	2.1%	5.0%	10%
First Incidence (days)	NA	670	704	454
Hemangiosarcomas/hemangiomas - All organs	1/50	1/50	4/50	5/50
Overall rate	2%	2%	8%	10%
Rate adjusted for intercurrent mortality	2.2%	2.1%	10%	12.4%
First Incidence (days)	NA	670	704	454
Hepatocellular Carcinoma	10/50	11/50	16/49	21/49
Overall rate	20%	22%	33%	43%
Rate adjusted for intercurrent mortality	20.8%	22.9%	35.9%	45.9%
First Incidence (days)	374	621	430	312
Hepatocellular Adenoma or Carcinoma	30/50	24/50	31/49	30/49
Overall rate	60%	48%	63%	61%
Rate adjusted for intercurrent mortality	61.9%	48.9%	67.5%	64.8%
First Incidence (days)	374	549	430	312

[8]Poly-3 test estimate taken from NTP, 2000 technical report series 484

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Table 4 - Concordance Table Showing the Relationship of Proposed Mode of Action for Formation of Liver Hemangiosarcomas & Hepatocellular Carcinomas in Male Mice to Humans[9]

Event	Relation to Animal Tumors			Overall Weight of Evidence[5]	Qualitative Relation to Humans	Quantitative Relation to Humans
	Specificity[6]	Dose/ Temporal Relation	Biological Plausibility			
1. EGBE metabolism to BAA by alcohol dehydrogenase	High	Lower/ Earlier	Moderate	Moderate to Strong[10]	High	Moderate
2. RBC hemolysis by BAA	High for Hemolysis	Lower/ Earlier	Moderate	Moderate to Strong[11]	Low	Not Likely
3. Buildup of Hemosiderin in Kupffer cells of liver	Low	Lower/ Earlier	Moderate	Moderate to Strong[12]	Not Likely	Not Likely
4a. Production of reactive oxygen species by Fenton or Haber-Weiss reactions	Low	Higher/ Earlier	High	Weak[13]	Not Likely	Not Likely
4b. Kupffer cells activated and release cytokine	Low	??	Moderate	Weak	Not Likely	Not Likely
5. Reactive oxygen species results in oxidative DNA damage to endothelial cells	Low	Higher/ Earlier	High	Weak[12]	Not Likely	Not Likely
6. Modulation of endothelial cell gene expression	Low	??	Moderate	Weak	Not Likely	Not Likely
7. Endothelial cell proliferation	Low	??	Moderate	Strong	Not Likely	Not Likely
8. Promotion of initiated endothelial cells	Low	??	High	Weak	Not Likely	Not Likely
9. Neoplasm formation	Low	??	High	Strong	Not Likely	Not Likely

[9]Event, weight of evidence and specificity columns were adopted from Klaunig and Kamendulis (2005)

[10]EGBE is metabolized to BAA in rats and mice, but the tumor is only increased in male mice.

[11]Hemolysis is observed in both sexes of rats and mice, but the tumor is only increased in male mice.

[12]Hemosiderin is observed in Kupffer cells of both sexes of rats and mice, but the tumor is only increased in male mice. Early (subchronic) hemosiderin buildup is only observed in male mice, however.

[13]These effects have been observed to be more pronounced in mice (Siesky et al, 2002), which are also more susceptible to EGBE induced liver tumor formation than rats (NTP, 2000).

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ATTACHMENT 1

Forestomach Tumors in Female Mice

Background

A significant increase over controls (experimental and historical) of papillomas and one carcinoma of the forestomach (6/50; 12%) were reported by NTP (2000) in female mice exposed for two years to 250 ppm EGBE by inhalation. Significant increases in forestomach papillomas and carcinomas were not observed at other exposure levels in female mice, nor at any exposure level in male mice or in rats of either sex. The historical range of forestomach tumors in female control mice from NTP inhalation carcinogenicity studies averaged 0.3% (3/1,092) and ranged from 0% to 2% for carcinomas and averaged 1.6% (17/1,092) and ranged from 0% to 6% for papillomas (Haseman et al. 1998). NTP (2000) reports that 8% is the highest incidence of forestomach neoplasms that has been observed in any contemporary historical control group.

Mode of Action

Researchers from several laboratories, including the National Institute for Environmental Health Studies (Ghanayem et al., 1987a,b; Ghanayem and Sullivan, 1993; Lee et al., 1998; Dill et al., 1998; NTP, 2000), Battelle (Poet et al., 2003; Corley et al., 1994; 1997), Cantox Environmental Inc. (Cantox, 2000), and Syngenta Central Toxicology Laboratory (Green et al., 2002; Bennette, 2001) have made significant contributions towards resolving the mode of action for the development of forestomach tumors (papillomas and one carcinoma) observed in female mice following chronic inhalation exposure to EGBE. The following is a seven step summary of a proposed mode of action that is consistent with the research and reports of these authors.

1. *Deposition of EGBE/BAA in stomach and forestomach* via consumption or reingestion of EGBE laden mucous, salivary excretions, and fur material.
2. *Retention of EGBE/BAA in food particles of the forestomach* long after being cleared from other organs.
3. *Metabolism of EGBE to 2-butoxyacetaldehyde (BAL), which is rapidly metabolized to BAA systemically and in the forestomach.*
4. *Irritation of target cells* leading to hyperplasia and ulceration.
5. *Continued injury and degeneration* leading to high cell proliferation and turnover.
6. *High cell proliferation and turnover* leads to clonal growth of spontaneously initiated forestomach cells.

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Strength, Consistency and Specificity of Association of Tumor Response with Key Events

The proposed nonlinear MOA is consistent with the lack of direct genotoxicity that has been demonstrated for EGBE and its metabolites. Of the steps listed above, the event with the strongest association to the tumor response is step 5, continued injury and degeneration leading to high cell proliferation and turnover. The following is a discussion of the strength and consistency of the database supporting each step in the proposed MOA.

Several studies in rats and mice indicate that EGBE and its principle metabolite BAA deposit in the forestomach and are selectively retained there following whole body inhalation (Poet et al., 2002; Green et al., 2002) and nose-only inhalation (Poet et al., 2002), intravenous (iv) (Poet et al., 2002; Green et al., 2002; Bennette, 2001), intraperitoneal (ip) (Corley et al., 1999; Poet et al., 2002), subcutaneous (sc) (Corley et al., 1999) and gavage (Poet et al., 2002; Ghanayem et al., 1987a,b; Green et al., 2002) exposures. Upon iv administration to mice, EGBE metabolites rapidly accumulate in salivary secretions and are swallowed (Bennette, 2001; Green et al., 2002), and the same phenomena likely occurs following inhalation and ip injection, which cause forestomach lesions similar to those observed in gavage studies (Corley et al., 1999; Green et al. 2002). Findings which strengthen the case for deposition through swallowing EGBE laden material and retention of EGBE and BAA in the contents of the mouse forestomach (Steps 1 and 2) include (1) EGBE or a metabolite rapidly distributes to the oral cavity (buccal and oesophagus) following ip, iv and gavage dosing (Poet et al., 2003; Green et al., 2002); (2) a small but significant amount (9-10 mg/kg) of neat EGBE is available for oral consumption via daily grooming following the NTP inhalation exposures (Corley et al., 1999; Green, 2000); (3) direct, neat exposure to EGBE without first-pass liver metabolism can cause forestomach lesions similar to those observed by the NTP (2000) following inhalation exposure (Corley et al., 1999); (4) the forestomach is poorly vascularized and the cells of the epithelium are separated from capillaries by substantial diffusion distances (Bueld and Netter, 1993; Browning et al., 1983); (5) several hours after ip dosing EGBE levels were two orders of magnitude higher in forestomach contents than forestomach tissue, and (6) 24 hours after ip and oral dosing levels of both EGBE and BAA remained high in forestomach tissue, but were nondetectable in any other tissue, including blood, after 30 minutes (Poet et al., 2003).

The metabolism of EGBE to BAA (Step 3) is well established in multiple in vivo and in vitro tests involving both sexes of several species, including rats, mice, rabbits, guinea pigs, dogs, monkeys, and humans (U.S. EPA, 1999). In addition, the irritation and the hyperplastic effects observed in the forestomach following EGBE exposure are more severe when BAA is administered directly (Green et al., 2002). Poet et al. (2003) have suggested that the small amount of food remaining in the forestomach acts as a storage compartment for EGBE providing a continual source for EGBE to forestomach tissue where it is locally metabolized to BAA. At 3-6 hours after *ip* injection of 250 mg EGBE/kg, EGBE levels were 3-fold higher than BAA levels in stomach contents, but an order of magnitude lower than BAA levels in forestomach tissue. At 9 hours after *ip* injection, the levels of EGBE in stomach contents were reduced to approximately the same levels

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as BAA, supporting the hypothesis that food stored in the forestomach serves as a source of EGBE for forestomach tissue where EGBE is locally metabolized to BAA. Further support for the importance of local metabolism is provided by the work of Corley (2003), who extended a previously described EGBE PBPK model (Corley et al., 2003) to include the metabolism of EGBE to BAL via alcohol dehydrogenase and the subsequent metabolism of BAL to BAA via aldehyde dehydrogenase as an intermediate step in the metabolism of EGBE to BAA (Figure A1-1 and A1-2). Using rate constants derived from mouse stomach fractions (Green et al., 2002) and making several assumptions about the use of these enzyme activity data (see discussion below under "*Biological Plausibility and Coherence of the Database*"), Corley (2003) estimated that 250 ppm EGBE would result in peak C_{max} concentrations of 48 EGBE, 1.1 BAL and 3,200 BAA μM in GI tissue of female mice at the end of a 6 hour exposure period (see Figure A1-3). These estimates are supported by a recent gavage study (see discussion below of Deisinger and Boatman, 2004).

Irritation of target cells leading to hyperplasia and ulceration (Step 4) is well documented following EGBE exposure to both sexes of B6C3F1 mice (Poet et al., 2003; Green et al., 2002; NTP, 2000). NTP (2000) reported a dose-related increase in epithelial hyperplasia (1/50, 7/50, 16/49 and 21/48 in males; 0/50, 6/50, 27/50 and 42/49 in females) and ulceration (1/50, 2/50, 9/49 and 3/48 in males; 1/50, 7/50, 13/49 and 22/50 in females) following chronic inhalation exposure to 0, 62.5, 125 and 250 ppm EGBE. However, NTP (2000) only observed forestomach tumors in female mice (see Table 1 of Executive Summary).

An indication of the importance of continued damage and cell turnover (step 5) towards tumor formation following EGBE exposure is given by the fact that tumors were only observed to increase in female mice, which had more extensive and severe forestomach lesions than male mice and rats (NTP (2000) observed epithelial hyperplasia and ulcers in rats, but the incidence in exposed groups of both sex were not significantly increased over controls). Green et al. (2002) reported that the number of cells in S-phase (an indication of cell turnover) increased in a dose dependent fashion within dose-groups following EGBE (7.71 ± 2.50, 9.33 ± 2.55 and 12.88 ± 2.60) and BAA (8.72 ± 4.97, 9.01 ± 2.32 and 16.22 ± 5.61) exposure at 50, 150 and 500 mg/kg, though none of the changes were significantly increased because of the high value reported for the control group (12.06 ± 2.41).[14] The fact that similar frequencies of H-*Ras* gene mutations were detected in DNA isolated from forestomach neoplasms from treated (8/14) and untreated (5/11) mice (Sill et al., 2000) is consistent with a nongenotoxic mechanism such as increased cell turnover and does not support a mechanism involving direct mutation by EGBE or its metabolite(s).

[14]The authors did not speculate or provide any reason for what they refer to as a"high value" in the control group. However, only four or five animals were used per dose group and all but the high dose responses were within a standard deviation of any other value.

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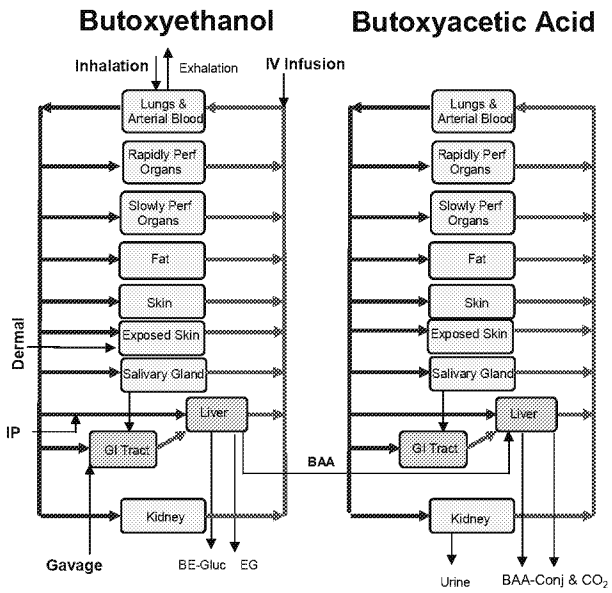


Figure A1-1: EGBE published model
(Corley et al., 2003)

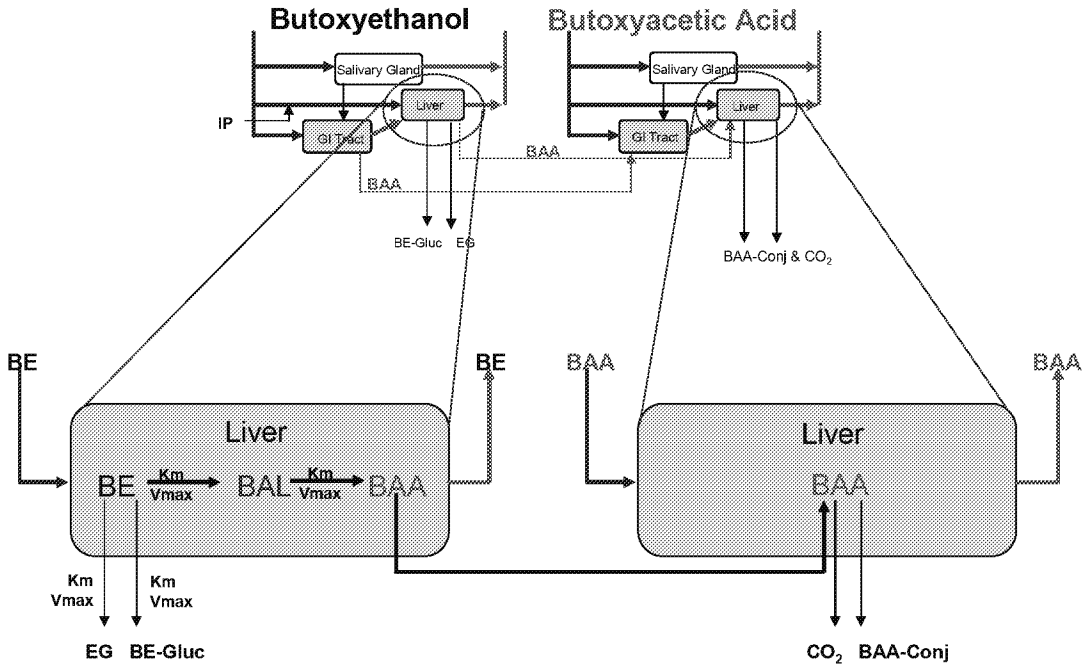
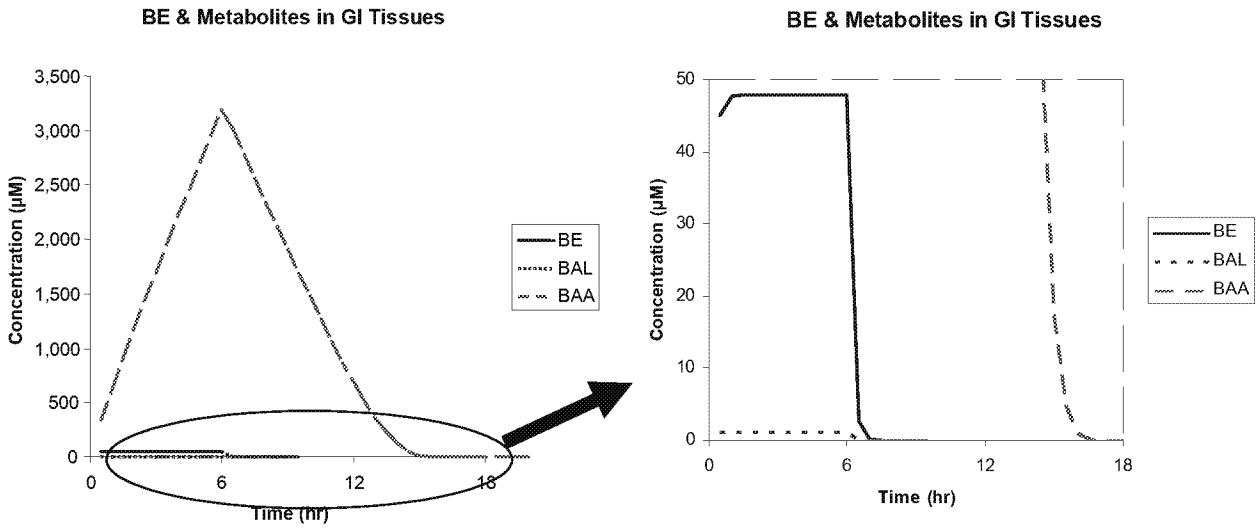


Figure A1-2: Modified version of Corley et al. (2003) model, incorporating BAL intermediate in liver and GI tissues



	Cmax (µM)
BE	48
BAL	1.1
BAA	3,200

Note: GI tissues include BE and BAA in saliva that is swallowed, but not BE from grooming of fur or from muco-ciliary clearance

Figure A1-3: Concentrations of BE, BAL and BAA in GI tissues of female mice exposed to 250 ppm

Over the past two decades, nonlinear modes of action involving cytotoxicity and increased cell turnover have been proposed for the carcinogenic activity of a number of other nongenotoxic forestomach carcinogens (Ghanayem et al., 1994; Ghanayem et al., 1986; Hirose et al., 1986) and for chemicals that cause cell proliferation in other organ systems (Cohen and Ellwein, 1990; Popp and Marsman, 1991). Like many of these compounds, mutagenicity studies generally indicate that EGBE is nonmutagenic with or without activation systems (see discussion under "*Other Possible Modes of Action...*" below) and is not considered to be a tumor-initiator. In support of this contention is the temporal relationship between the noncancerous forestomach lesions which indicate persistent cell damage (i.e., epithelial hyperplasia and ulceration) and tumor formation.

Temporal Association

All of the steps in the proposed mode of action have been observed to occur in female mice prior to tumor formation. NTP (2000) reported that female mice experienced epithelial hyperplasia (1/10, 5/10, 9/10 and 10/10) after just 13 weeks of exposure at the same exposure levels used in the

chronic study, 0, 62.5, 125, and 250 ppm. The first reported incidence of a forestomach papilloma or carcinoma in female mice was 731, 731 and 582 days in the 62.5, 125 and 250 ppm exposure groups, respectively. This is consistent with the findings of Ghanayem et al. (1986; 1993; 1994), who have investigated the temporal relationship between the induction of this type of forestomach lesion by another nongenotoxic irritant, ethyl acrylate (EA), and the development of squamous cell papillomas and carcinomas. They observed cell proliferation/hyperplasia in the forestomach of all rats that received EA by gavage (200 mg/kg, 5 days/wk) for 6 or 12 months. All of these potentially precancerous forestomach lesions regressed in animals treated with EA for 6 months and allowed 2 or 15 months of recovery, and no forestomach neoplasms were observed. However, treatment of rats with EA for 12 months followed by 2 months of recovery resulted in the development of forestomach papillomas in 2 of 5 rats, and treatment of 13 rats for 12 months followed by 9 months of recovery resulted in 1 rat developing a papilloma and 3 rats developing carcinomas of the forestomach. This is an indication that some of these foci or lesions had already developed into the stage of progression (Pitot, 2002). Although EA, an unsaturated aldehyde, is not a metabolite of EGBE, it is an analog of BAL and a much more potent carcinogen (Gold et al., 1993).

The high incidence of forestomach hyperplasia, the relatively lower incidence of papillomas and the late occurrence of a single carcinoma in the high, 250 ppm exposure group is suggestive of a temporal relationship and tumor progression following EGBE exposure to female mice. The presence of a single carcinoma merely probably indicates the spontaneous transition from cells in the stage of promotion (papilloma) to those in the stage of progression (carcinoma).

Male mice may show the beginnings of tumorogenic effects as the incidence of papillomas was observed to increase, but not significantly over concurrent or historical controls. No hyperplasia and no tumors were observed in inhalation studies of rats (NTP, 2000) and in drinking water studies of mice (NTP, 1993a), supporting the need for these steps prior to tumor formation.

Dose-Response Relationships

Forestomach tumors were only increased over controls at dose levels above those that caused significant hyperplasia. The dose-response curve for the tumor response was nonlinear. All key events and tumor effects depend on the dose rate.

Noncancer forestomach effects observed in EGBE exposed female mice, epithelial hyperplasia (6/50, 27/50, 42/49, 44/50) and ulceration (1/50, 7/50, 13/49, 22/50), were dose related, and were significantly increased over controls (concurrent and historical) at lower dose levels than forestomach tumors were observed (Table 1). The incidence of epithelium hyperplasia and ulceration were increased in male mice at all exposure levels, but not as severely.

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Biological Plausibility and Coherence of the Database

Both genotoxic and nongenotoxic chemicals have been shown to induce forestomach tumors in rodents (Kroes and Wester, 1986; Huff et al., 1991; NTP, 2000; Ghanayem et al, 1986; 1993, 1994). Nongenotoxic substances that cause such tumors appear to require long term contact with the forestomach epithelium leading to irritation, cell proliferation and neoplasia. The overstimulation of repair processes and enhancement of growth promoting factors are believed to be involved (Harrison, 1992). Promotion and other activities associated with the stimulation of cell proliferation have been observed for many of these compounds (Clayson et al., 1991; Ghanayem et al., 1994). High concentrations of EGBE and its BAA metabolite sequestered in the forestomach are assumed to be the cause of chronic irritation and the more serious damage observed in the forestomach lining of female mice. Incidence of ulcers consisting of a defect in the forestomach wall that penetrated the full thickness of the forestomach epithelium were significantly increased in all exposed groups of females. NTP (2000) suggests that EGBE exposure-induced irritation caused inflammatory and hyperplastic effects in the forestomach and that "the neoplasia [papillomas and 1 carcinoma] was associated with a continuation of the injury/degeneration process."

Other substances that have caused forestomach hyperplasia in male and female mice following inhalation exposure include acetonitrile (NTP, 1996), 1,3-butadiene (NTP, 1993b) and chloroprene (NTP, 1998). Both propionic and butyric acid have been shown to induce proliferative responses in forestomach epithelium after only seven days, and long-term propionic acid exposure has produced papillomas in the rat forestomach (Kroes and Wester, 1986). Since high levels of EGBE and BAA have been observed in the stomachs of mice following iv, ip, oral gavage and inhalation, it is apparent that the chemical partitions to the forestomach via multiple routes, including grooming of fur, systemic blood circulation, ingestion of salivary excretions and respiratory tract mucus and possibly repartitioning from the stomach contents (Poet et al., 2003; Green et al., 2002).

Because the forestomach functions as a storage organ, there is a reduced requirement for vascularization. The planar capillary network within the epithelial layers of the rodent forestomach contrasts strongly with the thick mucosal network of capillaries in the glandular stomach of rodents (Browning et al., 1983). The cells of the forestomach epithelium, especially the more superficial squamous cells, are separated from capillaries by substantial diffusion distances (Bueld and Netter, 1993; Browning et al., 1983). In addition, the glandular stomach contains a complex mucosal protection and buffering system necessary to withstand the high acidity of the digestion process. As a result, acidic substances that concentrate in the forestomach tend to act as irritants to the forestomach, but not to the glandular stomach or other gastrointestinal tissue (Browning, 1983; Cantox, 2000). The reduced vasculature of the forestomach also suggests that EGBE and its metabolites are delivered to the forestomach by ingestion rather than systemically.

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While there is a significant amount of recent laboratory research that supports the proposed mode of action, several questions remain. Regardless of whether these questions are resolved, however, the EPA should have enough information from the current literature to make the important determinations relative to EGBE induced forestomach tumors, their relevance to humans and the application of a linear or nonlinear assessment.

- *Is BAA the toxic moiety responsible for the irritant effects of EGBE?* EGBE is metabolized to BAL via alcohol dehydrogenase, which is quickly metabolized to BAA via aldehyde dehydrogenase (Green et al., 2002; Ghanayem et al., 1987a). While some have suggested that some of the cytotoxic effects of EGBE may be attributable to BAL (Dartsch et al., 1999; Ghanayem et al., 1987b), recent studies indicate that BAA is largely responsible for the irritant effects observed in the forestomachs of mice following EGBE exposure. In a 10-day gavage study, Green et al. (2002) showed that BAA was significantly more potent than EGBE at inducing hyperkeratosis of the forestomach lining of female mice. EGBE induced minimal hyperkeratosis in 0/5 and 2/5 mice at 150 and 500 mg/kg, respectively. However, BAA exposure caused minimal hyperkeratosis in 3/5 mice at 150 mg/kg and more severe hyperkeratosis in 4/4 mice at 500 mg/kg. In addition, in vitro studies indicate that BAA is known to have other effects on cells that are thought to be important for tumor formation such as altered cell membrane permeability (Udden, 2002; Ghanayem, 1989).

  Available pharmacokinetic information also suggests that BAA is the principal metabolite responsible for the irritant effects of EGBE. Using glandular and forestomach tissue from mice and rats, Green et al. (2002) measured the kinetic constants K_m and V_{max} for enzymes that metabolize EGBE to BAA (an alcohol dehydrogenase) and BAL to BAA (an aldehyde dehydrogenase) (see Table A1-1). For both rats and mice, the rate constant for conversion (V_{max}/K_m) of BAL to BAA via the aldehyde dehydrogenase was significantly higher than the rate constant for conversion of EGBE to BAL via the alcohol dehydrogenase in rats and mice, suggesting that far more BAA would accumulate in the stomach than BAL.

Table A1-1: Dehydrogenase enzyme activities in rat and mouse stomach fractions (Green et al. (2002)

Species/Tissue	Alcohol Dehydrogenase			Aldehyde Dehydrogenase		
	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Rat Forestomach	0.29	1.627	5.61	0.037	3.738	101
Rat glandular Stomach	0.73	2.170	2.97	0.029	5.624	194
Mouse Forestomach	46.59	17.094	0.37	0.056	8.576	153
Mouse glandular Stomach	87.01	13.986	0.16	0.135	8.950	66.3

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A recent attempt has been made to quantify the amount of EGBE/BAA/BAL that would be present in the liver and GI tissues of female mice following a 250 ppm inhalation exposure to EGBE (Corley, 2003) using the forestomach rate constants provided by Green et al. (2002) (Table A1-1). This work extends an earlier model developed by Dr. Corley (Corley et al., 2003) to include the intermediate formation of BAL in target tissues (liver and GI tract). Given the limitations of the available data, Corley was required to make several assumptions, including:

- Rate constants for metabolism of EGBE to BAL in the forestomach of rats are similar to in vitro publications of liver metabolism (Aasmoe et al., 1998; Johanson et al., 1986)
- Forestomach rate constants apply to the entire GI tract
- Forestomach rate constants apply to the liver
- In vivo rate constants from current PBPK model correspond to the first, rate-limiting step in metabolism (EGBE to BAL)
- Ratio of in vitro BAL to BAA/EGBE to BAL can be used (parallelogram approach) to estimate in vivo V_{max} for BAL to BAA
- BAL does not leave tissue where formed while BE and BAA circulate in the body

Given these assumptions and the rate constants derived from mouse stomach fractions (Green et al., 2002), Corley (2003) estimated that 250 ppm EGBE would result in peak C_{max} concentrations of 48 EGBE, 1.1 BAL and 3,200 BAA μM in GI tissue of female mice at the end of a 6 hour exposure period (see Figure A1-3). A recent gavage study performed by Deisinger and Boatman (2004) provides support for the Corley (2003) model and the predicted low levels of the BAL metabolite in GI tissue.[15]

- *Why are there no effects in the glandular stomach of rodents?* If BAA is indeed the toxic moiety, the higher dehydrogenase activity per volume of forestomach tissue versus glandular stomach tissue (Table A1-1) could largely explain this difference in susceptibility. In addition, the nature and the function of the forestomach must be considered. The rodent forestomach is separated from the glandular stomach by a prominent limiting ridge. Material entering the forestomach is stored without being digested prior to entering the glandular stomach. Residence time in the forestomach is considerable. In fact, during rodent bioassays it is rarely empty (Poet et al., 2003; Green et al., 2002). In contrast, the increased vascularity and digestive processes of the glandular stomach cause it to empty relatively quickly. The combination of high dehydrogenase activity and prolonged contact with the substrate is considered to be the principle reason for the forestomach specificity of EGBE (Green et al., 2002).

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[15] The Corley (2003) model predicts that the concentrations of BAL in gastrointestinal tract tissues of male and female mice would be 19 and 33 μM, respectively, following oral gavage exposure to 600 mg/kg EGBE. This compares remarkably well with the levels of BAL actually observed in forestomach tissue of male and female mice, 18 and 33 μM, respectively, following oral gavage exposure to EGBE at 600 mg/kg (Deisinger and Boatman, 2004).

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- *Why were there no forestomach effects observed in the NTP (1993a) subchronic drinking water study of mice?* There is no clear answer to this question at this point in time. No signs of forestomach irritation were observed in mice at dose levels as high as 1400 mg/kg/day in 2-week and 13-week drinking water studies conducted by NTP (NTP, 1993a). It has been suggested that such oral non-bolus dosing of EGBE does not result in high enough local concentrations of EGBE and BAA (Poet et al., 2003). Studies with other nongenotoxic forestomach carcinogens have demonstrated that forestomach effects are dependent not only on the dose but also on the chemical concentration in the dosing solution (Ghanayem et al., 1985) and other effects of EGBE appear to be highly dependent on the concentration attained (Nyska et al., 1999a; Long et al., 2000; Ghanayem et al., 2000; 2001). In addition, first-pass liver metabolism of orally administered EGBE may effect the extent to which EGBE reaches the forestomach via the route that has been proposed following iv injection, distribution to salivary glands followed by the swallowing of EGBE laden saliva (Green et al., 2002; Poet et al., 2003).

- *Why are mice more sensitive to forestomach effects of EGBE than rats?* Green et al. (2002) have suggested that, since the rate limiting step in the metabolism of EGBE to BAA is the slower metabolism of EGBE to BAL via alcohol dehydrogenase, the order of magnitude higher alcohol dehydrogenase activity (Vmax) in mice versus rats provides a possible explanation for this relative mouse sensitivity. However, while the higher V_{max} for this enzyme indicates that mice may have a higher maximal capacity for metabolizing EGBE to BAL, the higher affinity constant (K_m) in mice indicates that this enzyme is less "efficient" in mice versus rats. A comparison of the metabolic rates for this step in rats and mice is a more valid approach. Table A1-2 compares predicted rates of BAL formation between rats and mice under substrate concentrations ranging from 0.001 mM to 150 μM. Rates were estimated using the Michaelis-Menten rate equation (V_{max} * [substrate] / (K_m + [substrate]) and kinetic constant values for forestomach tissue alcohol dehydrogenase provided in Table 1 of Green et al. (2002).

  The results in Table A1-2 indicate that similar rates of BAL formation would be observed at approximately 4.5 mM EGBE. At EGBE concentrations below 4.5 mM, rates of BAL formation in the rat will occur at higher rates, roughly 11-fold higher at an EGBE concentration of 0.1 mM. Concentrations of EGBE in the stomach above 4.5 mM are considered unlikely based on Corley (2003) and Figure A1-1. Thus, it does not appear that the apparent greater sensitivity of mice to the forestomach effects of EGBE can be explained by species differences in metabolism. It should be noted, however, that mice have a faster breathing rate than rats and were exposed to 2-fold higher concentrations of EGBE (high of 250 ppm) than rats (high of 125 ppm) in the NTP study (NTP, 2000), factors that would likely lead to higher target organ doses in mice.

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Table A1-2: Alcohol Dehydrogenase-catalyzed conversion of EGBE to BAL in rats and mice - rates predicted from published kinetic constants (Green et al., 2002)

[EGBE] (mM)	Predicted Metabolic Rate		
	Rats	Mice	Rat:Mouse
0.001	0.005	0.0004	15.24
0.01	0.054	0.004	14.78
0.05	0.239	0.018	13.06
0.1	0.417	0.037	11.39
0.5	1.029	0.182	5.67
1	1.261	0.359	3.51
4.5	1.528	1.505	1.02
5	1.538	1.657	0.93
50	1.618	8.849	0.18
100	1.622	11.661	0.14
150	1.623	13.043	0.12

- *Why were no tumors in male mice observed?* As has been discussed, it is likely that the mode of action for the formation of these tumors involves chronic tissue injury as forestomach effects were preceded or accompanied by marked signs of irritation, including hyperplasia and ulceration. Female mice in the NTP study (NTP, 2000) were clearly more susceptible to inflammation and hyperplasia than male mice at similar exposure levels. The incidence of hyperplasia of the epithelium in the forestomach was 54% at 62.5 ppm, 86% at 125 ppm and 88% at 250 ppm in female mice, and 14% at 62.5 ppm, 32% at 125 ppm and 42% at 250 ppm in male mice (NTP, 2000). Female rats and mice were also more sensitive than males to the hematological effects of EGBE. The reasons for the apparent female rodent sensitivity to these lesions are not clear. However, the hemolytic sensitivity of female rats is well correlated with the longer residence time for BAA in the blood of female versus male rats (Dill et al., 1998).

Other Possible Modes of Action for Forestomach Tumor Development in Female Mice

Though the evidence favors the hypothesis that BAA is the principle toxic metabolite of EGBE, roles for BAL (Dartsch et al., 1999; Ghanayem et al., 1987b) and butyric acid (Cantox, 2000) have been suggested. Neither BAL nor butyric acid have been identified in vivo following EGBE

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exposure, but they are recognized as required metabolites of EGBE (NTP, 2000; Green et al., 2002).

It is not likely that butyric acid plays a significant role in the toxicity of EGBE, particularly at environmentally relevant concentrations. High concentrations of butyric acid have caused ulceration and other preneoplastic lesions in mice (Harrison et al., 1991). However, low concentrations of butyric acid do not appear to be harmful as it naturally occurs in the diet through the fermentation of fiber and starch and constitutes a significant (up to 10 mol%) portion of total bovine milk fatty acid (Smith and German, 1995).

In vitro studies without enzyme (dehydrogenase) activation have shown that BAL is a less potent hemolytic agent, but causes similar hemolytic effects at 0.5 mM (Ghanayem et al., 1989) and is more cytotoxic (Dartsch et al., 1999; Ghanayem, 2003) than EGBE or BAA. Also, EGBE was as toxic to rat and more toxic to mouse hepatocyte cell cultures (no enzyme activation) than BAA as measured by LDH release at comparable concentrations (25 and 50 μM) (Park et al., 2002a). However, the analysis performed by Corley (2003) indicates that BAL concentrations in the liver and stomach would not have risen much above 1 μM, even in the highest exposure group, and that BAA concentrations would likely have been at least 3 orders of magnitude higher.

In vivo studies have indicated that pretreatment of rats with an alcohol dehydrogenase inhibitor, pyrazole, prior to a single 125 mg EGBE/kg gavage exposure protected against hemolysis (Ghanayem et al., 1987b), presumably by blocking the production of both BAL and BAA. Pretreatment of rats with an aldehyde dehydrogenase inhibitor, cyanamide, prior to a single 125 mg EGBE/kg gavage exposure reduced hemolytic responses, but did increased RBC swelling, increased mortality, decreased BAA formation and excretion in the urine, and increased the urinary excretion of EGBE conjugates with glucuronide and sulfate (Ghanayem et al., 1987b). This hematotoxicity in the presence of cyanamide may be due to BAL, but it may also be due to residual BAA. Inhibitors such as cyanamide and pyrazole are not very specific and may cause other effects. In addition, Ghanayem et al., (1990) found that while EGBE + cyanamide decreased BAA concentrations in rats, some BAA was formed and the BAA half-life was increased. Further, when Ghanayem et al. (1987) administered a gavage dose of 125 mg BAL/kg + cyanamide to rats they observed almost no hemolytic activity (Ghanayem et al., 1987). Also, gavage administration to rats of 125 mg EGBE/kg and the molar equivalent of BAL and BAA resulted in no significant difference between the hemolytic effects of the three chemicals between 2 and 24 hours after exposure (Ghanayem et al., 1987). These facts suggest that EGBE's hemolytic activity (without co-exposures) is due to BAA, and that the metabolism of EGBE and BAL to BAA takes place rapidly and completely.

Another possible alternative mode of action could exist if EGBE or one of its metabolites were to have the capability of damaging a cell through direct interaction with its DNA. EGBE has been extensively tested in a variety of short-term genotoxicity assays. Recent reviews of available in vitro and in vivo genotoxicity assays are in agreement that EGBE is not likely to be genotoxic (Commonwealth of Australia, 1996; Elliot and Ashby, 1997; U.S. EPA, 1999; NTP, 2000). Known

or proposed structure-activity relationships do not suggest that EGBE would be expected to be genotoxic (Tennant and Ashby, 1991). EGBE was non-genotoxic in bacteria with or without microsomal activation. Cytogenetic assays on mammalian cells in vitro and assays that demonstrate the ability for a chemical to introduce mutations into mammalian cells in in vitro culture systems showed no evidence of activity associated with EGBE. EGBE also showed no evidence of clastogenicity in several in vitro chromosome aberration assays (NTP, 1993a; Villalobos-Pietrini et al., 1989). Sister chromatid exchange assay results were mixed. EGBE did not affect CHO cells (NTP, 1993a; Slesinski and Weil, 1989), but was reported to be "weakly positive " in V79 cells (Elias et al., 1996) and positive in peripheral human lymphocytes (Villalobos-Pietrini et al., 1989). These positive results may be secondary effects associated with cell cycle delay induced by the cytotoxic effects of EGBE (Elliot and Ashby, 1997). The Syrian hamster embryonic cell transformation (SHE) assay has provided negative (Elias et al., 1995; 1996; Park et al., 2002b) or uncertain (Kerckaert et al., 1996) results for EGBE. In vivo tests of EGBE's genotoxicity, including a mouse and rat micronucleus assay and a ^{32}P-postlabelling assay (Kieth et al., 1996) were negative. Similar frequencies of *H-ras* mutations were detected in DNA derived from forestomach neoplasms from treated (57%, 8/14) and untreated (45%, 5/11) mice, prompting the authors to suggest that EGBE may act as a promoter that stimulates clonal growth of initiated cells present spontaneously in forestomach tissue (Sill et al., 2000).

The major metabolite of EGBE, BAA, was negative in the *Salmonella*/microsome assay with or without activation by rat liver homogenate (S9) (Hoflack et al., 1995). A number of tests were reported by Elias et al. (1996), but the authors generally provided insufficient data to confirm their conclusions (Elliot and Ashby, 1997). BAA was negative in an in vivo bone marrow micronucleus assay in the CD-1 mouse; but there was evidence of toxicity and a reduction in polychromatic erythrocytes (Elias et al., 1996), a finding that is consistent with BAA's known ability to cause erythrocyte membrane fragility. Overall, there is no clear evidence that BAA is genotoxic, but the database is very limited.

BAL, a short-lived metabolite of EGBE, has also been studied in several genotoxicity assays. BAL was negative for bacterial mutation in several strains in the *Salmonella*/microsome assay (Hoflack et al., 1995) and did not induce mutations in the CHO AS52 cell line at up to 0.2% (v/v) (Chiewchanwit and Au, 1995). A recent Comet Assay performed by Klaunig and Kamendulis (2004; 2005) found that BAL did not induce DNA single strand breaks at concentrations three orders of magnitude higher than BAL concentrations estimated to occur by PBPK modeling in liver and forestomach (see discussions of Corley, 2003 above). However, it has been reported to be clastogenic in in vitro assays without enzyme activation at concentrations ranging from 0.2 to 1 mM (Elliot and Ashby, 1997; Ghanayem, 2003). In Chinese hamster lung (V79), BAL and other alkoxyacetaldehydes (methoxtacetaldehyde and ethoxyacetaldehyde) appear to be mitotic poisons which seem to interfere with several different components responsible for cell division in a dose-dependent fashion at concentrations ranging from 0.08 to 0.69 mM (Elliot and Ashby, 1997). The specific cellular targets, the mechanisms and biochemical bases of their interactions are not known.

Unpublished in vitro data submitted by Ghanayem (2003) indicates that 0.5 mM BAL (no enzyme activation) can cause sister chromatid exchange (twice control levels) in human lymphocyte cells. Ghanayem (2003) also reported that BAL was cytotoxic to human lymphocyte cells, causing a 50% reduction in cell number and viability at 0.5 mM.

As discussed previously, Green et al. (2002) and Corley (2003) have suggested that BAL is very short-lived, being metabolized further to BAA due to a high aldehyde dehydrogenase activity in the mouse forestomach. There are other lines of evidence that indicate that direct interaction of BAL with the DNA molecules does not play a significant role in the carcinogenic activity of EGBE. First, BAL causes cytotoxicity at levels associated with chromosome effects and cytotoxicity itself can have effects which result in chromosome damage such as reduction in the repair of SCE. Second, acetaldehyde is recognized as "weakly mutagenic" and structural comparisons of acetaldehydes demonstrate that a longer-chain aldehyde such as BAL would be less likely to interact with DNA than a shorter chain aldehyde such as acetaldehyde (Dellarco, 1988). Third, if BAL was a stable mutagenic metabolite in any of the in vitro assays exposed to butoxyethanol, one would expect them to give positive results; the results were generally negative. The Elias et al. (1996) paper suggests that the V79 cells possess neither alcohol dehydrogenase nor aldehyde dehydrogenase. The relevance of these studies, or any systems that lack these enzymes, are of limited value in elucidating the mode of action of toxicity in biological systems which possess these enzymes. Finally, chemicals for which mutagenesis/genotoxic effects play a significant role generally induce more tumors at earlier time points, rather than near the end of the conducted bioassays, due to their ability to both initiate and promote tumor pathogenesis. The mutagenic compound ethylene dibromide, for instance, was reported to induce forestomach tumors in all dose groups 168 to 280 days from the start of exposure (NCI, 1978). As was discussed above under "*Temporal Association*," EGBE is consistent with other nongenotoxic forestomach carcinogens such as EA in that observed tumors were not as severe (generally did not progress to carcinoma) and were not observed until well into the study, after long periods of forestomach cell damage and repair. The first reported incidence of forestomach papilloma or carcinoma in female mice was 731, 731 and 582 days in the 62.5, 125 and 250 ppm EGBE exposure groups, respectively. In summary, evidence from *in vivo* and *in vitro* genotoxicity assays do not support the idea that BAL would have any significant genotoxicity *in vivo*.

In general, aldehydes such as formaldehyde and acetaldehyde are irritants and have been found to possess some genotoxic activity. The former has been found to be carcinogenic in rat nasal tissue (Swenberg et al., 1983). With increasing carbon length, the primary aldehydes appear to exhibit less to no genotoxic potential. While the opinion is not universal, mutagenicity is not believed to be the driving force in the toxicity of two other analogous aldehydes, formaldehyde and acetaldehyde. For acetaldehyde, the apparent cytogenetic damage is best described as a intracellular reduction in pH by acetic acid. The chromosome damaging effect of lowered pH has been clearly demonstrated by Morita et al. (1992) and Morita, (1995).

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It does not appear that EGBE, BAL or BAA preferentially bind to stomach tissue macromolecules (Poet et al., 2003; Green et al., 2002). Poet et al. (2003) found that high levels of EGBE concentrate in the food content of the forestomach following *ip* exposure (Poet et al., 2003), indicating that the observed sequestering of EGBE in the forestomach is related to its retention in the food that remains there, not to preferential binding to proteins within forestomach tissue.

In summary, a nonlinear mode of action involving forestomach damage and cell epithelial proliferation is likely to be responsible for the increased forestomach tumor incidence reported by NTP (2000). A metabolite of EGBE, BAL, has been found to cause chromosome damage in some *in vitro* studies at cytotoxic levels of exposure. However, available evidence from a published EGBE PBPK model that has been modified to include kinetics for the metabolism of the BAL intermediate (Corley, 2003) and dosimetry data from gavage studies that confirm these model estimates (Deisinger and Boatman, 2004) suggest that the conditions of these *in vitro* assays (e.g., no metabolic activation; high, cytotoxic concentrations of BAL) are of little relevance to expected target organ (forestomach) environment (e.g., high metabolic activity; low concentrations of BAL).

Relevance of Female Mouse Forestomach Tumors to Humans

Pending a definitive determination concerning the role of BAL genotoxicity, EGBE appears to be one of a group of non-genotoxic compounds that can indirectly cause forestomach tumors through the sustained cytotoxicity and cell regeneration brought about by irritation and breakdown of the forestomach's gastric mucosal barrier. According to the EPA's Science Advisory Panel, quantification of the cancer risk for such compounds should be a nonlinear threshold approach based on the forestomach tumors. Ideally, such an approach would take into account differences in the tissue doses and pharmacokinetics of EGBE in humans versus rodents (see also "Relevance of Mouse Liver Hemangiosarcomas to Humans" in Attachment 2 of this paper; and U.S. EPA, 1999) and certain unique characteristics of the rodent forestomach (Green et al., 2002; Poet et al., 2003) in order to make a reasonable approximation of the human exposure that could result in a target organ dose roughly equivalent to the dose presented to the mouse forestomach in the NTP study (NTP, 2000). Selection of a target organ is made difficult by the fact that humans do not have an organ directly comparable to the forestomach. However, there are histological similarities between rodent forestomach tissue and the lower part of the human esophagus, and humans do suffer from conditions (e.g., Barrett's esophagus) where chronic irritation caused by acid reflux or other pathological influences can cause severe histological damage that may progress to a neoplastic result.

Relevance to Susceptible Subpopulations, Including Children

Differences in susceptibility to gastric irritation - Infants (especially those less than three months in age) do not have fully developed digestive systems. This can lead to problems related to the infant stomach's high pH and inability to destroy certain stomach bacteria (U.S.EPA, 1991). Adults have low pH (high acidity) stomach acid that tends to destroy bacteria. The impact this

would have on the susceptibility of the infant stomach to irritation caused by EGBE is unknown at this time. However, the reduced pH in adults might increase their susceptibility to the genotoxic effects of EGBE or its metabolites due to the chromosome damaging effect of lowered pH (Morita et al., 1992; Morita, 1995). Chronic irritation at the gastroesophageal junction induced by acid reflux is increasing in incidence (Voutilainen et al., 1999) as is Barrett's Esophagus and the causes of these human conditions are similar to that seen in the rodent forestomach following EGBE exposure, i.e. chronic inflammation and induced cell proliferation. However, for individuals so affected, it is unlikely that the small levels of EGBE in question would significantly exacerbate this condition.

Genetic differences - Other potentially susceptible subpopulations include individuals with enhanced metabolism or decreased excretion of BAA. Polymorphisms in alcohol and aldehyde dehydrogenases could lead to differences in the metabolism and elimination of EGBE. Human genetic polymorphisms in alcohol dehydrogenase and aldehyde dehydrogenase are prevalent in certain ethnic groups (Chan, 1986) and these polymorphisms have been shown to alter rates of metabolism and elimination of ethanol and acetaldehyde (Agarwal and Goedde, 1992). For instance, native Americans and approximately 50% Asian people are deficient in aldehyde dehydrogenases. Aldehyde dehydrogenases comprises more than nine isoforms in humans (Hsu et al., 1994). A deficiency or loss of one of them (ALDH2) can lead to a nearly complete loss of enzymatic activity (Crabb et al., 1989; Kitagawa et al., 2000). Individuals with atypical and/or deficient alcohol dehydrogenase appear to be more susceptible to adverse effects from increased levels of acetaldehyde including facial flushing, general discomfort, acetaldehyde-protein adducts and alcohol-induced liver diseases (Agarwal and Goedde, 1992). However, the PBPK model developed by Corley et al. (2004) indicates that individuals with low aldehyde dehydrogenase activity (½ Vmax) would not accumulate significant BAL in the liver or forestomach,[16] even following inhalation exposure to a theoretical maximum of 1160 ppm EGBE for 6 hours.

Haufroid et al. (1997) conducted a human study on workers exposed to EGBE to test the possible influence of genetic polymorphism for CYP 2E1 on urinary BAA excretion rate. One exposed individual exhibited a mutant allele with increased cytochrome P450 oxidative activity that coincided with a very low urinary BAA excretion. However, the researchers did not measure BAA conjugated to glutamine, an alternative pathway for BAA excretion in humans. Further investigations on the influence of genetic polymorphism for CYP 2E1 on urinary BAA excretion rate are needed before any firm conclusions can be drawn.

Gender differences - As discussed, Dill et al. (1998) have reported that female rats and mice metabolize EGBE to BAA faster and female mice clear BAA slower than males. A number of effects on the rat liver, kidneys, spleen, and bone marrow and, to a lesser extent, the thymus,

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[16]Predicted BAL concentrations were below 0.001 mM in the liver and 0.004 mM in the GI tract following inhalation exposure to saturated air concentrations of EGBE. These concentrations are considerably lower than concentrations of BAL shown to be clastogenic (0.2 mM) or hemolytic (0.5 mM: Ghanayem et al., 1989) in vitro.

particularly secondary effects of hemolysis such as anemia, infarctions, and thrombosis, are more pronounced in females (NTP, 1993a; Nyska et al., 1999a,b; Ghanayem et al., 2000; 2001; long et al., 2000) and female mice experienced a higher incidence and higher severity of forestomach lesions following chronic exposure (NTP, 2000). Slight gender differences have been noted in other rodent (Carpenter et al., 1956; Dodd et al., 1983; NTP, 1993a; NTP, 2000), rabbit (Tyler, 1984), dog, monkey, and human studies (Carpenter et al., 1956), with females being consistently more susceptible to the primary hemolytic effects of EGBE. In the process of studying and comparing the metabolic and cellular basis of EGBE-induced hemolysis, Ghanayem (1989) observed that the blood from female human volunteers showed a slightly greater sensitivity to hemolysis following incubation with BAA than male blood.

Age differences - A number of factors may differentially affect children's responses to toxicants. The only information available on the toxicity of EGBE to children is from the case study by Dean and Krenzelok (1991), who observed 24 children, age 7 mo to 9 years, subsequent to oral ingestion of at least 5 mL of glass window cleaner containing EGBE in the 0.5% to 9.9% range (potentially 25 to 1500 mg EGBE exposures). No symptoms of EGBE irritation, poisoning or hemolysis were reported. While the effects reported in adult poisonings have been more severe than those reported in these children, adults tended to consume larger volumes and different concentrations of EGBE, making a comparison of toxic effects observed to age sensitivity of the human extremely difficult.

The effect of age on EGBE-induced hematotoxicity was studied in male F344 rats by Ghanayem and co-workers (1987a, 1990). These studies also demonstrated the time course for the onset and resolution of hematological and histopathological changes accompanying hemolysis. Adult (9-13 wk) male F344 rats were significantly more sensitive to the hemolytic effects of EGBE than were young (4-5 wk) male rats following administration of a single gavage dose of EGBE at 32, 63, 125, 250, or 500 mg/kg. In concurrent metabolism studies, increased blood retention of EGBE metabolite BAA (as measured by increased C_{max}, AUC, and $T_{½}$) was observed. Additionally, young rats eliminated a significantly greater proportion of the administered EGBE dose as exhaled carbon dioxide (CO_2) or as urinary metabolites as well as excreting a greater proportion of the EGBE conjugates (glucuronide and sulfate) in the urine (Ghanayem et al., 1987a,b; 1990). These researchers suggested that the pharmacokinetic basis of the age-dependent toxicity of EGBE may be due to a reduced ability by older rats to metabolize the toxic metabolite BAA to CO_2 and a diminished ability to excrete BAA in the urine.

NTP (2000) also found that young mice (6-7 weeks) eliminated BAA 10-times faster than aged (19 months) following a 1-day of inhalation exposure to 125 ppm EGBE. This difference was not as apparent after 3 weeks of exposure, suggesting that factors other than age may have been involved (Dill et. al., 1998).

Developmental studies, which may also be of possible relevance to this issue, have been conducted using rats, mice, and rabbits dosed orally, by inhalation or, in one study, dermally (Hardin et al., 1984; Heindel et al., 1990; Nelson et al., 1984; NTP, 1993a; Sleet et al., 1989; Tyler,

1984; Wier et al., 1987). Maternal toxicity related to the hematologic effects of EGBE and relatively minor developmental effects such as delayed skeletal ossification were reported in most studies. No teratogenic toxicities were noted in any of the studies. It can be concluded from these studies that EGBE is not significantly toxic to developing fetuses of laboratory animals.

Older rats have been shown to have a reduced ability to metabolize the toxic metabolite BAA to CO_2 and a diminished ability to excrete BAA in the urine (Ghanayem et al.,1987a, 1990). However, the relevance of this finding to the possible susceptibility of elderly humans is uncertain due to the fact that humans have conjugation pathways for the excretion of BAA (BAA-Glutamine and BAA-Glycine) that are not available to the rat.

Summary

Available data establish a plausible nonlinear, nongenotoxic mode of action for the moderate increase observed by NTP (2000) in the incidence of forestomach tumors in female mice following chronic inhalation exposure to EGBE. EGBE appears to be one of a group of non-genotoxic compounds that can indirectly cause forestomach tumors through the sustained cytotoxicity and cell regeneration brought about by irritation and breakdown of the forestomach's gastric mucosal barrier. While this mode of action may be of qualitative relevance to humans, the exposure concentrations that would be necessary to cause hyperplastic effects and tumors in humans, if attainable, are likely to be much higher than the concentrations necessary to cause forestomach effects in mice, primarily because humans lack a comparable organ for storage and long term retention of EGBE. However, even if this fact is ignored, the analysis in Attachment 3 indicates that the exposure concentrations necessary to cause hyperplastic effects in humans would be much higher than the existing RfD and RfC for EGBE. Given these considerations, it appears reasonable to assume that the RfC and RfD developed for EGBE (EPA, 1999a) are sufficient for the prevention of hyperplasia and associate tumors in humans.[17]

[17]These analyses are consistent with the nonlinear assessment approach described in existing interim (U.S. EPA, 1999a) and draft (2003) cancer guidelines.

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ATTACHMENT 2

Liver Hemangiosarcoma and Hepatocellular Carcinoma in Male Mice

The framework for a cancer mode of action analysis proposed by EPA (U.S. EPA, 1999a; 2003) will be used here to summarize what is currently known about EGBE's mode of action. In general, the current draft of the cancer guidelines requires discussion of the proposed mode of action, the strength of its supporting database, whether it is believed to be operative in humans, and whether any human subpopulation is apt to "qualitatively respond to the mode of action differently than the general population." The framework for this approach is applied here to assess the mode of action for EGBE-induced liver hemangiosarcomas and hepatocellular carcinomas in male mice and their relevance to humans.

Postulated Mode of Action

In the EGBE IRIS assessment (U.S. EPA, 1999b), EPA determined that available information did not allow for a definitive statement regarding the mode of action for the increase in the incidence of hemangiosarcomas in the livers of male mice. Since that time, considerable research has been completed in this area, and several scientists have postulated a hemolysis-mediated mode of action for the formation of these tumors (Klaunig et al., 1998; Kamendulis et al, 1999; Xue et al., 1999; Siesky et al., 2002; Foster, 2000; Boatman, 2000; Park et al., 2002a,b; Klaunig, 2002). In addition, the Agency's 1999 assessment did not consider a role for EGBE in the formation of hepatocellular carcinomas, largely due to the statement in the NTP (2000a) report that "the increased incidence of hepatocellular carcinoma in 250 ppm males and the decreased incidences of hepatocellular adenoma in 125 and 250 ppm females were interpreted as normal variations based upon chance rather than effects associated with exposure to 2-butoxyethanol [EGBE]." However, NTP also described these findings as "uncertain," and given the dose-response trend observed(10/50, 11/50, 16/49, 21/49) along with increased early mortality in the mid and high-exposure groups, it was considered prudent to consider EGBE's potential role in the induction or promotion of this tumor type as well. The following is a nine step summary of the mode of action that has been proposed for the formation of these tumors.

1. *EGBE is metabolized* to 2-butoxyacetaldehyde (BAL) which is subsequently oxidized to 2-butoxyacetic acid (BAA).
2. *BAA causes hemolysis* of red blood cells and an increase in hemoglobin levels.
3. *Hemosiderin* released from the excess hemoglobin is taken up by and stored in phagocytic (e.g., Kupffer) cells of the spleen and liver.
4. *Increased DNA synthesis* of endothelial cells[18] occurs due to one or more of the following:

[18]Endothelial cells are epithelial cells that line the sinusoidal cavities of blood and are proximate to Kupffer cells; hemangiosarcomas are derived from these cells.

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a. Generation of reactive oxygen species (ROS) from iron within Kupffer cells (Siesky et al., 2002; Park et al, 2002a,b) and perhaps from within hepatocytes and sinusoidal endothelial cells (Foster, 2000)[19] by Fenton or Haber-Weiss reactions.

b. Activation of Kupffer cells to produce cytokines/growth factors which suppress apoptosis and promote cell proliferation (Siesky et al., 2002).

5. *Oxidative DNA damage* in endothelial cells is produced by reactive oxygen species.
6. *Modulation* of endothelial cell gene expression occurs.
7. *Proliferation* of endothelial cells occurs.
8. *Promotion* of endothelial cells to tumor forming cells.
9. *Neoplasms* form.

This hypothesized mode of action would suggest that the pathology of hemangiosarcoma and hepatocellular carcinoma development from EGBE exposure is nonlinear and dependent upon the level of hemolysis, the amount of iron build-up within the target cell population, and the DNA repair capacity of that cell population.

Strength, Consistency, and Specificity of Association of Tumor Response with Key Events

Steps 1 and 2, the metabolism of EGBE to BAA and the association of BAA with hemolytic effects, have been clearly established in multiple in vivo and in vitro tests involving both sexes of several species, including rats, mice, rabbits, guinea pigs, dogs, monkeys, and humans (U.S. EPA, 1999b). A possible indication of the importance of hemolysis to tumor formation is given by the fact that splenic hematopoietic cell proliferation in the mice with liver hemangiosarcomas was reported to be more severe (average severity grade of 3.2 for all dose groups) than in mice that did not develop hemangiosarcomas (average severity grade of 2.3).

Step 3 has been verified through the observation of hemosiderin (iron) within Kupffer cells (phagocytic cells that line the walls of the sinusoids), and hepatocytes (epithelial cells of the liver) following prolonged breakdown of the red blood cells in both sexes of rats and mice exposed to EGBE (NTP, 2000a; Ghanayem and Sullivan, 1993; Ghanayem et al., 1987a,b; Krasavage, 1986; Kamendulis et al., 1999; Siesky et al., 2002). Of the steps listed above, this one has been found to have the strongest association with tumor response. Two recent analyses of carcinogenicity studies of B6C3F1 mice at NTP found a highly significant ($p<0.001$) association between liver hemangiosarcoma and Kupffer cell hemosiderin pigmentation, particularly when pigmentation is observed subchronically, that is limited to male mice (Nyska et al., 2004; Gift, 2005).

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[19]It is possible that a continued cycle of hemolysis and hemoglobin release can eventually overwhelm normal phagocytic mechanisms causing iron to appear within cells which normally would not contain significant amounts of hemosiderin, such as the hepatocytes and sinusoidal endothelial cells. However, the necessity and extent of such build-up are not clear (see discussion under *"Strength, Consistency, and Specificity of Association of Tumor Response with Key Events"*).

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A recent gavage study (Siesky et al., 2002) provides strong support for steps 4 and 5 by providing evidence of increased oxidative DNA damage (8-hydroxyguanosine, OH8dG), increased lipid peroxidation (malonaldehyde) and decreased antioxidant (Vitamin E) levels and increased endothelial cell DNA synthesis in rats and mice subchronically exposed to EGBE. The more pronounced response to these effects in mice observed by these authors is consistent with the NTP (2000) observation of hemangiosarcomas in mice but not rats.[20] Other investigators (Kamendulas et al., 1999; Park et al., 2002a) provide additional *in vitro* evidence that hepatic oxidative stress by EGBE is mediated through reactive oxygen species formed from increased iron deposition resulting from hemolysis, rather than through a compound- or metabolite-specific mechanism. The role of oxidative damage resulting from iron exposure was also supported by results demonstrating an inhibition of endothelial cell DNA damage by supplementation with the antioxidant vitamin E (Reed et al., 2003; Klaunig and Kamandulis, 2005; Siesky et al., 2002). The activation of Kupffer cells (step 4b), either through red blood cell hemolytic components and/or iron accumulation in the Kupffer cell, is proposed to result in the production of cytokines, possibly including vascular endothelial growth factor, a growth factor whose importance has recently been implicated in the induction of hemangiosarcomas in rodents (Klaunig and Kamendulis, 2005).

Step 6, has not been shown directly for endothelial cells, but the induction of oxidative stress and oxidative damage has been shown to modify gene expression in mammalian cells. In addition to inducing DNA damage and lipid peroxidation, the production of reactive oxygen species can alter gene expression, resulting in stimulation of cell proliferation and/or inhibition of apoptosis (Klaunig and Kamandulis, 2005; Nyska et al., 2002; Muller et al., 1997; Manna et al., 1998).

Step 7, endothelial cell proliferation is required prior to the formation of hemangiosarcomas. The induction of endothelial cell proliferation by 2-butoxyethanol has been demonstrated in vivo in the mouse but not in the rat (Siesky et al., 2002) at doses that produced hemangiosacromas in the mouse liver (NTP, 2000).

The final two steps (8 and 9) are consistent with the lack of direct genotoxicity demonstrated for EGBE and the high background of spontaneous endothelial neoplasms in the male mouse liver relative to the rat (Klaunig and Kamendulis, 2005). The premise that the effects seen in the male mouse are the result of tumor promotion mechanisms is also supported by data suggesting a relationship between early onset hemosiderin buildup in the Kupffer cells and tumor formation in male mice (see discussion of *Temporal Association* below).

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[20] The fact that NTP (2000) exposed rats to lower concentrations of EGBE could also be a factor.

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Table A2-1: Incidence of Liver Hemangiosarcomas in NTP Chemicals Causing Increased Hemosiderin in Kupffer Cells						
SEX/SPECIES/CHEMICAL/NTP TR	HEMOSIDERIN	SC[3]	HEMANGIOSARC.	HEPATO. CARC.	H. CARC. or	TYPE
MALE RATS (F344)						
2-Butoxyethanol (EGBE) - TR-484	23/50, 30/50, 34/50, 42/50	yes	0/50, 0/50, 1/50, 0/50	0/50, 1/50, 0/50, 1/50	1/50, 3/50, 0/50, 2/50	Inh.
Butyl benzyl phthalate - TR-458	2/60, 1/60, 6/60, 6/60	no	None Reported	0/60, 0/60, 1/60, 0/60	2/60, 1/60, 1/60, 4/60	Feed
p-Chloroaniline Hydrochloride - TR-351	1/49, 0/50, 0/49, 26/49	no	None reported	1/49, 1/50, 1/49, 0/49	1/49, 1/50, 1/49, 0/49	Gav.
o-Nitroanisole - TR-416	0/20, 1/20, 18/20	no	None Reported	1/20, 0/20, 0/20	1/20, 0/20, 0/20	Feed
Pyridine - TR-470	4/50, 11/49, 20/50, 25/50[1]	no	None Reported	0/50, 0/49, 1/50, 0/50	1/50, 1/49, 1/50, 3/50	Water
FEMALE RATS (F344)						
2-Butoxyethanol (EGBE) - TR-484	15/50, 19/50, 36/50, 47/50	yes	None Reported	None Reported	None Reported	Inh.
Butyl benzyl phthalate - TR-458	4/60, 1/60, 6/60, 10/60	no	None Reported	None Reported	0/60, 0/60, 2/60, 2/60	Feed
CI Pigment Red 3 - TR-407	0/50, 3/50, 14/50, 41/50	no	None Reported	None Reported	0/50, 0/50, 1/50, 10/50	Feed
o-Nitroanisole - TR-416	8/20, 2/20, 20/20	no	None Reported	None Reported	None Reported	Feed
Pyridine - TR-470	6/50, 2/50, 6/50, 17/50	no	None Reported	None Reported	None Reported	Water
MALE MICE (B6C3F1)						
2-Butoxyethanol (EGBE)	0/50, 0/50, 8/49, 30/49	yes	0/50, 1/50, 2/49, 4/49	10/50, 11/50, 16/49, 21/49	30/50, 24/50, 31/49, 30/49	Inh.
p-Chloroaniline Hydrochloride - TR-351	0/50, 0/49, 0/50, 50/50	yes	2/50, 2/49, 1/50, 6/50	3/50, 7/49, 11/50, 17/50	11/50, 21/49, 20/50, 21/50	Gav.
p–Nitroaniline - TR-418	1/50, 1/50, 8/50, 50/50	yes	0/50, 1/50, 2/50, 4/50	10/50, 12/50, 13/50, 6/50	25/50, 26/50, 25/50, 13/50	Gav.
Pentachloroanisole - TR-414	1/50, 50/50, 50/50[2]	yes	2/50, 8/50, 10/50	9/50, 16/50, 12/50	26/50, 34/50, 24/50	Gav.
CI Pigment Red 3 - TR-407	0/50, 5/50, 30/50, 41/50	no	0/50, 1/50, 1/50, 0/50	5/50, 10/50, 8/50, 4/50	12/50, 16/50, 16/50, 19/50	Feed
o-Nitroanisole - TR-416	0/50, 0/50, 3/50, 16/50	no	2/50, 2/50, 1/50, 0/50	7/50, 12/50, 11/50, 7/50	21/50, 32/50, 45/50, 32/50	Feed
FEMALE MICE (B6C3F1)						
2-Butoxyethanol (EGBE) - TR-484	0/50, 5/50, 25/49, 44/50	yes	0/50, 0/50, 1/49, 0/50	10/50, 12/50, 13/49, 10/50	10/50, 12/50, 13/49, 10/50	Inh.
p-Chloroaniline Hydrochloride - TR-351	0/50, 0/50, 1/50, 46/50	yes	1/50, 0/50, 0/50, 1/50	1/50, 2/50, 0/50, 3/50	6/50, 8/50, 6/50, 11/50	Gav.
CI Pigment Red 3 - TR-407	2/50, 1/50, 1/49, 29/50	no	0/50, 1/50, 0/49, 0/50	4/50, 8/50, 2/49, 1/50	10/50, 14/50, 4/49, 9/50	Feed
p–Nitroaniline - TR-418	1/50, 1/50, 4/50, 39/50	no	1/50, 1/50, 0/50, 0/50	7/52, 6/50, 10/51, 9/51	17/52, 17/50, 21/51, 16/51	Gav.
Pentachloroanisole - TR-414	0/50, 37/50, 48/50	yes	0/50, 0/50, 1/50	4/50, 2/50, 2/50	11/50, 10/50, 14/50	Gav.

[1] Pigment identified as hemosiderin, but not explicitly reported in Kupffer Cells

[2] Authors reported that Kupffer cell pigment did not contain iron, bile or PAS-positive materials according to "appropriate staining procedures." They speculate that it may consist of porphyrins known to be produced from exposure to chlorinated hydrocarbons, the possibility that it may have consisted of hemosiderin "was not entirely eliminated."

[3] Chemicals that caused hemosiderin accumulation in Kupffer cells following subchronic exposure are identified with a "yes" in this column.

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Temporal Association

Key steps in the proposed mode of action - hemolysis, hemosiderin build-up, and oxidative damage - have all been observed in subchronic or shorter duration rat and mouse studies (NTP, 2000a; Kamendulis et al., 1999; Siesky et al., 2002) well in advance of tumor formation. In mice, increased endothelial cell DNA synthesis was observed at exposure days 7 and 14 in mice, and increased hepatocyte DNA synthesis was observed at 90 days. No increase in the DNA synthesis of either cell type was observed in rats at any time point. This may be an indication of the importance of early life stage damage to the DNA of these cell types. In addition, mice show evidence of a more sustained hemolytic response to EGBE than rats. Mice experienced an increase in liver and splenic hematopoietic cell proliferation throughout the 2-year NTP (2000a) study, while rats did not show evidence of a sustained hemolytic response[21] and do not develop hemangiosarcomas.

Table A2-1 presents the incidence of liver hemangiosarcoma, hepatocellular carcinoma and hepatocellular carcinoma or adenoma for all chemicals identified in NTP studies[22] as causing hemosiderin pigmentation of the Kupffer cells following exposure to EGBE.[23] A potentially important difference between the four chemicals that caused liver hemangiosarcoma and/or hepatocellular carcinoma in male mice (EGBE; p-chloroaniline; p-nitroaniline; and pentachloroanisole[24]) and the two that did not (CI pigment red 3 and o-nitroanisole) is that only the former four chemicals appear to have induced hemosiderin buildup in the Kupffer cells early, by week thirteen. In the studies of the latter two chemicals, hemosiderin buildup was not as prominent and was not observed until the end of the 2-year study. In the case of EGBE, it appears that early buildup of hemosiderin combined with early increases in endothelial cell and hepatocyte DNA synthesis results in a longer exposure of cells to oxidative damage via iron-generated radicals (step 4). This would be consistent with a mechanism involving a continuing cycle of damage and repair and accumulation of DNA mutations (steps 5 and 6). Thus, it is possible that the two chemicals did not induce liver tumors because they are not as potent hemolytic agents and because of temporal differences in their initiation of hemosiderin buildup.

Dose-Response Relationships

Liver tumors were only increased over controls at doses that caused significant hemosiderin buildup in Kupffer cells within the first 13 weeks of exposure. The dose-response curves for the tumor and hemosiderin endpoints are both nonlinear. All

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[21]Though rats are initially more sensitive to hemolysis than mice, and were used to derive the EPA RfC for EGBE, they tend to compensate for the effects of EGBE after a few months. This increased tolerance is documented in the IRIS file for EGBE and is evidenced by a lack of induction of splenic hematopoiesis at the end of the 2 year NTP (2000a) study.

[22]Data for Tables A2-1 were obtained via a search of NTP's Post-PWG TDMS database (http://ntp-apps.niehs.nih.gov/postpwg/webapp/open.cfm).

[23]Chemicals were not included if they caused pigmentation that was not believed to be hemosiderin, such as bile or porphyrin pigmentation, and that was not observed in Kupffer cells. Chemicals that caused Kupffer cell pigmentation were included if an association with hemosiderosis could not be ruled out.

[24]It is not clear that the "yellow-brown granules" found in Kupffer cells of rats and mice exposed to pentachloroanisole contained hemosiderin (NTP, 1993a). "Appropriate staining procedures" did not reveal iron, and there was no evidence of hemolytic activity. The authors suggested that they may consist of one or more porphyrins, but none were identified. Thus, the possibility that the pigmentation consisted of hemosiderin "was not entirely eliminated."

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key events and tumor effects depend on the dose rate.

Hemangiosarcomas - As can be seen from the EGBE-specific data in Table A2-1, an increase in liver hemangiosarcoma was observed in chronic studies of male mice for EGBE and the three other chemicals (p-chloroaniline, p-nitroaniline and pentachloroanisole) that have been shown to cause a hemosiderin buildup in the Kupffer cells of mice after just 13 weeks of exposure. The tumor response data for these four chemicals were modeled using the Agency's benchmark dose software (BMDS), and each represents a significant dose-response trend. In each case, the highest response was significantly different from both concurrent and historical controls. For all but pentachloroanisole, only the highest dose was significantly increased over controls. The dose-responses for endpoints describing alleged precursor effects, splenic hematopoietic cell proliferation, and liver hemosiderin accumulation are coincident to these tumor effects and dose-related (U.S.EPA, 1999b), as would be expected if these endpoints are representative of precarcinogenic effects.

Hepatocellular carcinomas - An increasing dose-response trend was also observed for the incidence of hepatocellular carcinomas following EGBE chronic exposure. In the EGBE study, hemangiosarcomas and hepatocellular carcinomas were only increased over concurrent controls at the high dose level. Dose-responses for several hemolytic effects were observed in rats, but liver tumors were not increased in rats at any dose. However, the high dose in the rat study was half the high dose in the mouse study.

Biological Plausibility and Coherence of the Database

Oxidative damage is increasingly recognized as playing an important role in the pathogenesis of several diseases, including cancer and cardiovascular disease (Lesgards et al., 2002). In support of the proposed hypothesis, increased reactive oxidative stress is known to accompany the release of large amounts of iron from hemolysis (Ziouzenkova et al 1999). If EGBE causes oxidative stress by this mode of action, one would expect to observe the production of protein and DNA damage, some of which will occur via the production of adducts of OH8dG, and a decrease in antioxidant (e.g., Vitamin E) levels following EGBE exposure (Yamaguchi et al 1996; Wang et al 1995; Houglum et al 1997). This was verified by both Kamendulas et al. (1999) and Siesky et al. (2002) who measured a dose-dependent increase in levels of OH8dG and MDA and a decrease in vitamin E levels in the livers of mice acutely and subchronically exposed to EGBE.

Liver hemangiosarcomas develop from the endothelial cell component of the vascular sinusoidal cells of the liver (Frith and Ward, 1979). The fact that iron (hemosiderin), which is known to accumulate in cells of rodent livers following EGBE exposure, can produce hydroxyl radicals in combination with oxidative by-products via the Fenton reaction (Kamendulis et al., 1999) has led several scientists (Xue et al., 1999; Siesky et al., 2002; Foster, 2000, Boatman, 2000; Kamendulis et al., 1999; Bachowski et al, 1997; Klaunig et al., 1995) to suggest that male mouse liver hemangiosarcomas result from increased oxygen radical damage and an associated increase in endothelial cell DNA synthesis caused by excess iron from hemolysis. The damaging effects of iron overload to liver sinusoidal cells in rats following a single ip injection of 200 mg iron/kg (Junge et al., 2001) lend support to this hypothesis. Additional support for this hypothesis is provided by the fact that endothelial cells do appear to be more susceptible than other cells (e.g., hepatocytes or Kupffer cells) to oxidative stress (DeLeve, 1998; Spolarics, 1999).

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While largely supported by recent laboratory research, several questions remain concerning the postulated mode of action. The questions outlined below may be answerable through further research.

- *Is the severity and incidence of observed Kupffer cell pigmentation at terminal sacrifice sufficient to suggest that oxidative stress from hemosiderin buildup in these cells is solely responsible for the increased incidence of liver hemangiosarcoma in the male mouse?* NTP (2000a) has suggested that hemosiderin buildup is not related to the formation of liver hemangiosarcomas because Kupffer cell pigmentation was only of minimal severity and only observed in three of the four high-dose male mice that developed this tumor. However, given the apparent susceptibility of male mouse endothelial cells, it is reasonable to hypothesize that the reported minimal pigmentation observed over such a large percentage of high-dose animals (61% in the 250 ppm exposure group versus 0% in controls) could have caused the marginal increase in hemangiosarcomas reported for this dose group (8% versus 0% in controls, with a 2.5% mean historical background rate). Additionally, the fact that Kupffer cell pigmentation was not reported for one of the four mice with liver hemangiosarcomas does not preclude the proposed mechanism because (a) the Perls Prussian blue stain for iron performed to determine the presence of hemosiderin is not a particularly sensitive test (Ghio, 2002; Ali et al, 2003) and (b) the observation of a liver hemangiosarcoma in 1/50 without hemosiderin buildup is not inconsistent with the rate of incidence in historical controls, reported to be 0-6% (mean of 2.5%) over all NTP studies of male mice (NTP, 2000b).

- *What is the relation between Kupffer cell pigmentation and the hepatocyte and sinusoidal endothelial cells, that demonstrate no pigmentation and from which the hemangiosarcomas arise?* While the Siesky et al. (2002) study does suggest a temporal association between liver DNA adduct formation and increased endothelial cell DNA synthesis (both having been observed on the 7th exposure day), it does not confirm whether observed endothelial cell DNA synthesis was the result of direct DNA damage or Kupffer cell activation leading to increased endothelial cell proliferation, both of which can lead to unscheduled DNA synthesis. Indicators of oxidative damage (increased OH8dG and malondialdehyde levels) were only recorded for total liver, and iron was not observed within the endothelial cells. Siesky et al. (2002) and Rose et al. (1997; 1999) have suggested that increased hepatocyte and endothelial cell DNA synthesis and oxidative damage can occur without direct iron involvement in the endothelial cells through indirect Kupffer cell activation leading to the release of cytokines and growth regulatory molecules that contribute to the induction of DNA synthesis and damage. In addition, reactive oxygen species generated in the Kupffer cells may impacting endothelial cell DNA due to the proximity of these two cells within the liver. Given the apparent predisposition of male mouse hepatocytes and endothelial cells to carcinogenic transformation, these indirect factors may be enough to trigger mutations within endothelial cell DNA and tumor formation. However, Perl's iron staining is a relatively insensitive technique (Ghio, 2002)[25], and redox active iron can associate with protein, often in one of the forms of ferritin, in many different kinds of cells (Knutson and Wessling-Resnick, 2003), leaving open the possibility that iron stores located directly within endothelial cells may also be contributing to the observed DNA synthesis and damage.

- *If hemosiderin buildup in Kupffer cells is important for the development of EGBE-induced liver hemangiosarcomas, why was the incidence of this tumor not increased in the female mouse for which hemosiderin pigmentation was at least as high as in the males?* This observation is not

[25] A newly developed fluorescent calcein method is a more sensitive measure of LMW iron in biological fluids (Ali et al., 2003).

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unique to EGBE. In fact, all NTP chemicals (Table A2-1) that induced early onset hemosiderin buildup in conjunction with an increased incidence of this tumor exhibited this apparent male specificity. Inherent differences between the antioxidant capacity of male versus female mice related to hormonal modulation of antioxidative enzymes has been offered (Foster, 2000; Nyska et al., 2004) as a possible explanation for the lack of a hepatic tumorogenic response in the females. Though there is no direct evidence to support this hypothesis, the historical incidence of hemangiosarcomas does appear to be considerably higher in male versus female mice. NIH/NTP has observed liver hemangiosarcomas in 105 of 4183 (2.51%) male versus just 35 of 4177 (0.84%) female historical controls (NTP, 2000b; Klaunig, 2002). A more definitive answer to this question could come from additional research, perhaps involving measurement of the relative capabilities of the antioxidant systems of male and female mouse liver endothelial cells.

- *Is the proposed relationship between the buildup of hemosiderin in Kupffer cells and the development of liver hemangiosarcoma and hepatocellular carcinoma supported by studies of other chemicals?* NTP (2000a) reported that they did not find an association between hemosiderin deposition in the liver and "liver neoplasms (adenomas, carcinomas, or hemangiosarcomas)" in the 79 male and 103 female mice that had chemically related liver neoplasms at the end of an NTP study. However, the NTP analysis did not focus on the specific relationship between hemosiderin buildup in the Kupffer cells of male mice and liver hemangiosarcoma and/or hepatocellular carcinoma. A more recent analysis of 130 two-year carcinogenicity studies of B6C3F1 mice at NTP found a highly significant ($p<0.001$) association between liver hemangiosarcoma and Kupffer cell pigmentation that is limited to male mice (Nyska et al., 2004). Nyska et al. (2004) report that a chemical associated with Kupffer cell pigmentation related to hemosiderosis has a high likelihood (3/4 or 75%) of producing liver hemangiosarcoma, compared with a very low likelihood (3/126 or 2%) in the absence of such pigmentation. The four chemicals identified by Nyska et al. (2004) as causing this Kupffer cell pigmentation are EGBE, p-chloroaniline hydrochloride, p-nitroaniline and o-nitroanisole. Table A2-1 identifies two other chemicals pentachloroanisole and CI pigment red 3, for which Kupffer cell pigmentation has been reported, but the relationship to hemosiderosis has been questioned.[26] As is indicated in Table A2-1, there appears to be no association between hemosiderin in Kupffer cells and liver hemangiosarcomas for rats and female mice.

- *If hemosiderin deposition and increased hematopoietic cell proliferation can lead to tumor formation, why were no neoplasms noted in the spleen, where these effects were also observed?* Cells of the spleen, such as phagocytes, serve to rid the peripheral circulation of damaged red blood cells and would be expected to encounter high levels of hemoglobin and hemosiderin in the process, thereby requiring the need for greater protection against any harmful effects from such buildup. The fact that phagocytic cells seem to have a higher antioxidant capacity than sinusoidal endothelial cells lends some support to this hypothesis (DeLeve 1998; Foster, 2000). Thus, the proposed mode of action is not inconsistent with the fact that hemosiderin buildup in splenic cells did not lead to the type of tumor formation observed in the liver, particularly given the very slight increase in liver tumor incidence observed.

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[26]Authors of the NTP Pentachloroanisole study (TR-414) did not identify iron in the Kupffer cell pigmentation, but stated that an association with hemosiderosis could not be ruled out. Author of the NTP CI pigment red 3 study (TR-407) stated that Kupffer cell pigmentation was related to hemosiderosis, but Nyska et al. (2004) attributed it to a compound metabolite. Pentachloroanisole exposure was associated with an increased incidence of hemangiosarcomas, but CI pigment red 3 did not cause an increased incidence of hemangiosarcomas.

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Other Possible Modes of Action for Liver Tumor Development in Male Mice

Although certain key events in EGBE's mode of action for the development of liver tumors in male mice are fairly well described and verified, (induction of hemolysis by BAA, induction of cell proliferation, and clonal expansion), some alternate considerations (also supported by scientific literature) may be involved. Reactive oxygen species can potentially be derived from two sources: iron overloading in the liver (through Fenton and Haber-Weiss reactions) and/or from Kupffer cell activation. Via either source, oxygen radicals can induce oxidative damage to DNA and lipids as documented in liver following EGBE treatment (Seisky et al., 2002). The activation of Kupffer cells (through phagocytosis of red blood cell hemolytic components and/or iron in the Kupffer cell), results in the production of cytokines, possibly including vascular endothelial growth factor that may elicit a growth response on endothelial cells. In addition to the production of oxidative DNA damage, reactive oxygen species, whether derived from Kupffer cell activation or other biological processes, can alter gene expression (e.g. MAP kinase/AP-1, and NFKB) resulting in stimulation of cell proliferation and/or inhibition of apoptosis (Klaunig and Kamendulis, 2004).

Because of the high background rate for hemangiosarcomas in male mice and the fact that the relationship between hemosiderin buildup and hemangiosarcomas is only apparent for male mice, it is reasonable to hypothesize that endothelial cell proliferation arise from the promotion of preexisting (spontaneously) initiated cells. However, this has not been established. The increased DNA synthesis and/or oxidative DNA damage caused by EGBE could result in the acquisition of new mutations in endothelial cells (tumor initiation) rather than a selective clonal expansion of initiated endothelial cells (tumor promotion).

Another well recognized mechanism for the development of chemically induced liver hemangiosarcomas involves direct interaction with DNA. This is the mode of action that is recognized for vinyl chloride and thorotrast, two chemicals that are known to induce hemangiosarcomas in humans. As discussed in Attachment 1, BAL is the EGBE metabolite considered to have the greatest potential to interact with DNA as it has been shown to cause in vitro SCE at concentrations ranging from 0.2 to 1 mM. However, high aldehyde dehydrogenase activity in the liver, as in the forestomach, is expected to results a very short residence time and low Cmax liver tissue concentrations of BAL. The Corley (2003) model discussed in Attachment 1 includes the metabolism of EGBE to BAL via alcohol dehydrogenase and the subsequent metabolism of BAL to BAA via aldehyde dehydrogenase in both the liver and forestomach. Using rate constants derived from mouse stomach fractions (Green et al., 2002) and making several assumptions about the use of these enzyme activity data (see discussion in Attachment 1 under "*Biological Plausibility and Coherence of the Database*"), Corley (2003) estimated that 250 ppm EGBE would result in peak C_{max} concentrations of 7 EGBE, 0.5 BAL and 3,250 BAA μM in liver tissue of male mice at the end of a 6 hour exposure period (Figure A2-1). Thus, the Corley (2003) PBPK model suggests that the conditions of the in vitro assays which show BAL to be clastogenic (e.g., no metabolic activation; high, cytotoxic concentrations of BAL) are of little relevance to expected target organ (liver) environment (e.g., high metabolic activity; low concentrations of BAL). A recent gavage study performed by Deiringer and Boatman (2004) provides support for the Corley (2003) model and the predicted low levels of the BAL metabolite in liver

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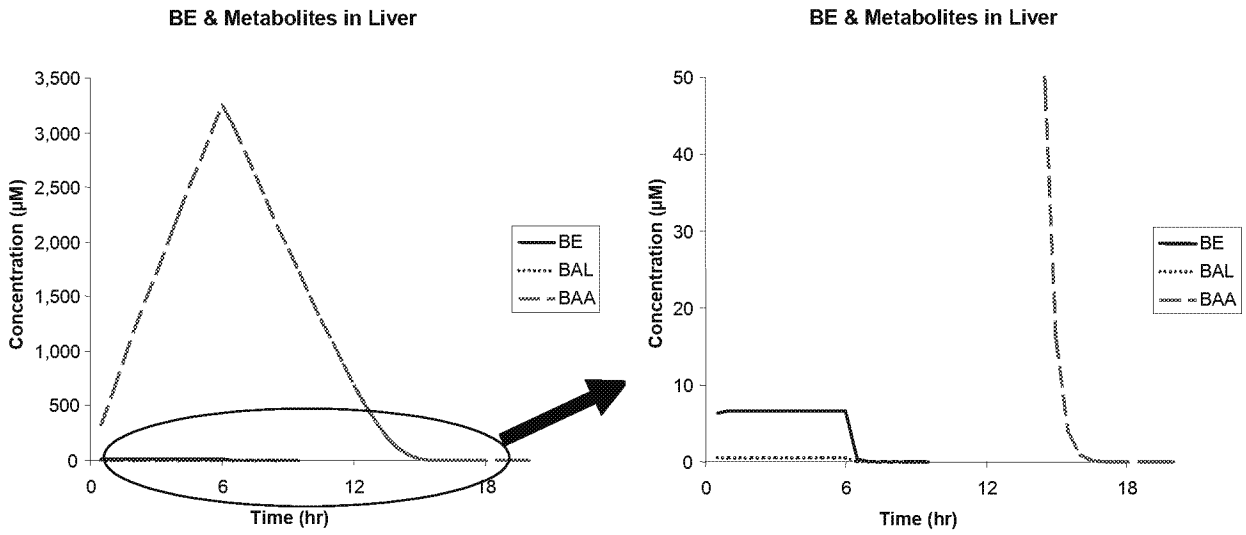

	Cmax (µM)
BE	7
BAL	0.5
BAA	3,250

Figure A2-1: Concentrations of BE, BAL and BAA in liver tissues of female mice exposed to 250 ppm

tissue.[27] Also, as is discussed in detail in Attachment 1 under *Other Possible Modes of Action for Forestomach Tumor Development in Female Mice*, evidence from *in vivo* and *in vitro* genotoxicity assays do not support the idea that BAL would have any significant genotoxicity *in vivo*.

Further, the mode of action for hemangiosarcoma induction by genotoxic chemicals such as vinyl chloride and thorotrast involves the initiation of hepatocellular and sinusoidal cell hyperplasia and sinusoidal compression leading to the development of fibrous septa, generally in the peri-portal area, out of which eventually develop multiple areas of angiosarcomas (Foster, 2000). EGBE exposure does not generate this same pattern of effects prior to the development of cancer in mice. The main non-neoplastic effect of EGBE on the liver is an accumulation of hemosiderin (iron) within the Kupffer cells following a chronic elevated breakdown of the red blood cells with few other precursor lesions reported.

[27] The Corley (2003) model predicts that the concentrations of BAL in liver tissues of male and female mice would be 17 and 29 µM, respectively, following oral gavage exposure to 600 mg/kg EGBE. The levels of BAL actually observed in the liver tissue of male and female mice, following oral gavage exposure to EGBE at 600 mg/kg 3 and 4 µM, respectively, were even lower than the predicted values (Deiringer and Boatman, 2004).

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Relevance of Mouse Liver Hemangiosarcomas and Hepatocellular Carcinomas to Humans

According to the latest research findings, it is quite likely that hemolysis represents a key event in the formation of liver tumors in male mice following EGBE exposure. If the increased incidence of male mouse liver hemangiosarcoma and hepatocellular carcinoma are indeed secondary effects of hemolysis, they are not likely to be relevant to humans. Iron overload in humans either exposed to excess iron or genetically susceptible due to defects in iron metabolism has been associated with the induction of liver tumors (Mandishona et al., 1998; Stevens et al., 1994). However, humans are not likely to experience significant iron buildup from EGBE exposure because they are comparatively less sensitive to the hemolytic toxicity of EGBE than rats and mice. Human volunteers experienced no hemolysis from controlled laboratory acute inhalation exposures (up to 195 ppm) that caused significant erythrocyte fragility in rats (Carpenter et al., 1956); and only mild, reversible hemolytic effects have been observed in humans acutely exposed to oral doses EGBE (400 to 1500 mg/kg) that have been shown to cause marked, and in some cases irreversible, hemolytic effects in rats (Ghanayem et al., 1987b; Grant et al., 1985; U.S. EPA, 1999b). In vitro testing suggests that blood concentrations of the hemolytically active metabolite BAA must reach levels in human blood in excess of 7.5 mM for even minimal prehemolytic changes to occur. This blood level of EGBE is 15-fold higher than the blood level at which comparable effects occur in rats (Udden, 1995; 2002) and is significantly higher than the maximum blood concentrations of approximately 2 mM predicted by PBPK modeling for the highest, theoretically saturated (~1160 ppm), air concentrations of EGBE (Corley et al, 1994).

As is discussed below, some attempts have been made to identify susceptible human subpopulations (Udden, 1994; 1995, 2002). While no sensitive human subpopulation has been identified to date, there is some reason to believe that humans may vary considerably in their ability to metabolize and excrete EGBE and in their response to EGBE exposure . Both alcohol and aldehyde dehydrogenase enzymes are polymorphically distributed in humans. As discussed below under *Metabolic and genetic differences*, these polymorphisms have been shown to alter rates of metabolism and elimination of other alcohols and aldehydes (Agarwal and Goedde, 1992). As work in this area continues, further information on the metabolic or structural differences that result in the lower sensitivity of human RBCs compared to rat RBCs may eventually illuminate characteristics in the human population that may indicate increased susceptibility. However, existing work indicates that the usual human subpopulations of concern, including aged, sickle-cell anemia, hereditary spherocytosis patients (Udden, 1994) and children (Udden, 2002) are not be sensitive to the hemolytic effects of EGBE. It should be recognized, however, that effects in humans from chronic exposure to EGBE have not been studied, and the case reports of acute exposures and short-term (4 hour on average) in vitro tests discussed above may not accurately reflect the potential for cumulative damage from low-level chronic EGBE exposure.

In addition to the pharmacodynamic differences described above, there are also pharmacokinetic differences between the rodent and human response to EGBE exposure (Figure A2-2). The two main oxidative pathways of EGBE metabolism observed in rats are alcohol dehydrogenase and O-dealkylation by a cytochrome P450 dealkylase (CYP 2E1) (Medinsky et al., 1990). The former pathway, which involves the production of the toxic metabolite BAA, is applicable to both rats and humans. However, unlike rats, approximately two-thirds of the BAA formed by humans is conjugated with glutamine and glycine (Corley et al., 1997; Rettenmeier et al., 1993). Thus, the glutamine and glycine detoxification pathways may provide humans with some measure of added protection from the harmful effects of BAA.

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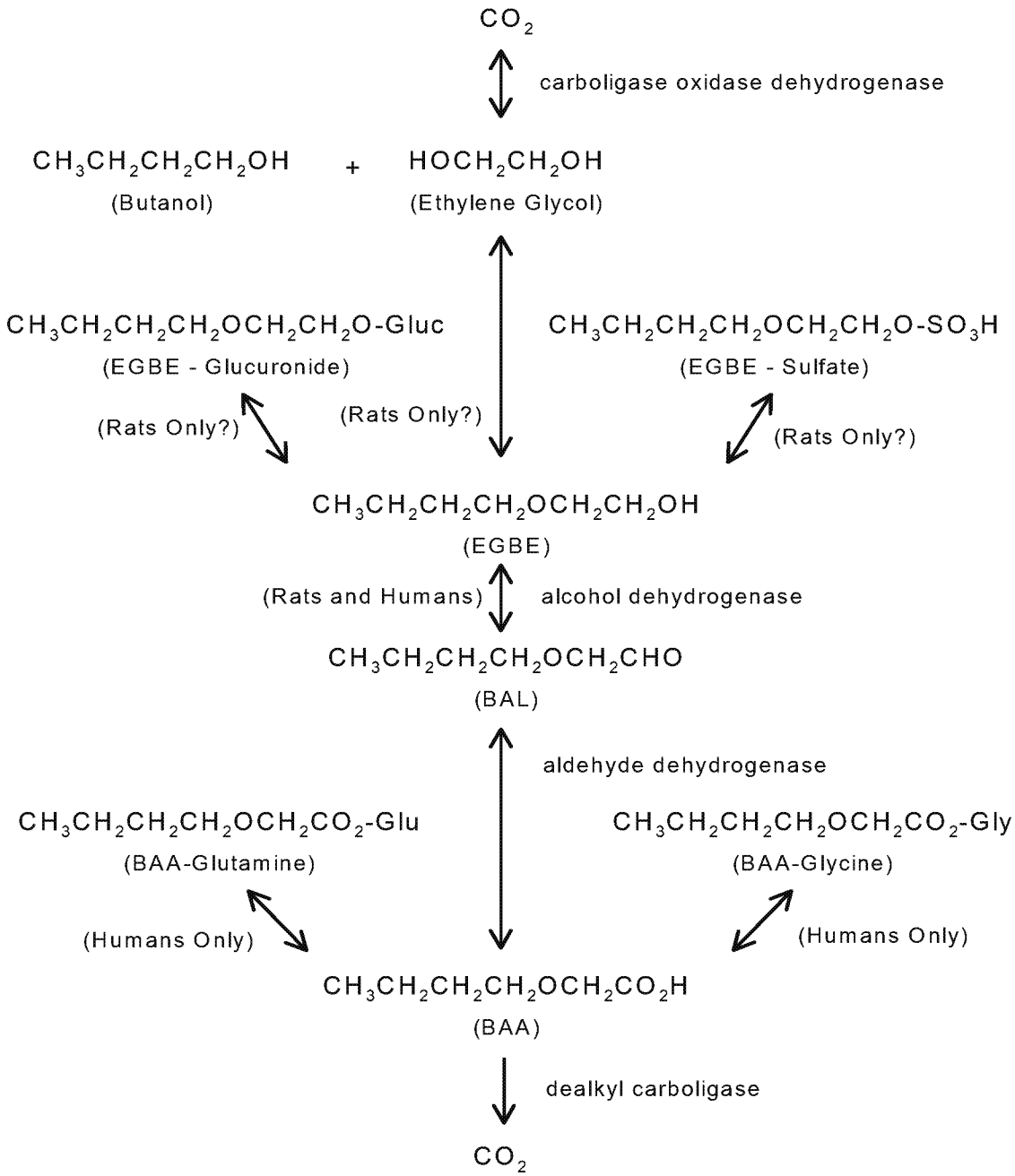

Figure A2-2: Proposed metabolism of EGBE in rats and humans (Adapted from Medinsky et al., 1990 and Corley et al., 1997)

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Relevance to Susceptible Subpopulations, Including Children

Differences in susceptibility to hemolysis - The primary noncancer effect of EGBE is hemolysis of red blood cells, which is caused by its primary metabolite, BAA. There is no evidence for the existence of human subpopulations with increased susceptibility to red blood cell lysis caused by BAA. However, Udden (1994) has shown that the RBCs of normal and aged patients and patients with sickle-cell anemia and hereditary spherocytosis are all equally resistant to the hemolytic effects of BAA.

Metabolic and genetic differences - Other potentially susceptible subpopulations include individuals with enhanced metabolism or decreased excretion of BAA. Polymorphisms in alcohol and aldehyde dehydrogenases could lead to differences in the metabolism and elimination of EGBE in some humans. Human genetic polymorphisms in alcohol dehydrogenase and aldehyde dehydrogenase are prevalent in certain ethnic groups (Chan, 1986) and these polymorphisms have been shown to alter rates of metabolism and elimination of ethanol and acetaldehyde (Agarwal and Goedde, 1992). For instance, native Americans and approximately 50% Asian people are deficient in aldehyde dehydrogenases. Aldehyde dehydrogenases comprises more than nine isoforms in humans (Hsu et al., 1994). A deficiency or loss of one of them (ALDH2), can lead to a nearly complete loss of enzymatic activity for structurally similar aldehydes (e.g., methoxyacetaldehyde), in human liver mitochondria fractions (Crabb et al., 1989; Kitagawa et al., 2000). Individuals with atypical alcohol dehydrogenase and/or deficient aldehyde dehydrogenase appear to be more susceptible to adverse effects from increased levels of acetaldehyde including facial flushing, general discomfort, acetaldehyde-protein adducts and alcohol-induced liver diseases (Agarwal and Goedde, 1992). However, the PBPK model developed by Corley et al. (2004) indicates that individuals with low aldehyde dehydrogenase activity (½ Vmax) would not be expected to accumulate significant BAL levels in the liver or forestomach,[28] even following inhalation exposure to a theoretical maximum of 1160 ppm EGBE for 6 hours (U.S. EPA, 2004).

Haufroid et al. (1997) conducted a human study on workers exposed to EGBE to test the possible influence of genetic polymorphism for CYP 2E1 on urinary BAA excretion rate. One exposed individual exhibited a mutant allele with increased cytochrome P450 oxidative activity that coincided with a very low urinary BAA excretion. However, the researchers did not measure BAA conjugated to glutamine, an alternative pathway for BAA excretion in humans. Further investigations on the influence of genetic polymorphism for CYP 2E1 on urinary BAA excretion rate are needed before any firm conclusions can be drawn.

Gender differences - Slight gender differences have been noted in rodent (Carpenter et al., 1956; Dodd et al., 1983; NTP, 1993b; NTP, 2000a), rabbit (Tyler, 1984), dog, monkey, and human studies (Carpenter et al., 1956), with females being consistently more susceptible to the primary hemolytic effects of EGBE. A number of secondary effects resulting from the hemolytic toxicity of EGBE, such as effects on the rat liver, kidneys, spleen, and bone marrow and, to a lesser extent, the thymus, are more pronounced in females (NTP, 1993b). In the process of studying and comparing the metabolic and cellular basis of EGBE-induced hemolysis, Ghanayem (1989) observed that the blood from female human volunteers showed a slightly greater sensitivity to incubation with BAA than male blood.

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[28]Predicted BAL concentrations were below 0.001 mM in the liver and 0.004 mM in the GI tract following inhalation exposure to saturated air concentrations of EGBE. These concentrations are considerably lower than concentrations of BAL shown to be clastogenic (0.2 mM) or hemolytic (0.5 mM: Ghanayem et al., 1989) in vitro.

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Age differences - It is generally recognized that children have fewer risk factors for anemia than adults (Berliner, et al. 1995; Hord and Lukens, 1999), and Udden (2002) has shown that the red blood cells of children are no more sensitive to EGBE induced hemolytic effects than adult red blood cells. Childhood exposures can be important determinants of certain cancers (Anderson et al., 2000), including early childhood bacterial infections (e.g. *Helicobacter pylori*) (Rowland and Drumm, 1998); and because bacterial infection (*Helicobacter hepaticus*) has also been associated with the development of liver hemangiosarcomas in mice (Nyska et al., 1997), it is not unreasonable to consider whether early childhood exposure to EGBE might also be more important than adult exposures towards the formation of EGBE induced liver hemangiosarcomas. However, if the proposed mode of action for EGBE's involvement in the formation of these tumors in male mice is correct, there is no reason to believe that exposed human children would experience significant buildup of iron from hemolysis unless large toxicokinetic or toxicodynamic differences between adults and children are identified.

The only human toxicity information available on the toxicity of EGBE to children is from the case study by Dean and Krenzelok (1991), who observed 24 children, age 7 mo to 9 years, subsequent to oral ingestion of at least 5 mL of glass window cleaner containing EGBE in the 0.5% to 9.9% range (potentially 25 to 1500 mg EGBE exposures). No symptoms of EGBE irritation, poisoning, or hemolysis were reported.

Adult (9-13 wk) male F344 rats were significantly more sensitive to the hemolytic effects of EGBE than were young (4-5 wk) male rats following administration of a single gavage dose of EGBE at 32, 63, 125, 250, or 500 mg/kg. In concurrent metabolism studies, increased blood retention of EGBE metabolite BAA (as measured by increased C_{max}, AUC, and $T_{½}$) was also found. Additionally, young rats eliminated a significantly greater proportion of the administered EGBE dose as exhaled carbon dioxide (CO_2) or as urinary metabolites as well as excreting a greater proportion of the EGBE conjugates (glucuronide and sulfate) in the urine (Ghanayem et al., 1987c; 1990). These researchers suggested that the pharmacokinetic basis of the age-dependent toxicity of EGBE may be due to a reduced ability by older rats to metabolize the toxic metabolite BAA to CO_2 and a diminished ability to excrete BAA in the urine.

NTP (1998) also found that young mice (6-7 weeks) eliminated BAA 10-times faster than aged (19 months) following a 1-day exposure to 125 ppm EGBE. This difference was not as apparent after 3 weeks of exposure. Dill et al. (1998) have suggested that this may be related to a greater sensitivity to the acute toxicity of EGBE in older animals that appears to be compensated within 2-3 weeks.

Developmental studies, which may also be of possible relevance to this issue, have been conducted using rats, mice, and rabbits dosed orally, by inhalation or, in one study, dermally (Hardin et al., 1984; Heindel et al., 1990; Nelson et al., 1984; NTP, 1993b; Sleet et al., 1989; Tyler, 1984; Wier et al., 1987). Maternal toxicity related to the hematologic effects of EGBE and relatively minor developmental effects were reported in most studies. No teratogenic toxicities were noted in any of the studies. It can be concluded from these studies that EGBE is not significantly toxic to developing fetuses of laboratory animals.

Older rats have been shown to have a reduced ability to metabolize the toxic metabolite BAA to CO_2 and a diminished ability to excrete BAA in the urine (Ghanayem et al.,1987b, 1990). However, the relevance of this finding to the possible susceptibility of elderly humans is uncertain due to the fact that humans have conjugation pathways for the excretion of BAA (BAA-Glutamine and BAA-Glycine) that are not available to the rat.

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Summary

Available data establish a plausible nonlinear, nongenotoxic mode of action for the moderate increase observed by NTP (2000) in the incidence of liver tumors in male mice following chronic inhalation exposure to EGBE. The proposed mode of action suggests that the endothelial cells and hepatocytes of male mice are sensitive to the formation of the subject neoplasms (as evidenced by the relatively high background rate of these tumors in male mice) and that excess iron from EGBE-induced hemolysis can result in sufficient iron-induced oxidative stress to cause the observed, marginal increase in the incidence of liver hemangiosarcomas and hepatocellular carcinomas in these animals (NTP, 2000). Given the relatively low sensitivity of humans, including subpopulations such as children, to the hemolytic effects of EGBE, it appears reasonable to assume that the EGBE RfC and RfD (EPA, 1999a), which were based on hemolytic effects in female rats, are sufficient for the prevention of hemolysis and associate tumors in humans.[29] However, this determination assumes a nonlinear mechanism that requires exposure levels to be high enough to cause certain lesions that are considered to be precancerous. Information is currently inadequate to dismiss the potential contribution of a linear mechanism associated with the possible mutagenic metabolite BAL. A definitive determination regarding the appropriateness of a nonlinear approach can not be made until questions regarding the role of BAL are resolved. As discussed above, additional research (e.g., verification of existing PBPK modeling results and improved genotoxicity assays) would assist the Agency in making a more informed decision concerning the potential for BAL to contribute to the adverse effects seen in animals following EGBE exposure and use of the proposed nonlinear assessment approach.

[29]These analyses are consistent with the nonlinear assessment approach described in existing interim (U.S. EPA, 1999a) and draft (2003) cancer guidelines.

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ATTACHMENT 3

Benchmark Dose Assessment of Forestomach Lesions in Female Mice Using PBPK Models to Estimate Human Equivalent Exposures

Several PBPK models have been developed for EGBE, all of which are capable of estimating internal doses. These models are summarized briefly in the EGBE IRIS file (EPA, 1999). Consistent with the EPA (1999) IRIS assessment, the Lee et al. (1998) was used to estimate internal dose levels from inhalation exposures to the experimental animal, in this case the female mouse.

C_{max} (peak concentration in the blood during the exposure period) of BAA was used in the existing IRIS file (EPA, 1999) for the derivation of the RfD and RfC from hematological endpoints because of (a) convincing evidence that BAA is the causative agent for EGBE-induced hemolysis and (b) EGBE-induced hemolysis appears to be highly dependent upon the BAA concentration attained. Similarly, BAA is believed to be the toxic moiety responsible for the forestomach effects observed following EGBE exposure (Attachment 1) and concentration appears to critical in the development of these effects as well. No signs of forestomach irritation were observed in mice at very high dose levels of 1400 mg/kg/day in 2-week and 13-week drinking water studies conducted by NTP (NTP, 1993b). It has been suggested that such oral non-bolus dosing of EGBE does not result in high enough local concentrations of EGBE and BAA (Poet et al., 2003). Previous studies with other nongenotoxic forestomach carcinogens demonstrated that forestomach effects are dependent not only on the dose but also on the chemical concentration in the dosing solution (Ghanayem et al., 1985) and other effects of EGBE appear to be highly dependent on the concentration attained (Nyska et al., 1999; Long et al., 2000; Ghanayem et al., 2000; 2001). Use of blood concentrations as a common dose metric is also justifiable because, as is discussed in Attachment 1, systemic distribution of inhaled EGBE via the blood to the salivary glands makes an important contribution to target organ dose in mice (via the swallowing of saliva) and because humans would not be expected to receive exposure via the other major distribution route in the mouse, oral ingestion through the grooming of fur.

The endpoint used in this analysis was epithelial hyperplasia of the female mouse forestomach as it was the most sensitive forestomach effect observed in the NTP (2000) study. Consistent with the 1999 IRIS assessment, four steps were employed to estimate human equivalent oral and inhalation benchmark exposures from this endpoint: (1) a $BMDL_{10}$ value was estimated using modeled "end of the week" internal dose (C_{max} BAA in blood) levels; (2) verify that steady state was achieved (e.g., no change in BAA C_{max} as a result of prolonging the exposure regimen); (3) simulate the internal dose surrogate (C_{max} BAA in blood) for humans (continuous air exposure; drinking water assumption was that a 70-kg human consumes an average of 2 liters of water during a 12-hour awake cycle); and (4) calculate the human equivalent dose/concentration that resulted in the same internal dose (C_{max} BAA) simulated for the animal in Step 1.

STEP 1: ESTIMATION OF $BMDL_{10}$ (C_{max}) DOSE

C_{max} for BAA in arterial blood was determined using the PBPK model of Lee et al. (1998). The results of this model and incidence data for the endpoint of concern are summarized in Table A3-1.

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Table A3-1. PBPK Model estimates of BAA C_{max} blood levels and incidence of forestomach epithelial hyperplasia in female mice.

Water Conc. (ppm)	C_{max} BAA (uM/L)	Incidence of Forestomach Hyperplasia
0	0	0/50
62.5	529	6/50
125	1200	27/50
250	2620	42/49

BMD and $BMDL_{10}$ estimates were derived using the available models in version 1.3.2 of the EPA benchmark dose software.[30] The estimates for each model, along with statistical goodness-of-fit information are provided in Table A3-2.

Table A3-2. BMDS model estimates of C_{max} BMD_{10} and $BMDL_{10}$ values for forestomach epithelial hyperplasia in female mice.

BMDS Model	BMD (uM/L)	BMDL (uM/L)	AIC (Lowest ≈best fit)	P-value (>0.1 = adequate fit)
Gamma	420.56	266.87	151.16	0.5287
Logistic	544.757	444.896	162.191	0.0067
Log-Logistic	462.513	329.04	150.153	0.8717
Multistage (1st degree)	177.442	145.713	156.244	0.0648
Multistage (2nd degree)	338.483	202.437	152.681	0.0976
Multistage (3rd degree)	338.485	197.436	152.681	0.2535
Probit	525.521	430.612	161.304	0.0086
Log-Probit	470.876	344.412	150.163	0.8673
Weibull	376.085	238.952	151.855	0.3807

Considering all goodness-of-fit parameters, including chi-square residuals at low doses and visual inspection of plots, the Log-Logistic model was chosen as the model that best describes the dose-response for this endpoint. Graphical results of this model are provided in Figure A3-1. A textual description (model output) of these results is provided in Appendix A. The $BMDL_{10}$ was determined to be 329 μM/L, using the 95% lower confidence limit of the dose-response curve expressed in terms of the C_{max} for BAA in blood.

[30] A copy of the BMDS can be obtained from the Internet at www.epa.gov/ncea/bmds.htm.

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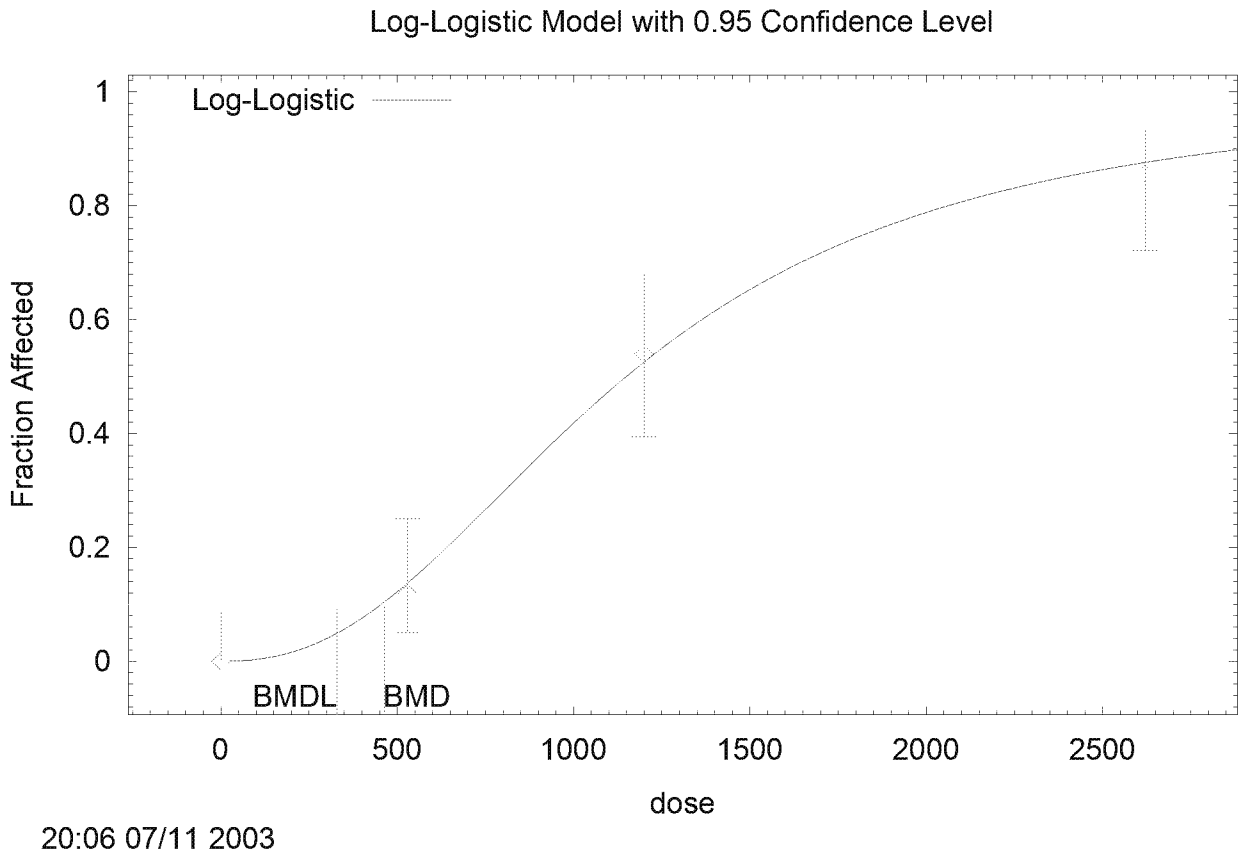

Figure A3-1. BMD plot of fraction of female mice with forestomach epithelial hyperplasia following inhalation exposure(NTP, 2000) vs. internal dosimetric (BAA C_{max}, µM/L).

Step 2: Verification of Steady State

As can be seen from Table A3-3, C_{max} levels are relatively constant through 6 months, then increase at and beyond 12 months, presumably due to clearance problems in aging animals. However, the earlier steady state levels are appropriate for use in this assessment because that is the more conservative approach and because similar effects were observed during the subchronic portion of the NTP (2000) study at the same dose levels, indicating that the higher internal doses at and beyond 12 months were not required for the effects to appear.

Table A3-3: Female Mouse C_{max} Levels for Various Time Points of NTP (2000) Study Estimated by the Lee et al. (1998) Model

Months on Study	62.5 ppm		125 ppm		250 ppm	
	Male	Female	Male	Female	Male	Female
1	403	529	921	1200	2080	2620
3	402	527	925	1202	2120	2652
6	399	523	914	1184	2071	2582
12	484	639	1079	1414	2349	2951
16	643	849	1443	1839	2798	3501
18	756	995	1625	2102	3067	3803

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Step 3: Simulation of Internal Human Doses

The tables below summarize the results of model simulations of the internal dose surrogate (C_{max} BAA in blood) for a 70-kg human who consumes an average of 2 liters of drinking water during a 12-hour awake cycle (Table A3-4) or is continuously exposed to air concentrations (Table 5) of EGBE.

Table A3-4: Estimated C_{max} for BAA in blood for humans continuously exposed to varying drinking water concentrations of EGBE (Corley et al, 1994; 1997).

EGBE concentration in water (ppm)	Calculated dose of EGBE from drinking water (mg/kg/d)	C_{max} BAA in blood (μM/L)
24	0.7	9
48	1.4	18
94	2.7	36
188	5.4	73
375	10.7	147
750	21.4	299

Table A3-5: Estimated C_{max} for BAA in blood for humans continuously exposed to varying concentrations of EGBE (Corley et al, 1994; 1997).

Concentration of EGBE in air (ppm)	C_{max} BAA in blood (μM/L)
1	2.6
5	13.0
10	26.1
20	52.9
50	137.1
100	295.0
200	733.7

Step 4: Calculate the Human Equivalent Dose/concentration

The Corley et al. (1994; 1997) PBPK model was used to "back-calculate" a human equivalent oral dose of 23.6 mg/kg-day from the C_{max} $BMDL_{10}$ of 320 μM/L estimated in Step 1, assuming that rats and humans receive their entire dose of EGBE from drinking water over a 12-hr period each day.

The Corley et al. (1997) PBPK model was used to "back-calculate" human equivalent air concentration of 113 ppm (551 mg/m^3) from the C_{max} $BMDL_{10}$ of 320 μM/L estimated in Step 1, assuming continuous exposure (24 hr/day).

These results indicate that the RfD and RfC values for EGBE, which were based on hemolytic effects in female rats and lower human equivalent $BMDL_{10}$ estimates for both water (5.1 mg/kg/day) and air (380 mg/m^3), should be adequate for the prevention of gastrointestinal hyperplastic effects.

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APPENDIX A

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==============================================================================
        Logistic Model $Revision: 2.1 $ $Date: 2000/02/26 03:38:20 $
        Input Data File: F:\BMDS\DATA\EGBE\F_MOUSE_HYP_LOG-LOGIST.(d)
        Gnuplot Plotting File:  F:\BMDS\DATA\EGBE\F_MOUSE_HYP_LOG-LOGIST.plt
                                                    Fri Jul 11 19:53:31 2003
==============================================================================

BMDS MODEL RUN
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

  The form of the probability function is:

  P[response] = background+(1-background)/[1+EXP(-intercept-slope*Log(dose))]


  Dependent variable = F_Hyperplasia
  Independent variable = Dose
  Slope parameter is restricted as slope >= 1

  Total number of observations = 4
  Total number of records with missing values = 0
  Maximum number of iterations = 250
  Relative Function Convergence has been set to: 1e-008
  Parameter Convergence has been set to: 1e-008


  User has chosen the log transformed model


            Default Initial Parameter Values
              background =          0
               intercept =     -16.768
                   slope =     2.36735


       Asymptotic Correlation Matrix of Parameter Estimates

       ( *** The model parameter(s)  -background
           have been estimated at a boundary point, or have been specified by the user,
           and do not appear in the correlation matrix )

         intercept       slope

intercept          1          -1

    slope         -1           1
```

Parameter Estimates

Variable	Estimate	Std. Err.
background	0	NA
intercept	-16.7132	2.64108
slope	2.36545	0.372243

NA - Indicates that this parameter has hit a bound implied by some inequality constraint and thus has no standard error.

Analysis of Deviance Table

Model	Log(likelihood)	Deviance	Test DF	P-value
Full model	-72.9391			
Fitted model	-73.0765	0.274637	2	0.8717
Reduced model	-131.841	117.804	3	<.0001

AIC: 150.153

Goodness of Fit

Dose	Est._Prob.	Expected	Scaled Observed	Size	Residual
0.0000	0.0000	0.000	0	50	0
529.0000	0.1324	6.622	6	50	-0.2596
1200.0000	0.5145	25.725	27	50	0.3608
2620.0000	0.8705	42.653	42	49	-0.2777

Chi-square = 0.27 DF = 2 P-value = 0.8717

Benchmark Dose Computation

Specified effect = 0.1

Risk Type = Extra risk

Confidence level = 0.95

BMD = 462.513

BMDL = 329.04

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ATTACHMENT 4
External Peer Review—Summary of Comments and Disposition

The following are summaries of the recent external reviews that this position paper has undergone, a 2003 Letter Review of the draft position paper and a 2004 panel review of the interim final position paper and technical reports submitted in response to the Agency's November, 2003 proposal to delist EGBE from the list of CAA hazardous air pollutants. The focus of the summaries and EPA responses is on the comments which stated an opinion contrary to the draft or interim final position paper.

2003 Letter Review Comments and Responses (Blue)

Genotoxicity of EGBE or its Metabolites

1. Has the position paper drawn appropriate conclusions from the available literature on the genotoxicity of EGBE and its metabolites?

Three reviewers agreed that Butoxyacetaldehyde (BAL) is the most likely of the metabolites to pose genotoxic risks. However, they did not consider this risk to be significant for the following stated reasons:

1. Rapid conversion of BAL to BAA in vivo
2. Lack of evidence for BAL in rodent tissues
3. Studies indicating that aldehydes are rapidly cleared by metabolism
4. Negligible macromolecular adducts formed in vivo following EGBE exposures
5. Negative results for EGBE in in vitro w/ metabolic activation & in vivo genetox assays
6. General lack of genetox activity by other members of the glycol ether class
7. Largely negative results of in vitro genetox assays of BAL
8. Studies suggesting that DNA adducts formed by aldehydes are relatively easily repaired
9. Historical evidence of forestomach tumor formation by other non-genotoxic agents
10. Historical evidence that aldehydes carcinogenic by direct application to sensitive tissues (*e.g.* in gas phase, respiratory system) are not carcinogenic when metabolically generated

One reviewer suggested expanding the statement on page A1-13, line 7 to say "…genotoxicity assays are in agreement that neither EGBE nor its metabolites are likely to be genotoxic in vivo."

One reviewer agreed with conclusions regarding EGBE and BAA, but was not certain about BAL.

One reviewer felt that the likely modest genotoxicity of BAL was not fairly and adequately evaluated. This reviewer felt that EPA cannot exclude that some portion of the observed carcinogenic responses in the forestomach and liver is attributable to a genetic mechanism and recommended a combined "linear/nonlinear" approach.

RESPONSE: All reviewers agreed that the evidence supports the contention that EGBE and BAA are not genotoxic. Reviewers disagreed with respect to the primary genotoxicity issue identified, the potential for the EGBE metabolite BAL to directly interact with DNA following in vivo EGBE exposure. The position paper has been enhanced to address the potential role of

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BAL, and an effort to model the concentrations of EGBE, BAL and BAA in the stomach and liver of mice is included. Corley (2003) estimated that 250 ppm EGBE would result in peak C_{max} concentrations of 48 μM EGBE, 1.1 μM BAL and 3,200 BAA μM in GI tissue and 7 μM EGBE, 0.5 μM BAL and 3,250 BAA μM in liver tissue of mice at the end of a 6 hour exposure period (see Figures A1-1 and A2-1). This modeling effort is limited by the minimal metabolic rate information available for the formation and distribution of BAL, but does indicate that expected in vivo BAL concentrations in the subject target organs would be significantly lower than BAL concentrations found to be clastogenic in vitro assays (without metabolic activation). Nevertheless, the Agency agrees with two of the five reviewers that information is currently inadequate to completely dismiss the potential contribution of a linear mechanism associated with the possible mutagenic metabolite BAL. A definitive determination regarding the appropriateness of a nonlinear approach can not be made until questions regarding the role of BAL are resolved. Additional research (e.g., verification of existing PBPK modeling results and improved genotoxicity assays) would assist the Agency in making a more informed decision concerning the potential for BAL to contribute to the adverse effects seen in animals following EGBE exposure and use of the proposed nonlinear assessment approach. The role of BAL towards the effects observed in mice following EGBE exposure is addressed further below.

2. *Is there additional information (e.g., from other studies or studies of related compounds) that would suggest an alternative conclusion?*

Three reviewers said no. One of these reviewers stated that the only direct evidence to the contrary is the small number of equivocally positive results in clastogenicity experiments [with EGBE in vitro], but that this type of experiment is notoriously susceptible to direct toxic effects at the cellular level, which are not reflective of the situation in vivo.

One reviewer suggested that the mutagenic/clastogenic potency of butoxyacetaldehyde needs to be studied in relation to analogous activities by other aldehydes—e.g. ethoxyacetaldehyde, methoxyacetaldehyde, formaldehyde, acetaldehyde, propionaldehyde, and possibly butyraldehyde

One reviewer offered that BAL causes cytotoxicity (30% increase) and SCE (2-fold increase) in vitro in human lymphocyte cells at 0.5 mM (Ghanayem and Thompson, unpublished data).

RESPONSE: The new data concerning the cytotoxicity and clastogenicity of BAL to human lymphocyte cells in vitro (obtained from Ghanayem, 2003) and its relevance to the effects observed following in vivo exposure to EGBE are discussed in the revised position paper. Some additional discussion of the expected toxicity of BAL relative to other aldehydes has been added as well as it is recognized that similar issues have been raised regarding the extent to which other aldehydes such as the acetaldehyde metabolite of ethanol contribute to genotoxicity (Lipscomb, 2003; Dewoskin, 2003). BAL has been shown to be cytotoxic and clastogenic to various cells in vitro and it is possible that the weak positive results seen in some of the in vitro assays of EGBE (Elias et al., 1996, Table 1) are due to a low rate of metabolism of the alcohol to BAL. However, cytotoxicity itself is a recognized contributor (promoter) to carcinogenesis, and can effect natural events in the cell cycle and cause a reduction in the repair of SCE, increases in SCE. Given the low concentrations of BAL that are expected in vivo following EGBE exposure (see discussions in revised position paper and below) and the fact that increases in SCE were only observed following cytotoxic, in vitro exposures to BAL, it is unlikely that the above hypothesis regarding the role of BAL in vitro would hold true for in vivo exposures to EGBE. However, the available data do not allow for a definitive statement in this regard. An

understanding of the potential role of BAL in the formation of the noncancer and cancer lesions observed in mice (NTP, 2000) is key to this determination and is discussed further below and in the revised position paper.

Role of Butoxyacetaldehyde (BAL)

Elliot and Ashby (1997) concluded that EGBE and butoxyacetic acid (BAA) are not genotoxic, but that BAL, an intermediate oxidation product, shows some evidence of clastogenicity in vitro. Dartsch et al. (1999) attributed the cytotoxic effects of EGBE observed in vitro to BAL. However, BAL has not been identified in vivo, and studies of EGBE in forestomach and glandular stomach tissue supernatant (Green et al., 2002) and liver (Medinsky et al., 1990; Corley et al, 1997; Ghanayem et al., 1987) shows that it is rapidly metabolized to BAA by aldehyde dehydrogenase. In addition, despite the fact that metabolism of EGBE to BAL is reversible and metabolism of BAL to BAA is irreversible, BAA is significantly more toxic to the blood and forestomach of rodents than EGBE (U.S. EPA, 1999; Green et al., 2002).

3. *Is the position paper justified in determining that BAL did not significantly contribute to the EGBE induced noncancer and cancer effects observed in rats and mice (NTP, 2000)?*

Three reviewers responded yes. One reviewer stated while this conclusion is not strictly supported by the available data, the weight of evidence clearly supports this conclusion.

Two reviewers responded no. These reviewers offered the following facts as support of the potential contribution of BAL to the noncancer and cancer effects associated with EGBE exposure.

1. Pretreatment of rats with cyanamide, an aldehyde dehydrogenase inhibitor, reduced EGBE-induced hemolytic responses, but increased RBC swelling, increased the mortality caused by EGBE exposure, decreased BAA formation and excretion in the urine, and increased the urinary excretion of EGBE conjugates with glucuronide and sulfate.
2. Pretreatment of rats with pyrazole, an alcohol dehydrogenase inhibitor, protected against EGBE-induced hemolysis
3. In vitro studies demonstrated that:
    a. While BAA is more potent, the effects of BAA and BAL on RBCs are qualitatively similar.
    b. At 0.25-4.0 mM, BAL decreased viability and survival of peripheral human lymphocytes in a dose-dependent manner.
    c. At 0.5 mM, BAL doubled the number of SCE/cell vs. the vehicle (DMSO)
    d. No significant effects were observed using BAA at equimolar concentrations.
4. EGBE was more cytotoxic than BAA (as measured by LDH release) at comparable concentrations (25 and 50 μM) in cell culture (Park et al., 2002).

They also offered the following observations/suggestions:

1. Individuals with genetic polymorphisms in EGBE metabolizing enzymes may accumulate BAL and represent a sensitive subpopulations to EGBE exposure.
2. Extend existing PBPK models for EGBE to at least address the quantitative issue of how much concentration X time of the two metabolites can be expected to have been present under the conditions of dosage of the bioassay experiments.

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RESPONSE: The limitations and value of the in vitro and inhibition studies are addressed extensively in the revised position paper. The Elliot and Ashby (1996) review of the genetic toxicity studies relevant to EGBE published through 1996 concluded that EGBE is nongenotoxic, but that "the corresponding aldehyde [BAL] presents some evidence for clastogenicity in vivo, and that the derived acid [BAA] is non-genotoxic." This conclusion is supported by what is know about chemicals of similar structure (e.g. ethanol, acetaldehyde and acetic acid) and unpublished data showing that BAL causes cytotoxicity (30% increase) and sister-chromatid exchanges (2-fold increase) in vitro in human lymphocyte cells at 0.5 mM (Ghanayem and Thompson, unpublished data).

The primary issue of greatest relevance to this position paper is BAL's contribution to the carcinogenic effects of EGBE. However, the concern expressed by two reviewers regarding the contribution of BAL to noncancer effects (hemolytic and forestomach irritation) will be addressed first. The paper by Ghanayem et al. (1987) does show that some significant hemolytic activity remains when animals exposed to EGBE are pretreated with an aldehyde dehydrogenase inhibitor, cyanamide. However, several factors suggest that this activity is more likely due to residual BAA rather than BAL. Inhibitors such as cyanamide and pyrazole are not very specific. Thus, some BAA will be formed and, in fact, Ghanayem et al., (1990) found that while EGBE + cyanamide decreased BAA concentrations in rats, it also increased the half-life of BAA. In addition, when Ghanayem et al. (1987) administered a gavage dose of 125 mg BAL/kg + cyanamide to rats they observed almost no hemolytic activity (Ghanayem et al., 1987). Also, gavage administration to rats of 125 mg EGBE/kg and the molar equivalent of BAL and BAA resulted in no significant difference between the hemolytic effects of the three chemicals between 2 and 24 hours after exposure (Ghanayem et al., 1987). These facts suggest that EGBE's hemolytic activity is due almost entirely to BAA, and that the metabolism of EGBE and BAL to BAA takes place rapidly and completely. The ATSDR (1998) profile for EGBE states that:

> **Incubation of rat erythrocytes with butoxyacetaldehyde or 2-butoxyacetic acid caused time- and concentration-dependent swelling of red blood cells followed by hemolysis, which has also been observed in vivo (Ghanayem et al. 1990b). Butoxyacetaldehyde was less effective in causing hemolysis than was 2-butoxyacetic acid, suggesting that, although whole rat blood may contain enough aldehyde dehydrogenase to cause some conversion of the aldehyde to 2-butoxyacetic acid, the 2-butoxyacetic acid is the hemolytic agent. Addition of aldehyde dehydrogenase and its cofactors, followed by butoxyacetaldehyde, resulted in a significant increase in hemolysis, which could be decreased with the addition of cyanamide, an aldehyde dehydrogenase inhibitor, thus supporting the evidence that production of 2-butoxyacetic acid is the important step in 2-butoxyethanol-specific hemolysis (Ghanayem et al. 1989).**

A recent attempt has been made to quantify the amount of EGBE/BAA/BAL that would be present in the liver and GI tissues of female mice following a 250 ppm inhalation exposure to EGBE (Corley, 2003) using the forestomach rate constants provided by Green et al. (2002). This work extends an earlier model developed by Dr. Corley (Corley et al., 2003) to include the intermediate formation of BAL in target tissues (liver and GI tract). Given the limitations of the available data (outlined in Attachment 1), Corley (2003) estimated that 250 ppm EGBE would result in peak C_{max} concentrations of 48 μM EGBE, 1.1 μM BAL and 3,200 BAA μM in GI tissue and 7 μM EGBE, 0.5 μM BAL and 3,250 BAA μM in liver tissue of mice at the end of a 6 hour exposure period (see Figures A1-1 and A2-1). This limited analysis does not prove

that BAA is the responsible for the observed forestomach effects, but it strengthens the hypothesis considerably.

The data available for the determination of BAL's impact on the carcinogenicity of EGBE are limited. BAL does not appear to cause gene mutations at in vitro concentrations below 2 mM (Elliot and Ashby, 1997). However, Elias et al. (1986) and (Ghanayem and Thompson, unpublished data) determined that BAL is more cytotoxic and clastogenic than BAA, and can cause SCE in Chinese hamster lung (V79) and human lymphocyte cells at lower (.1 to 1 mM) in vitro concentrations. Clastogenic effects were not observed at concentrations below 0.1 mM for both acetaldehyde and BAL in V79 cells (Elliott and Ashby, 1997; Elias et al., 1986).

The relevance of in vitro data to in vivo exposure to EGBE is questionable. None of the in vitro studies of BAL have involved application of activating enzymes encountered in vivo and, as is discussed above, data from Green et al. (2002) and Corley (2003) suggest that BAL is rapidly metabolized to BAA in vivo due to a high aldehyde dehydrogenase activity in the mouse forestomach. Further, the BAA that is produced would tend to build up in vitro without the detoxification/elimination mechanisms present in vivo, resulting in a decreased pH level in the cell culture. Low pH is known to reduce the DNA repair capacity of cells (Xiao et al., 2003; Elliot and Ashby, 1997), and this could enhance the impact of a clastogen or a weak mutagen such as BAL. This is a mechanism that has been proposed for the SCE caused by structurally related acetaldehyde, which has also been determined to be weakly mutagenic (Dellarco, 1988; He and Lambert, 1995; Grafstrom et al., 1994), and produces chromosome damage in test systems using cells from humans and research animals.

Other lines of evidence that suggest that direct interaction of BAL with the DNA molecules would not play a significant role in the carcinogenic activity of EGBE are discussed further in Attachment 1 of the position paper. They include the fact that BAL causes cytotoxicity at levels associated with chromosome effects and cytotoxicity itself can have effects which result in chromosome damage, acetaldehyde is recognized as "weakly mutagenic" and structural comparisons of acetaldehydes demonstrate that a longer-chain aldehyde such as BAL would be less likely to interact with DNA than a shorter chain aldehyde such as acetaldehyde, the fact that in vitro and in vivo assays of EGBE were generally negative and that chemicals for which mutagenesis/genotoxic effects play a significant role generally induce more tumors at earlier time points, rather than near the end of the conducted bioassays, due to their ability to both initiate and promote tumor pathogenesis. While the existing evidence is certainly suggestive, it contains important data gaps. Additional research (e.g., verification of existing PBPK modeling results and improved genotoxicity assays) would assist the Agency in making a more informed decision concerning the potential for BAL to contribute to the adverse effects seen in animals following EGBE exposure and use of the proposed nonlinear assessment approach.

One reviewer suggests the need for quantitative PBPK modeling combined with data on the relative potency for the toxic effects observed. A limited analysis has been done and is discussed in Attachment 1 (Corley, 2003), but the appropriateness of the use of this information for predicting the corresponding liver and systemic activity of this enzyme is questionable. Kinetic information for aldehyde dehydrogenase activity on BAL exists only in the forestomach (Green et al., 2002). Research to determine the aldehyde activity in organs other than the stomach and a paper on the relative potencies of aldehydes would be useful and informative.

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Another reviewer mentioned the importance of considering the impact of genetic polymorphism towards variance in the human response to EGBE exposure. The issue and impact of polymorphism is recognized as being important to the consideration of potentially sensitive human subpopulations and is discussed in the revised position paper in this context.

Potential for Hemolysis in Humans

4. *Has the position paper drawn appropriate conclusions from the available literature on the potential for EGBE induced hemolysis in humans?*

All five reviewers said yes. However,

1. A role of BAL in the toxicity of EGBE in vivo must be considered.
2. Human cases of hemolysis should be assessed via PBPK modeling, and reported human interindividual variability (e.g., Ghanayem and Sullivan, 1993; Ghanayem, 1989) should be considered.
3. A broad study of the quantitative variations in sensitivity to BAA among a substantial number of representative humans (in the hundreds, if that could be arranged) would help prevent EPA from making its choices in ignorance of the extent of additional sensitivity that might be seen in a minority of people.
4. Definitive data supporting such a conclusion for chronic exposure are not available. However, the data available on both inter-species differences in pharmacokinetics and sensitivities to hemolysis indicate that humans are more resistant than rodents to hemolysis, iron buildup, oxidative stress, and cell proliferation as a result of EGBE exposure.

RESPONSE: Regarding the first suggestion, the potential role of BAL in the toxicity of EGBE is now extensively discussed in the position paper. No cases of human hemolysis have ever been observed, so the suggestion in #2 above would not be possible to accomplish. Suggestion #3 is not likely to make a significant impact on the Agency's determination with respect to EGBE's ability to cause hemolysis in humans and a broad (in vitro or in vivo) study in hundreds of humans would be costly and time consuming. While polymorphisms have been shown to alter rates of metabolism and elimination of alcohols (Agarwal and Goedde, 1992) there is no EGBE-specific information available, and it is not clear how such polymorphisms would influence human susceptibility to EGBE. As described in this paper and the 1999 EGBE IRIS file, case studies and early controlled exposure studies (Carpenter et al., 1956) and in vitro studies involving aged, sickle-cell anemia, hereditary spherocytosis patients (Udden, 1994) and children (Udden, 2002) strongly suggest that human RBCs are considerably less sensitive than rodent RBCs to the hemolytic effects of EGBE/BAA. In addition, if the reviewer is suggesting controlled human exposure experiments, considerable legal problems could forestall this type of experimentation/approach. For the purposes of this assessment paper, the existing studies provide sufficient information to determine the human cancer risk from a mode of action involving EGBE induced hemolysis.

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5. *Is there additional information that would suggest an alternative conclusion?*

Two reviewers said yes. See the responses to question 4 above.

Three reviewers said no.

RESPONSE: See above.

Relevance of in vitro studies

In vitro studies referred to in the position paper as indicating human insensitivity to hemolysis were of short (generally 4 hour) duration, and the slight effects on RBC deformability were still increasing at the end of the assay (Udden, 1994; 2000; Ghanayem et al, 1989). While hemolytic effects have not been observed in humans at high acute doses, the human effects of long term exposure to EGBE are not well studied (U.S. EPA, 1999).

6. *Is the position paper justified in concluding that humans would not be expected to experience hemolysis leading to oxidative stress and cell proliferation, key events in the proposed mode of action for EGBE's role in the formation of the observed mouse liver tumors (NTP, 2000)?*

Two reviewers said maybe, and indicated that:

1. Humans should be referred to as"comparatively less sensitive" to hemolysis.
2. Unless the molecular mechanisms responsible for the hemolytic effects of EGBE are fully characterized and the molecular basis of the lower sensitivity of human erythrocytes are characterized, it is not possible to conclude that humans are not sensitive.
3. Again, these issues need to be quantitatively analyzed with the aid of both PBPK modeling and more extensive observations of the human interindividual variability.

Three reviewers responded yes, that the studies *in vitro* are in concordant with the lack of observed hemolytic toxicity in humans following acute exposures. Longer-term exposures at lower levels of EGBE are ubiquitous in industry (and even in the general population) and the effects of hemolytic toxicity are fairly distinctive and well-known to occupational physicians. Therefore, it is not unreasonable to expect that they would have been noted if present. IF hemolysis leading to oxidative stress is the mechanism for the formation of liver hemangiosarcomas and other tumors, these would not be expected to occur in humans.

RESPONSE: With respect to the first concern, humans are identified as comparatively less sensitive in the revised position paper (as much as 150-fold according to Udden 1994; 1995 in vitro data). The primary question is whether the level of hemolysis that humans can reasonably be anticipated to experience could trigger a carcinogenic effect. Studies have already been performed to examined the interindividual variability between humans with blood diseases, elderly and children (Udden, 1994; Udden et al., 2002). No susceptible subpopulation has been identified and, as AS points out above, it seems unlikely that such a subpopulation would not have been identified in the literature by now. While a better idea of the molecular mechanism may help to identify or exclude sensitive human subpopulations, complete knowledge of the mechanisms involved is not necessary to make a reasoned judgment that humans exposed to EGBE would not be expected to experience the level of hemolysis necessary to trigger a secondary carcinogenic effect from EGBE induced hemolysis. It is not clear why a full PBPK

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model is necessary to compare sensitivities. A simple one-compartment model might be adequate since the effect of interest is in the blood compartment, not a peripheral (solid) tissue. In any case, existing literature on the metabolism of EGBE in humans versus rodents suggests that humans have pharmacokinetic mechanisms to help remove BAA that are not present in rodents (U.S. EPA, 1999).

Mode of Action for Formation of Forestomach Tumors in Female Mice

7. *Has the position paper drawn appropriate conclusions from the available literature on the potential for EGBE to contribute to the formation of forestomach tumors reported in female mice?*

One reviewer said no, and felt that the document has not done a full quantitative pharmacokinetic/pharmacodynamic analysis of the possible contributions of genotoxic processes via the butoxyacetaldehyde (BAL) metabolite. Surely, as with formaldehyde, some local toxic/irritant cell proliferation response is also involved. However, the likely genetic action via aldehyde reactions with DNA, combined with some ongoing rate of background cell replication, should allow some rate of fixation of DNA lesions into permanent chromosomal changes. The contributions from this process would be expected to be linear at low doses, requiring risk assessment treatment via a linear/nonlinear paradigm. (Simple mathematics yields the result that when a highly nonlinear process with an upward turning dose response relationship is combined with a fundamentally linear process, the linear process will dominate the shape of the dose response relationship at low doses. The dose at which the linear mode of action takes over depends on the contribution of the linear process at high doses where the observations are made, and the rapidity with which the "nonlinear" process declines with dose relative to the linear process.)

Four reviewers said yes, but

1. The 7-step summary of the proposed mode of action must include BAL in steps 1, 2 and 3.
2. Lack of forestomach tumors in male mice are not fully explainable at this time.
3. Extrapolation from measured impacts on the cell membranes of erythrocytes is a stretch because erythrocytes are noticeably lacking in cellular apparatus other than a cell membrane; so the fact that the membrane is the target in those cells does not exclude the possibility of other cellular targets in more broadly functional cells.

RESPONSE: The reviewers make some good points, most of which have been incorporated into the position paper. However, the evidence for a nonlinear process in the formation of the EGBE induced forestomach tumors significantly outweighs existing evidence for a linear process. The relationship between the irritation effects/ulcers and tumors was stressed by NTP (2000) and makes sense when examined in relation to other compounds that cause similar forestomach effects. Also, no hyperplasia and no tumors were observed in inhalation studies of rats (NTP, 2000) and in drinking water studies of mice (NTP, 1993a), supporting the need for these steps prior to tumor formation. In addition, if a direct interaction of EGBE or a metabolite were involved and irritation events were not necessary, one might expect to see tumors earlier in the mice rather than near the end of the conducted bioassays as was observed (NTP, 2000). The mutagenic compound ethylene dibromide, for instance, was reported to induce forestomach tumors in all dose groups 168 to 280 days from the start of exposure (NCI, 1978). No biochemical event at low doses is known that would suggest linearity, and it is not clear how a full PBPK model would clarify the contribution of BAL, given the available kinetic

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data for EGBE and BAL metabolism. The available data support the necessity of a prior irritation event, which suggests a nonlinear mode of action for the formation of these tumors. However, the contribution of a linear component (e.g., BAL interaction with DNA) can not be completely ruled out at this time.

8. *Is there additional information (e.g., from other studies or studies of related compounds) that would suggest an alternative conclusion?*

All five reviewers said no, but the following comments were provided,

1. Inclusion of similar findings for other nongenotoxic carcinogens (e.g. ethyl acrylate) (Ghanayem et al., 1985, 1993, and 1994) is recommended to support the proposed mode of action.
2. Comparisons should be made with the local responses to other aldehydes.
3. Bring in information on clearly genetically-acting compounds such as epichlorhydrin, which has been the subject of PBPK models with detailed modeling of local effects such as glutathione reduction (e.g. Ginsberg, G. L., Pepelko, W. E., Goble, R. L., and Hattis, D. B. "Comparison of Contact Site Cancer Potency Across Dose Routes: Case Study with Epichlorohydrin," Risk Analysis Vol. 16, pp. 667-681, 1996.).
4. The extensive discussion in the scientific literature of the significance of forestomach tumors in rodents as indicators of potential human carcinogenicity should be addressed.
5. The paper presents a thorough and relatively convincing analysis of the "preferred" hypothesis, but does not adequately address alternatives.
6. Lack of a forestomach in humans is not a valid justification for suggesting that EGBE induced forestomach effects are not relevant to humans.
7. The fact that humans do not have an overall coat of hair, or extensive grooming behavior is a more convincing argument that the experimental carcinogenicity observation is an unlikely predictor of human risk in this particular case.

RESPONSE: It was not the paper's intention to suggest that EGBE-induced forestomach effects are not relevant to humans. This was stated in the summary, but was not emphasized in attachment 1. Additional language has been added to Attachment 1 to emphasize the potential, qualitative relevance of these effects to humans. The main point that the document makes is that due to a lack of grooming, the lack of a forestomach and a reduced sensitivity to the cytotoxic effects of EGBE, humans are not likely to achieve the dose necessary to trigger the irritation events necessary for the proposed mode of action.

9. *The position paper offers an explanation for the relative insensitivity of male mice to the formation of forestomach tumors following EGBE exposure. Is there an alternative explanation?*

Two reviewers said maybe, but:

1. If BAA is the responsible metabolite and the greater activity of alcohol dehydrogenase in female mice leads to more BAA formation, then one may conclude that more BAL was formed in female mice prior to conversion to BAA.
2. Need a quantitative analysis aided by PBPK modeling, and a theory of background interaction to provide a quantitative estimate of likely response for comparison with the observations.

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3. Further assessments could be conducted to investigate root causes (e.g., endocrine roles of estrogens & androgens), but there are clear gender differences for many of the potential etiological factors proposed to be responsible for tumor formation (e.g., greater sensitivity to irritation/hyperplasia, higher levels of alcohol dehydrogenase, greater female RBC sensitivities to the hemolytic effects, and lower plasma clearance rates of BAA).
4. The pharmacokinetic theory holds up as the principal available explanation, but this does not exclude the possibility that other unexplored effects contribute (perhaps substantially) to the difference.

RESPONSE: Comment #1 is certainly true. Some BAL will be formed in female mice following EGBE exposure. Unfortunately, we do not know the relative aldehyde dehydrogenase activity in the forestomachs of male versus female mice. The activity of hepatic alcohol dehydrogenase enzyme is greater in female mice than in males (Dill et al, 1998), but the relevance of this has not been fully assessed. The question of the basis for the sex specific sensitivity would benefit from further research and analysis.

Mode of Action for Formation of Liver Tumors in Male Mice

10. Has the position paper drawn appropriate conclusions from the available literature on the potential for EGBE to contribute to the formation of liver tumors in male mice?

Four reviewers said yes, but:

1. Add BAL in addition to BAA as causes of hemolysis because in vivo and in vitro evidence suggested that BAL causes hemolysis (Ghanayem 1989 and 1996).
2. Although contrary to the NTP's position in its cancer bioassay report, the rationale is reasoned and plausible. In addition, several studies that were conducted subsequent to the release of the NTP report support the conclusions of the position paper, as does the publication of an NTP scientist (Cunningham, 2002).

One reviewer was not sure and pointed out that:

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1. Need a quantitative analysis of how much hemolysis is expected on an ongoing basis in the different species/sexes, how much resulting accumulation of iron in affected cell types results from this hemolysis, and then how much additional free radical generation and clastogenic/mutagenic/carcinogenic change is likely to result from this pathway.
2. An attempt needs to be made to either qualitatively or quantitatively analyze the alternative theory of genetic action via BAA[L] with the aid of knowable rates of generation and metabolism of this compound and the hepatotoxic/carcinogenic activities of this and related aldehydes.
3. Hemolysis/iron accumulation theory could be strengthened by experiments in which the proliferative or early carcinogenic responses to EGBE were inhibited by administration of a suitable chelator to tie up and facilitate the excretion of iron and thereby reduce the accumulation in Kupffer cells or other cells of the liver.

RESPONSE: The Agency agrees with the majority of these comments. The potential contributions of BAL to the hemolytic effects of EGBE will be discussed and the papers provided by Dr. Ghanayem will be cited. With respect to comment #1, a quantitative analysis of the "expected" relative hemolytic activity and iron buildup in the different species/sexes

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could be done but would not be particularly informative as a number of in vivo studies have confirmed that rats and mice of both sexes experience these effects to a similar degree of severity, and occupational studies, case studies and in vitro studies indicate that humans would not be expected to experience significant iron buildup from EGBE induced hemolysis. In addition, predicting the amount of expected "free radical generation and clastogenic/mutagenic/carcinogenic change" is not considered feasible at this time because the actual mechanism of action for the induction of DNA damage (e.g., oxidative stress or induced endothelial cell replication) is not clear.

Regarding comment #2, a recent attempt has been made to quantify the amount of EGBE/BAA/BAL that would be present in the liver and GI tissues of female mice following a 250 ppm inhalation exposure to EGBE (Corley, 2003) using forestomach rate constants for EGBE -> BAL provided by Green et al. (2002). This work is an attempt to extend an earlier model developed by Dr. Corley (Corley et al., 2003) to include the intermediate formation of BAL in target tissues (liver and GI tract). The limiting assumptions and results of this analysis are described in the revised position paper.

Suggestion #3 might be an interesting and informative extension of the experiments performed by Siesky et al. (2002) and could strengthen the hemolysis/iron accumulation theory. While not directly answering the charge question, the reviewer appears to agree that the position paper has drawn appropriate conclusions from the *available literature* regarding the relationship between hemolysis, iron accumulation and oxidative stress.

11. Is there additional information (e.g., from other studies or studies of related compounds) that would suggest an alternative conclusion?

One reviewer said yes. See question 10.

Four reviewers said no. One pointed out that although various alternatives could be proposed, including the possibility of a direct genotoxic action via some undiscovered alternative metabolic activation, they were not aware of any experimental data to support such alternative explanations.

RESPONSE: See responses question 10 above.

12. Is there any evidence in the literature that would suggest that the increased incidence of liver hemangiosarcomas and hepatocellular carcinomas can be explained by mechanisms other than those proposed or described?

One reviewer responded that they were unsure.

One reviewer responded yes, and stated that they believed one could glean some fodder for a possible analogy with acetaldehyde from a careful review of the mechanisms by which alcohol causes cirrhosis (and liver cancer??).

Three reviewers said no.

RESPONSE: The mechanism of action for other aldehydes such as acetaldehyde was considered and included in the position paper's discussion of possible alternative mechanisms for the induction of these liver carcinomas.

13. The position paper offers an explanation for the relative insensitivity of female mice to the formation of liver tumors following EGBE exposure. Is there an alternative explanation?

Two reviewers responded maybe, but stated that:

1. A comparison of the effects of EGBE on DNA synthesis in male and female mice could confirm the hypothesis, and confirm the role of liver antioxidants in male vs. female mice.
2. A quantitative analysis aided by PBPK modeling, and a theory of background interaction is needed for comparison with the observations.

Three reviewers said no, but one reviewer stated that since female mice are apparently more liable to generate high intracellular levels of BAA than males (according to investigators seeking to explain the forestomach results), pharmacokinetic differences between the sexes appear less plausible as an explanation (although special situations and alternative routes of metabolism specific to the liver could be involved).

RESPONSE: Male mice appear to be more sensitive than female mice to the induction of liver hemangiosarcomas by all four chemicals that NTP has found to cause early onset hemosiderin buildup in liver Kupffer cells (EGBE, p-chloroaniline, p-nitroaniline, and pentachloroanisole). If, as proposed, these chemicals induce liver hemangiosarcomas through a mechanism involving oxidative stress, the documented high sensitivity of male mice to liver oxidative stress (Klaunig et al., 1995; 1998; 2000) offers a highly plausible explanation for this sensitivity. Further support for this explanation is provided by the fact that the background incidence of liver hemangiosarcomas is clearly higher in male versus female control mice. The proposed hypothesis that males have an increased sensitivity to oxidative stress over females is not well supported by direct experimental evidence, but does offer a reasonable explanation for both the historical data and the apparent increased sensitivity of male mice to the induction of liver hemangiosarcomas via an EGBE induced oxidative stress mechanism. This question would benefit from additional research. However, the explanation suggested in the position paper is the most reasonable explanation given the available data. No alternative explanation was offered by any of the reviewers and it is not clear how additional PBPK modeling could result in a more reasonable, alternative explanation.

Other

One reviewer commented that gender differences should be expanded to include most recent studies which suggested that female rats are more sensitive than males and mice and suffered from thrombosis, and infarction after inhalation and gavage exposure to EGBE and offered the following:

Nyska, A., Maronpot, R. R., Long, P. H., Roycroft, J. H., Haliy, J. R., Travlos, G. S., and Ghanayem, B. I. (1999). Disseminated Thrombosis and Bone Infarction in Female Rats Following Inhalation Exposure to 2-Butoxyethanol. Toxicol. Pathol. 27:287-294

Nyska, A., Maronpot, R. R., and Ghanayem, B. I. (1999). Ocular Thrombosis and Retinal Degeneration in Female Rats by 2-Butoxyethanol. Human and Exp. Toxicol. 18:577-582.

Ghanayem, B. I., Ward, S M, Chanas, B, and Nyska, A. (2000). Comparison of the acute hematotoxicity of 2-butoxyethanol in male and female F344 rats. Human Exp. Toxicol. 19:185-192.

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Long P. H, Maronpot, R. R., Ghanayem, B. I., Roycroft, J H., and Nyska A (2000). Dental pulp infarction in female rats following inhalation exposure to 2-butoxyethanol. Toxicol. pathol. 28:246-252.

Ghanayem, B. I., Long, P., Ward, S M, Chanas, B, and Nyska, A. (2001). Hemolytic Anemia, Thrombosis, and Infarction in Male and Female F344 Rats Following Gavage Exposure to 2-Butoxyethanol. Experimental and Toxicologic Pathology 53:97-105.

Another reviewer offered that similar observations with respect to temporal observations were made with other nongenotoxic forestomach carcinogens and will strengthen the present discussion.

Ghanayem, B.I, Maronpot, R.R. and Matthews, H.B. (1986). Association of Chemically-Induced Forestomach Cell Proliferation and Carcinogenesis. Cancer Letters 32:271-278.

Ghanayem, B. I., Sanchez, I. M., Maronpot, R. R., Elwell, M. R., and Matthews, H. B. (1993). Relationship Between the Time of Sustained Ethyl Acrylate Forestomach Cell Proliferation and Carcinogenicity. Env. Health Persp. 101 (Sppl. 5):277-280.

Ghanayem, B. I., Sanchez, I. M., Matthews, H B., and Elwell, M. R. (1994). Demonstration of a temporal relationship between ethyl acrylate induced forestomach hyperplasia and carcinogenesis. Toxicologic Pathology 22:497-509.

Regarding the question "why were there no forestomach effects observed in the NTP (1993b) subchronic drinking water study of mice, one reviewer pointed out that previous studies with other nongenotoxic forestomach carcinogens demonstrated that forestomach effects are dependent not only on the dose but also on the chemical concentration in the dosing solution (Ghanayem et al., 1985). This may explain the observed effect in mice but not in rats (mice were exposed to higher concentrations of EGBE than rats).

Ghanayem, B.I, Maronpot, R.R. and Matthews, H.B. (1985). Ethyl Acrylate-Induced Gastric Toxicity: II. Structure-Toxicity Relationships and Mechanism. Toxicol. Appl. Pharmacol. 80:336-344.

Nyska, A., Maronpot, R. R., and Ghanayem, B. I. (1999). Ocular Thrombosis and Retinal Degeneration in Female Rats by 2-Butoxyethanol. Human and Exp. Toxicol. 18:577-582.

Ghanayem, B. I., Ward, S M, Chanas, B, and Nyska, A. (2000). Comparison of the acute hematotoxicity of 2-butoxyethanol in male and female F344 rats. Human Exp. Toxicol. 19:185-192.

Long P. H, Maronpot, R. R., Ghanayem, B. I., Roycroft, J H., and Nyska A (2000). Dental pulp infarction in female rats following inhalation exposure to 2-butoxyethanol. Toxicol. pathol. 28:246-252.

Ghanayem, B. I., Long, P., Ward, S M, Chanas, B, and Nyska, A. (2001). Hemolytic Anemia, Thrombosis, and Infarction in Male and Female F344 Rats Following Gavage Exposure to 2-Butoxyethanol. Experimental and Toxicologic Pathology 53:97-1

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One reviewer commented that, for a more comprehensive understanding of the report's message that humans would need substantially higher exposures to EGBE to reach hemolytic risks, quantitative information should be provided in the report. Show how the RfC and RfD were estimated originally from rodent data, what were the safety factors used, what are the differences in BAA levels in rodents vs. humans following comparable exposures to EGBE, what would be the additional margin-of-safety if it is assumed that human RBCs are 100 times less sensitive to BAA, etc. While there is agreement with the conclusions made in the report, the exposure, disposition, and metabolism stories would be bolstered substantially by adding some quantitative information.

Another reviewer felt that it would be helpful to be more specific about the nature of the "increased DNA synthesis" proposed and observed in the studies cited. Does it result from DNA repair or from increased cell proliferation?

RESPONSE: In general, EPA agrees with these "other" comments made by the reviewers and the position paper has been revised to take them into consideration and to incorporate the suggested references.

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2004 Panel Review Comments and Responses (Blue)

1. Liver hemangiosarcomas observed by NTP (2000) in male mice exposed to EGBE.

a. Does enough information now exist to support an informed decision concerning the significance of the BAL metabolite to the formation of EGBE induced liver tumors?

All seven panel members agreed that enough information now exists to support an informed decision concerning the significance of the BAL metabolite to the formation of EGBE induced liver tumors. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

- Whether ALDH deficient people constitute a subpopulation that is more susceptible to BAL resulting from EGBE metabolism should be investigated or considered.

- The additional data supplied with the EPA position paper (Klaunig and Kamendulis, 2005; Deisinger and Boatman, 2004) should be included in the report as they provide important evidence that the BAL metabolite is not likely to contribute to the formation of EGBE induced liver tumors.

- Nyska et al. (2004), retrospectively evaluating the results of 130 two-year carcinogenicity studies conducted in B6C3FI mice at the NTP, have shown an overall association between liver hemangiosarcoma and Kupffer cell pigmentation to be highly significant ($p<0.001$) and limited to males.

- A comparison of the Corley et al (2004) EGBE model results and the gavage study of Deisinger and Boatman (2004) revealed that the predicted levels of BAL in both GI and liver tissue are within 101% of the measured values.

- The Corley et al. (2004) model has been used to predict BAL concentrations in the liver following oral and inhalation exposures in mice and in mice with aldehyde dehydrogenase metabolic rates (Vmax) set at ½ the initial values (R. Corley, personal communication) and for inhalation exposures up to the theoretical maximum of 1160 ppm for 6 hr, the prediction BAL liver levels are not going achieve concentrations above 0.001 mM in such low metabolizing individuals.

RESPONSE: EPA has addressed these concerns and suggestions in the revised position paper. As is discussed in the position paper, while it is recognized that individuals with atypical alcohol dehydrogenase and/or deficient aldehyde dehydrogenase may experience some increase, the Corley et al. (2004) model suggests that BAL levels are not be expected to accumulate significantly in the liver or forestomach, even if ALDH activity is half its normal rate and EGBE inhalation exposure levels are assumed to be at the theoretical maximum of 1160 ppm. The data from the recent gavage study by Deisinger and Boatman (2004) support the Corley et al. (2004) model results. These data, as well as other recent reports relevant to the genotoxicity (Klaunig and Kamendulis, 2005) and mode of action (Nyska et al, 2004) of EGBE have been considered in the revised position paper.

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b. Is the current information adequate to support the mode of action described in the position paper for the EGBE induced formation of hemangiosarcomas in male mice and the potential relevance of this finding to humans?

All seven panel members agreed that the current information is adequate to support the mode of action described in the position paper for the EGBE induced formation of hemangiosarcomas in male mice and the potential relevance of this finding to humans. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

- Iron bound to low molecular weight (LMW) chelators is redox active and, thus, is capable of producing oxidants. It could be of great importance if this fraction of iron has been measured in the animal studies. A newly developed fluorescent calcein method could measure LMW iron in biological fluids of the EGBE-exposed animals (Ali *et al.,* 2003).

- Protection by various antioxidants, such as vitamin E, supports the oxidative stress mechanism. However, a protection by specific iron chelators, such as deferoxamine, would greatly strengthen the role of iron in the mode of action induced by EGBE.

- It is difficult for this reviewer to believe that the iron deposition in the livers of these animals did not occur in Kupffer and endothelial cells, although the report suggests (footnote Figure A2-1) that there was not explicit mention of iron deposition in these cell types. If this point becomes important, it is readily checked by a reexamination of the histology and if there is any question, ultrastructural studies may be carried out, even on formalin-fixed tissues.

- If further studies were to be done in this area, they may be oriented towards the possible role of female sex hormones as a possible explanation for the fact that hemangiosarcomas were observed in males only and the neoplastic response to the chronic inflammation and cell proliferation in the forestomach was seen only in females.

- Another aspect that needs to be considered is that BAA is an acid, which may release iron from transferrin.

- Some alternate considerations (also supported by scientific literature) may be involved in the mode of action. In particular:

    - in addition to the production of oxidative DNA damage, reactive oxygen species can alter gene expression (e.g. MAP kinase/AP-1, and NFkB) resulting in stimulation of cell proliferation and/or inhibition of apoptosis.
    - If Kupffer cells are activated by iron as well as by phagocytosis of EGBE-induced hemolyzed RBC's, and the production of reactive oxygen species occurs through Kupffer cell derived reactions, the necessity for identifying iron in endothelial cells (presumably to produce reactive oxygen within the target cell?) is lessened.

RESPONSE: The first four bulleted comments above refer to additional research that could be conducted to confirm the mode of action and the explanation for the male mouse specificity that have been presented in the position paper. While these types of studies would likely help to clarify the proposed mode of action, they are not believed to be necessary for EPA to make an informed decision regarding the human risk from EGBE exposure. Key steps that indicate a nonlinear mode of action (e.g., the importance of hemolysis leading to hemosiderin buildup in liver Kupffer cells) are well

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documented. What is know about these key steps and their relevance to mice and humans is sufficient for the determination of the overall risk to humans and the characterization and, while other potential contributing factors such as the acidic nature of BAA, the alteration of gene expression, the necessity of iron directly in endothelial cells are considered in the revised position paper, verification of additional factors contributing to the promotion or activation of endothelial cells is not considered necessary at this time.

c. Does the available information support a nonlinear cancer assessment approach for the male mouse liver tumors observed following EGBE exposure (i.e., is it reasonable to expect that the prevention of hemolytic effects in humans would prevent the formation of liver tumors in humans)?

All seven panel members agreed that the available information supports a nonlinear cancer assessment approach for the male mouse liver tumors observed following EGBE exposure, and therefore it is reasonable to expect that a lack of hemolytic effects in humans would prevent the formation of liver tumors in humans. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

- The sex dependent effects might be explained by the fact that male mouse liver is well known to be more sensitive to the development of tumors than female mouse liver. Thus exposure to a similar concentration of EGBE may not have been quite sufficient to induce a similar increased incidence in female mice.

- The only possible group that might be affected (to this reviewer's knowledge) might be the hemochromatosis heterozygote that comprises some 12% of the human population (Barton and Bertoli, 1996). Smaller amounts of hemolysis in these individuals could lead, over extended periods, to some chronic iron deposition in hepatocytes, but it would seem unlikely that even such individuals would have any problem.

RESPONSE: None of the reviewers offered a linear mechanism for the formation of hemangiosarcomas. Some offered possible explanations for male mouse specificity and one offered that hemochromatosis heterozygotes might represent a sensitive subpopulation because smaller amounts of hemolysis in these individuals could lead, over extended periods, to some chronic iron deposition in hepatocytes. However, this same reviewer did not believe that these individuals could have an EGBE related iron overload problem.

2. ***Forestomach tumors observed by NTP (2000) in female mice following EGBE exposure.***

a. Does enough information now exist to support an informed decision concerning the significance of the BAL metabolite to the formation of EGBE induced forestomach tumors?

All seven panel members agreed that enough information now exists to support an informed decision concerning the significance of BAL genotoxicity to the formation of EGBE induced forestomach tumors. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

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- Levels of BAL in ALDH-deficient people may be a concern (see 1a).

- Available information indicates that BAL does not result in genetic alteration that leads to an increased incidence of forestomach tumors. However, it has not been determined that this relatively reactive metabolite does not account, at least in part, for the chronic irritation that results in an increased incidence of tumors.

- The female mice had particularly severe irritation in the forestomach relative to males treated with the same dose, which supports a non-genotoxic mode of action for the female forestomach carcinogenesis.

- The model of Corley et al (2004) has been used to predict BAL concentrations in the GI tract following oral and inhalation exposures in mice and in mice with aldehyde dehydrogenase metabolic rates (Vmax) set at ½ the initial values. Only after oral doses of 300 mg/kg are BAL levels comparable to those found to have an effect in vitro. For inhalation exposures up to the theoretical maximum of 1160 ppm for 6 hr, the prediction BAL liver levels are not going to achieve concentrations above 0.01 mM in these low metabolizing individuals (Table A4-1). This predicted maximal concentration is considerably lower than concentrations of BAL shown to be clastogenic (0.2 mM) or hemolytic (0.5 mM: Ghanayem et al., 1989) in vitro.

RESPONSE: It is recognized that BAL may be partially responsible for the irritation effects following EGBE exposure and that ALDH deficient populations may experience higher BAL levels. However, as one reviewer pointed out, while BAL has only been detected at very low levels in blood following high oral EGBE doses, BAA has been detected over time and at relatively high levels in the forestomach of female mice following in vivo exposures to EGBE through multiple routes. Another reviewer also pointed out that since it is not possible to administer EGBE or BAL to intact animals without their rapid metabolism to BAA conclusive determination of the contribution of BAL to the irritating effects of EGBE is probably not feasible. Nevertheless, the Corley et al. (2004) model and the Deisinger and Boatman (2004) gavage studies make it clear that BAL levels in the GI tract would be very low.

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Table A4-1. Dose-response simulations of the peak tissue concentrations (Cmax) of butoxyacetaldehyde (BAL) in female mice following either oral gavage or 6-hr inhalation exposures. To simulate a heterozygous population with lower aldehyde dehydrogenase activity, simulations with ½ the Vmax rate are also shown.

Route or Exposure (ppm)	Dose (mg/kg)	Cmax BAL Liver (μM)	Cmax BAL Liver @ ½ Vmax (μM)	Cmax BAL GI Tract (μM)	Cmax BAL GI Tract @ ½ Vmax (μM)
Oral	1	0.002	0.004	0.068	0.136
	10	0.021	0.043	0.686	1.399
	25	0.056	0.112	1.755	3.686
	50	0.123	0.247	3.644	8.090
	100	0.305	0.615	7.85	19.95
	150	0.584	1.187	12.57	38.22
	300	2.241	4.773	25.07	160.5
	500	4.211	9.502	31.61	419.2
	600	4.586	10.47	32.99	525.3
	900	4.991	11.53	34.96	725.6
Inhalation	1	0.000	0.001	0.003	0.006
	5	0.002	0.003	0.015	0.030
	10	0.003	0.006	0.030	0.061
	25	0.008	0.016	0.076	0.153
	50	0.016	0.032	0.153	0.307
	63	0.020	0.040	0.193	0.388
	100	0.032	0.064	0.307	0.619
	125	0.040	0.080	0.384	0.776
	150	0.048	0.096	0.462	0.935
	200	0.064	0.129	0.618	1.257
	250	0.081	0.162	0.775	1.583
	500	0.164	0.329	1.576	3.292
	750	0.249	0.502	2.404	5.141
	950	0.320	0.645	3.086	6.732
	1160	0.395	0.799	3.820	8.519

b. Is the current information adequate to support the mode of action described in the position paper for the EGBE induced formation of forestomach tumors in female mice and the potential relevance of this finding to humans?

All seven panel members agreed that the current information is adequate to support the mode of action described in the position paper for the EGBE induced formation of forestomach tumors in female mice and the potential relevance of this finding to humans. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

- Whether the increased DNA synthesis results in acquisition of new mutations or results in a selective clonal expansion of initiated cells (i.e. functions at the tumor promotion stage of carcinogenesis), has not been established.

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- More careful studies of the fate of EGBE in forestomach might prove helpful in determining just how much of each of the three chemicals, EGBE, BAL and BAA, are present in forestomach cells, how long they persist and why EGBE in drinking water did not induce similar lesions/tumors.

- The presence of a single carcinoma merely indicates the spontaneous transition from cells in the stage of promotion (papilloma) to those in the stage of progression (carcinoma).

- If further studies were to be done in this area, they may be oriented towards the possible role of female sex hormones in the neoplastic response to the chronic inflammation and cell proliferation in the forestomach seen in females.

- The lack of effect observed following drinking water studies may indicate a buffering of this irritating effect from the water vehicle or, more likely, a dose-rate effect. In addition, neither EGBE nor its major metabolite binds to stomach macromolecules.

RESPONSE: Though the data seem to be more consistent with forestomach effects being the result of clonal expansion of initiated cells, it is realized that this is not the only possible explanation. However, cell turnover resulting from irritation appears to play a key role for EGBE and other chemicals that cause an increase in forestomach tumors. Studies of the distribution of EGBE and its metabolites in the gut have been done and, as the reviewer points out, additional studies would probably still not conclusively resolve the question of whether EGBE or a metabolite accounts for most of the chronic irritation that lead to an increased incidence of tumors.

c. Does the available information support a nonlinear cancer assessment approach for the female mouse forestomach tumors observed following EGBE exposure (i.e., is it reasonable to expect that the prevention of hyperplastic effects in humans would prevent the formation of gastrointestinal tumors in humans)?

All seven panel members agreed that the available information supports a nonlinear cancer assessment approach for the female mouse forestomach tumors observed following EGBE exposure and therefore making it reasonable to expect that a lack of hyperplastic effects in the region of gastroesophageal junction in humans would prevent the formation of gastroesophageal tumors in humans. The following suggestions/comments were made regarding some additional information that could be included in the position paper:

- Esophageal and gastric emptying occur relatively rapidly within the human (a matter of minutes to a few hours) unlike the mouse and rat under the conditions of the assay.

- A potential alternative mode of action involving direct DNA reactivity by BAL was previously postulated. However, as the comments outlined under question 2a above indicate, the contribution of BAL to the induction of forestomach tumors in female mice is not likely to contribute to the observed neoplasia based on pharmacokinetic factors.

- The mode of action for the induction of forestomach tumors in mice would be expected to apply to humans (i.e., the key events could occur in humans). However, taking into account kinetic and dynamic factors, the key events in the mode of action is not likely to occur in humans.

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- There is a clear association between chronic irritation and an increased incidence of forestomach tumors, but it can not be conclusively determined if the parent chemical, EGBE, or one of its major metabolites, BAL and BAA, accounted for the chronic irritation.

- In the absence of intentional consumption, humans will not encounter similar exposures to EGBE and even then the exposures would be acute rather than chronic. Thus, it would appear that the forestomach tumors observed in female mice are not relevant to humans.

RESPONSE: EPA agrees with the reviewers on all points and these considerations are included in the revised position paper.

3. *Additional Comments and References*

- A description of how mice were housed would be helpful.

- It is a possible that an increased incidence of forestomach tumors were observed in the inhalation studies, but not in drinking water studies because the drinking water studies did not permit consumption of neat EGBE as a result of condensation on the airways and/or grooming. The inhalation study may have achieved both more concentrated and more prolonged exposure of the forestomach to EGBE than did the drinking water study.

RESPONSE: The Agency agrees with these additional comments provided by the reviewers. The new references listed below that were provided by the reviewers were obtained and evaluated, and the position paper has been updated accordingly.

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