Comments of

Ferro Corporation

on

HAZARD IDENTIFICATION DOCUMENT FOR BUTYL BENZYL PHTHALATE (BBP)

for consideration by the

State of California
Office of Environmental Health Hazard Assessment
Scientific Advisory Board Carcinogen Identification Committee

under

Proposition 65

November 18, 2013

EXECUTIVE SUMMARY

Ferro Corporation (Ferro) is submitting these comments on the hazard identification document (HID) for butyl benzyl phthalate (BBP), to assist the Carcinogen Identification Committee (CIC) in its evaluation whether BBP should be listed under Proposition 65 for carcinogenicity. Ferro is a major producer of BBP.

The data are not sufficient to list BBP as "known to the state to cause cancer." Under the State's Experts process, Proposition 65 provides for the listing of a substance only where "it has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer." Health & Safety Code §25249.8. As set forth below and in these comments, that standard is not met in the case of BBP.

The weight of the evidence is that BBP is not genotoxic. The primary evidence regarding tumors comes from three bioassay reports of National Toxicology Program (NTP, 1982; 1997a; 1997b). The weight of the evidence is that the observation of tumors in the NTP studies of BBP does not provide a basis for finding BBP clearly causes cancer. For each tumor type, the data is inconsistent across the NTP studies. Most of the observations of increased tumor incidence did not even achieve statistical significance and/or occurred in only one sex of one species in one study. Plainly, this evidence does not support a finding of clear evidence of carcinogenicity. The conclusion is supported by the reviews of IARC in 1982 and 1999, the latter of which considered all the same NTP studies.

Unfortunately, the HID obscures these plain facts by presenting the CIC with incomplete, erroneous, misleading, and/or extraneous information concerning the BBP data. Prejudicial summary statements are followed by voluminous pages of speculation, indicating that OEHHA has departed from its proper role of providing an objective summary of all relevant data, and instead seeks to influence the CIC in the direction of listing by giving it a biased and misleading document. As just one example, the HID Executive Summary and the Summary and Conclusion give a very misleading summary of the genotoxicity data, giving undue attention to selected positive tests while obscuring the greater number of negative tests – just the opposite of a proper weight-of-the-evidence approach. Nor does the HID acknowledge that NTP has never even considered BBP for listing in its Annual Report on Carcinogens, even though NTP sponsored all three bioassays and would ordinarily consider listing if it felt the findings in its studies warranted such consideration.

The overall rigor and quality of the HID is very disappointing, and it is particularly disappointing that OEHHA has failed even to include highly relevant information provided by Ferro in response to OEHHA's solicitation of information. The appearance of bias in the presentation of the data is strong, and undermines any reasonable reliance on the HID without careful review of underlying publications and data.

To be able to make a weight-of-the-evidence determination based on a fair review of all relevant evidence, the CIC will need to consider carefully both these comments and the underlying citations. The CIC obviously has a very limited amount of time to do so prior to its December 5 meeting. Therefore, we urge the CIC to consider deferring its determination of BBP and

requesting a revised, complete, and objective HID from OEHHA, with sufficient time for stakeholders to comment and the CIC to review both the HID and the comments.

If the CIC does consider BBP on December 5, then Ferro strongly believes it should find that the weight of the evidence does not support a conclusion that BBP clearly causes cancer, and that BBP should not be listed as "known to the State to cause cancer."

TABLE OF CONTENTS

Page

EXECUTIVE SUMMARY i

INTRODUCTION AND SUMMARY 1

I. THE HID IS BIASED AND INCOMPLETE; THE CIC MUST CAREFULLY WEIGH ALL THE EVIDENCE, DEFERRING CONSIDERATION OF BBP IF NECESSARY 4

A. The HID contains information that is extraneous but prejudicial 5
1. Exposure information 5
2. Speculation on mechanisms 6
B. The HID includes information that is erroneous and/or misleading 6
C. The HID is rife with unsupported speculation about the potential carcinogenicity of BBP 7
D. Principles of fairness and honesty dictate that the deficiencies of the HID be cured 8

II. THE WEIGHT OF THE EVIDENCE PROVIDES NO BASIS FOR DETERMINING THAT BBP CLEARLY CAUSES CANCER 9

A. Human cancer experience: Epidemiology studies show no association between breast cancer incidence and BBP exposure 9
B. Animal bioassays: Evidence from long-term animal bioassays does not provide clear evidence that BBP causes cancer 10
C. Genetic toxicity testing: The weight of the evidence is that BBP is not genotoxic 12
D. Mode-of-Action/Mechanism of tumorigenicity: The mechanisms proposed in the HID are purely speculative and not supported by the apical animal bioassays nor the existing epidemiology studies 12
1. Section 3.3.2 Genotoxicity 13
2. Section 3.3.5.1 In vivo differential cDNA microarray analysis 13
3. Section 3.3.5.2 In vitro differential cDNA microarray analysis 13
4. Section 3.3.5.3 Proteomic analysis 13
5. Section 3.3.11 Structure Activity Comparisons 13
6. Section 4.1 Genotoxicity 14
7. Section 4.2 Activation of PPARs 14
8. Section 4.4 Estrogen Receptor (ER) 14
9. Section 4.5 Androgen receptor (AR) and steroidogenesis 15
10. Postulated outcomes of the speculative mechanisms are not supported by apical animal studies or epidemiology studies 15
E. Structure activity comparisons: The HID provides no basis for finding a structural activity among phthalates 16

CONCLUSION 17

REFERENCES 18

EXHIBIT A Relevant Articles Cited in Materials Submitted to OEHHA that Are Not in the HID 21

ATTACHMENT A OPINION OF DR. ERROL ZEIGER 1

ATTACHMENT B OPINION OF DR. GENE MCCONNELL 2

ATTACHMENT C OPINION OF DR. TIM ZACHAREWSKI 3

ATTACHMENT D FERRO JANUARY 22, 2013 SUBMISSION 4

INTRODUCTION AND SUMMARY

Ferro Corporation (Ferro) is submitting these comments to assist the Carcinogen Identification Committee (CIC) in its evaluation whether butyl benzyl phthalate (BBP) should be listed under Proposition 65 for carcinogenicity.[1] Ferro is a major producer of BBP.

These comments and the attached expert opinions (Attachments A, B and C) address the hazard identification document (HID) for BBP posted by the Office of Environmental Health Hazard Assessment (OEHHA) on October 4, 2013.[2] Importantly, we provide herein pertinent information and citations not included in the HID, which are critical to the CIC's full understanding of the data for BBP. *Ferro believes that the weight of the evidence is that BBP has not been clearly shown to cause cancer and therefore should not be listed under Proposition 65 for cancer.*

Ferro provided materials in response to OEHHA's solicitation of information relevant to the assessment of the evidence of carcinogenicity for butyl benzyl phthalate (copy provided with these comments as Attachment D).[3] Ferro representatives also met with OEHHA staff on January 25, 2013 to present an overview of the BBP data. We are therefore severely disappointed to find numerous citations made in our submission missing from the HID. Exhibit A lists over 40 such citations that are readily available in the open literature and without question should have been included in the HID.

Further, much of the information in the HID is presented in a biased and misleading fashion, For example, from the HID one would conclude BBP is genotoxic, whereas the weight of evidence clearly shows that it is not (see Attachments A and D). The Executive Summary and the Summary and Conclusion state that "BBP is genotoxic in multiple *in vitro* and *in vivo* studies using mammalian cells" and then lists 5 or 6 putatively positive studies with prominent bullets. The next small paragraph concedes that BBP is not mutagenic, but fails to inform the reader that there are many more negative tests than supposed positive tests. Indeed, there are 8 negative *in vitro* gene mutation tests, 3 negative *in vitro* chromosome damage and cell transformation studies, and, importantly, 3 negative *in vivo* genetic toxicity tests, but the HID does not list any of these negative results with bullets as it did for positive results.

The animal cancer data are presented in a similarly incomplete and misleading fashion. Isolated findings that were not replicated in other studies are given undue weight, and the lack of replication is given inadequate attention. The HID relies on purported increases in certain

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[1] See Announcement of the Carcinogen Identification Committee Meeting Scheduled for December 5, 2013 and the Availability of Hazard Identification Documents for Butyl Benzyl Phthalate and Diisononyl Phthalate [10/04/13], http://www.oehha.ca.gov/prop65/public_meetings/100413MeetingHazIDmats.html .

[2] OEHHA, Evidence on the Carcinogenicity of Butyl Benzyl Phthalate (Oct. 2013), http://www.oehha.ca.gov/prop65/hazard_ident/pdf_zip/BBP_HID10042013.pdf .

[3] Announcement of Chemical Selected by OEHHA for Consideration for Listing by the Carcinogen Identification Committee and Request for Relevant Information on the Carcinogenic Hazards of Butyl Benzyl Phthalate [11/26/12], http://www.oehha.ca.gov/prop65/CRNR_notices/state_listing/data_callin/note112312.html .

tumors (adrenal medulla pheochromocytoma, urinary bladder transitional cell) that NTP itself did not consider significant, *without disclosing the contrary view of the NTP scientists who conducted the studies*. Indeed, nowhere does the HID acknowledge that NTP has never even considered BBP for listing in its Annual Report on Carcinogens, which speaks volumes given that NTP sponsored all three bioassays and would ordinarily proceed with a cancer classification if it considered any of the findings in its own studies significant.

Further, the HID provides a lengthy amount of information on mechanisms that might lead to formation of other types of tumors These sections amount to pure speculation and should be ignored by the CIC.

The CIC will have only about 2 weeks to review these comments and weigh them against the incomplete and misleading information in the HID. It therefore would be a reasonable decision for the CIC to defer consideration of BBP at the December 5, 2013 meeting. The CIC could direct OEHHA to provide a new version of the HID that incorporates the missing information and presents it in a more objective manner. Principles of scientific rigor through open exchange of information and views would then dictate that stakeholders have opportunity to comment on the revised HID, and that the CIC direct OEHHA to give it sufficient time to review both the HID and the stakeholder comments.

Part I of these comments provides additional information on the failings of the HID to present a complete and balanced summary of the evidence.

Part II of these comments presents a more complete overview of the key data, and then explains why the weight of the evidence is insufficient for a finding that BBP causes cancer.

The strong weight of the evidence is that *BBP is not genotoxic*. This is the conclusion of Dr. Errol Zeiger, a genetic toxicologist whose notable 40+ year career includes 21 years developing, implementing, and managing the genetic toxicology testing program for the NTP. His opinion is provided as Attachment A.

The weight of the evidence also is that the observation of tumors in the NTP studies of BBP do not provide a basis for finding BBP clearly causes cancer (NTP 1982; 1997a; 1997b). This is the conclusion of Dr. Gene McConnell, a pathologist whose 40+ year career includes heading the Pathology Branch of the NTP and directing the NTP Toxicology Research and Testing Program. His opinion is provided as Attachment B.

As discussed by Dr. McConnell and by Part II of these comments, *the data for all tumors is inconsistent* across the NTP studies:[4]

- Although there was a statistically significant increase in MNCL in female rats in NTP (1982), there was no such increase in female rats in NTP (1997a) or NTP (1997b). Increased MNCL was not seen in male rats in any of the studies, nor in either sex in the study of mice (NTP, 1982). Further, MNCL is a lesion of questionable significance for human health assessment.

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[4] Note that NTP found the male rat results in NTP (1982) inadequate for classification of any tumor.

- Although there was a statistically significant increase of pancreatic acinar cell tumors in male rats in NTP (1997a), there was no such finding in male rats in NTP (1997b), nor in females in any of the studies (NTP, 1982; 1997a; 1997b). Nor was there any such observation in mice of either sex (NTP, 1982).

- The HID asserts that there was an increase in adrenal medulla pheochromocytoma in female rats in NTP (1982), although this was not the finding of NTP itself. And no increase in these tumors was observed in female rats in the other two studies (NTP, 1997a; 1997b), nor in male rats in those studies, nor in mice of either sex (NTP, 1982).

- The HID asserts an increase in urinary bladder transitional cell tumors in female rats in NTP (1997b). However, the increase was not statistically significant, and the NTP did not consider this a positive finding. Again, no increases were observed for this tumor in female rats in the other studies, in male rats in any study, nor in male or female mice.

Thus, not one of the four tumor types is clearly a consequence of BBP treatment. In fact, the weight of the evidence across the genotoxicity data and all three NTP reports is that BBP does not cause cancer in rodents. Further, there is no evidence that BBP causes cancer in humans. Thus, the clear conclusion is that BBP does not meet the criteria for listing as "known to the State to cause cancer." This conclusion is consistent with NTP's assessments of its own studies, as reflected in the lack of any NTP cancer classification for BBP in the 17 years since it completed the last of its lifetime cancer studies. .This conclusion also is consistent with IARC's determination in 1999 not to classify BBP after considering all three NTP studie

I. THE HID IS BIASED AND INCOMPLETE; THE CIC MUST CAREFULLY WEIGH ALL THE EVIDENCE, DEFERRING CONSIDERATION OF BBP IF NECESSARY

The hazard identification document prepared by OEHHA to support the CIC's deliberations, while voluminous, is biased and incomplete to a significant and disturbing degree. *It does not provide a fair, accurate, complete and objective basis for the CIC's deliberations.* While the HID attempts to portray the data as providing a number of pieces of evidence of carcinogenicity, the fact of the matter is that *the several bioassays of BBP provide no consistency in observation of any tumor*, with most of the observations of increased incidence not even achieving statistical significance and/or occurring in only one sex of one species in one study. The HID obscures how lacking the evidence of carcinogenicity is with speculation, extraneous information, and misleading characterizations of the data.

Our concerns are compounded by the fact that many of the errors and omissions in the OEHHA document could have been avoided if OEHHA had considered and incorporated scientific information from materials previously provided by Ferro. Forty-four pertinent journal articles cited in those materials but not included in the HID are listed in Exhibit A. The omission of scientific information that does not support a decision against listing, repeated throughout the HID, is troubling and undermines the objectivity of the document.

Thus, to construct for itself a proper weight-of-the evidence assessment, as called for by its guidance criteria,[5] the CIC faces a heavy lift to review the information in these comments and expert reports, as well as that in the HID, and to consult underlying scientific publications and assessments by other authoritative bodies (including some not cited by the HID). There is little time between the due date of these comments and the December 5 meeting for the CIC to do so. We therefore recommend that the CIC consider deferring consideration of BBP at its December 5 meeting and instead request that OEHHA revise its HID to include all relevant information, presented in an objective manner, allow stakeholders sufficient opportunity to comment on that revised HID, and give the CIC itself sufficient time (4 weeks or more), to give the HID and stakeholder comments full review.

We do not suggest deferral lightly, but wish to emphasize that Ferro responded in a timely manner to OEHHA's request for information, and the current inadequate amount of time for the CIC to prepare and deliberate could easily have been avoided. We are aware that OEHHA earlier this year entered into a settlement—to which Ferro was not a party—in which it agreed to allow only 45 days for comment on HIDs for the next three years (compared to the past practice of 60 days). The very idea that OEHHA would agree to limit the comment period in all cases, regardless of the complexity of the issues or the volume of material that needs to be considered, is troubling. The saving of 15 days is immaterial to the overall pace of OEHHA listing activities, though highly prejudicial to interested parties that wish to comment. But more importantly, the settlement set no limit on the amount of time between the close of comments and the CIC meeting. That part of the schedule remains entirely within OEHHA's control. There is no justification for OEHHA producing an incomplete document, as it has done here, and not

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[5] Guidance Criteria for Identifying Chemicals for Listing as "Known to the State to Cause Cancer" (March 2001), http://www.oehha.ca.gov/prop65/policy_procedure/pdf_zip/revcriteria.pdf .

allowing CIC members adequate time to review comments and probe the literature to develop a proper weight-of-the-evidence assessment.

In support of its suggestion that consideration of BBP be deferred, and to alert the CIC of the need to be cautious in accepting the statements and conclusions in the HID if it does consider BBP on December 5, examples of the incompleteness and excessive tilt of the HID follow. Additional examples are evident in Part II. We also address in this part the requirement that governmental decisions be grounded in fair and objective review of all relevant data.

A. The HID contains information that is extraneous but prejudicial

1. Exposure information

OEHHA has stated many times that exposure to a chemical is not relevant to a Proposition 65 listing decision, which is based purely on hazard. The HID nevertheless provides information on uses of and exposure to BBP. This is not unusual background information for an HID, but here the information is presented in a highly prejudical and misleading manner, raising questions right at the outset about the objectivity of the HID.

This is particularly evident in the Executive Summary, which states, "Biomonitoring studies have found that the levels of MBzP in urine from *pregnant women* from California to be slightly higher than the levels in U.S. *females in general*."[6] This is highly misleading. Reference to Table 1 of the HID shows that with an apples to apples comparison, the urinary concentration for *pregnant* women sampled in California (7.73 ug/L) is slightly <u>less</u> than for *pregnant* women sampled from various US locations (9.3 ug/L).

Further, the HID gives no perspective to these values. Further, the HID gives no perspective to these values. Using information previously supplied to OEHHA by Ferro, the value for U.S. women converts to 0.24 ug/kg/day of BBP exposure The value for U.S. women converts to 0.24 ug/kg/day of BBP exposure.[7] That for pregnant women in California converts to approximately 0.31 ug/kg/day, a difference of 0.07 ug/kg/day.[8] Given the uncertainty inherent in such measurements, these two values probably should be considered identical, and they are millions of times below lowest effect doses administered in the NTP studies.[9] The focus on pregnant women, a sensitive subpopulation, and the misleading claim of increased exposure, appear to be

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[6] HID at 1 (emphasis added).

[7] This conversion is easily done using the method of David (2000) and Kohn et al. (2000), with the fractional urinary excretion value from Anderson et al. (2001). This methodology was described in Ferro's January 22, 2013 submission to OEHHA (see Section 5 of Attachment A to that submission found in Attachment D to this submission).

[8] It is not possible to do the conversion without a creatinine adjustment to the urinary concentration. That value is not provided for the California pregnant women. We have assumed that the relative difference between the ug/L value and the ug/g would be the same as for the U.S. women in general.

[9] More specifically, both values are several million times below the lowest BBP dose administered *ad libitum* that produced evidence (albeit equivocal evidence) of tumor formation in NTP (1997a). In that context a difference of 0.07 ug/kg/day is of no consequence at all.

included only for their prejudicial impact. An objective summary would have noted that biomonitoring data consistently show that while exposure is widespread, aggregate exposures from all sources are consistently shown to be very low for all tested populations, including potentially sensitive subpopulations.

2. Speculation on mechanisms

A very large proportion of the HID is dedicated to discussion of postulated mechanisms by which BBP theoretically might cause cancer. This is well and good for an academic exercise, but has no place in a proceeding meant to determine whether, "through scientifically valid testing according to generally accepted principles,"[10] BBP has not been "clearly shown … to cause cancer ."[11] Not one of the proposed mechanisms, based primarily on *in vitro* data, is shown through scientifically valid testing according to generally accepted principles to be applicable to BBP. And the proof of the pudding is *in vivo* testing, which, as discussed above does not clearly show BBP to cause cancer of any type. Thus, the Mechanisms section of the HID is purely speculative and should be completely disregarded by the CIC. Indeed, its inclusion is further evidence of the imbalance of the HID as a whole, and of the way that repeated selective and incomplete presentation of data can create an impression of the data as a whole that is greater than the sum of the parts.

As explained in Section II.D of these comments, the entire Mechanisms Section of the HID is irrelevant unless it is accompanied by functional, mechanistic studies that demonstrate or support some link between the changes reported in the in vitro assays and a toxic endpoint. In those few instances where the HID attempts to associate a BBP-induced tumor with a mechanism, the explanation fails.

Section 4.2. of the HID includes a discussion of the PPARα mode of action. It is not clear why this is even included in the HID. Section 4.2.1.2 of the HID, "The potential role of PPARa activation in BBP-induced tumors concludes by saying "Thus, there are no data to suggest that PPARα is involved in the induction of pancreatic cancer acinar cell tumors by BBP or in BBP tumorigenesis in general."

B. The HID includes information that is erroneous and/or misleading

The HID includes a number of statements that are outright erroneous. In other instances, the HID is misleading because important contrary information is not disclosed or the characterization of the information is framed in a misleading manner. Examples of erroneous statements, omissions of important information, and other misleading aspects of the HID include:

- The HID nowhere acknowledges that, even though all three cancer bioassays were conducted by NTP, NTP has never considered BBP for listing in its annual report on carcinogens, which it ordinarily would do for a commercially-important compound if it considered the findings significant. Further, while the HID does acknowledge the determination of IARC (1999) not to list, it buries this highly relevant bit of information

[10] Health and Safety Code §25249.8(b).

[11] *Id.*

in a Section 5 and leaves it out of the Executive Summary and the Summary and Conclusions.

- As discussed in the introduction of these comments, the HID summary of genotoxicity data is highly misleading. presentation of the data on genotoxicity fails to address important points presented in Ferro's prior comments. The overall weight of the evidence is that BBP is not genotoxic.

- The HID presents the information on MNCL incidence in F-344 rats in an incomplete and highly misleading way.

  - The HID fails to give proper recognition to the inconsistent MNCL findings across NTP studies.

  - Nowhere does the HID recognize that the spontaneous incidence of this finding in F344 rats is so great and variable that NTP has discontinued use of this strain of rat for its cancer testing programs.

  - The authors of the 1982 NTP study expressly judged the positive finding of MNCL in high dose female rats (but not other female rats or male rats) "to be of equivocal biological significance," but this is not disclosed in the OEHHA document.

  - Male rats in the NTP dietary restriction study had a statistically-significantly higher incidence of MNCL than weight-matched control rats, but critical information again is omitted from the HID. Due to the decreased incidence of MNCL in the weight-matched controls, NTP considered the finding not a treatment-related finding.

In some cases, the misstatements are subtle and discoverable only by close examination of tables. For example, the HID states that "BBP increases bladder transitional epithelial cell tumor incidence in rats;"[12] however, Table 7, p. 17, shows that the increase was not statistically significant.

C. The HID is rife with unsupported speculation about the potential carcinogenicity of BBP

A mere hypothesis has no place in determining whether a chemical "has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer."[13] Yet, as indicated above, where the data do not support a finding that BBP causes cancer, the HID falls back on speculation.

For example, the Executive Summary notes that two epidemiology studies found no association of BBP exposure with breast cancer, then states: "but both studies had limitations that may have

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[12] HID at 85.

[13] Safe Drinking Water and Toxic Enforcement Act of 1986 ("Proposition 65"), Health & Safety Code §25249.8(b).

prevented them from detecting excess risk."[14] This is purely gratuitous conjecture. Every study is of necessity subject to limitation and proof of a negative is impossible. A similar statement could be made for any study of any substance for which no effects were detected. An objective document would simply have stated that two limited epidemiology studies found no association of breast cancer with BBP, noted the absense of any mammary tumor findings in the animal cancer bioassays, and left it there.[15]

Breast, endometrial, ovarian, and testicular cancer are presented as potential outcomes of exposure to BBP. There is no mention in conjunction with this speculation that each of the bioassays completed on BBP does not support consideration of the breast, uterus, ovaries or testes as target organs for BBP toxicity nor do the many rodent reproduction studies (which routinely include pathological examination of the uterus and ovaries).

Much is made of results from in vitro and in vivo microarray assays and proteomic analysis. This speculation is highly misleading because it lacks demonstration of any mechanistic link between changes observed in the assay and causation of a toxic effect.

D. Principles of fairness and honesty dictate that the deficiencies of the HID be cured

It is fundamental that regulatory decisions that will impact the knowledge, health and economy of California citizens must be based on a complete, rigorous and objective consideration of all relevant information. In *CHMA v. IWC*, the California Supreme Court took for granted that agency action must be "reasonable, rather than arbitrary, capricious, or lacking evidentiary support."[16] As illustrated above, the HID contravenes that principle. It is arbitrary in the selection of information to present and of information to leave out. It is capricious and lacking in evidentiary support in relying heavily on speculation to try and overcome the fact that the weight of animal evidence does not support a determination that BBP should be listed as "know to the State to cause cancer."

The integrity of the listing process requires that OEHHA present the relevant scientific information in a fair and balanced manner, but it has not done so here. To cure the bias and incompleteness of the HID, the CIC must carefully review the information, expert opinions and citations provided herein. If the compressed time for review of comments and underlying information prevents the CIC from arriving at a weight of evidence decision that is based on all relevant evidence, it can cure the failings of the HID by deferring its consideration of BBP and requesting a fair and objective document from OEHHA. To further ensure that the determination regarding BBP is made in a fair manner, the CIC can also request that OEHHA provide a reasonable opportunity for stakeholder input on the revised HID and that OEHHA schedule the CIC meeting so that the CIC members have substantial time to review both the HID and the stakeholder comments.

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[14] HID at 1.

[15] Of note, as stated in the body of the document, one of the studies actually showed a negative dose-response between urinary level of the BBP metabolite and breast cancer. HID at 12.

[16] *Cal. Hotel & Motel Ass'n v. Indus. Welfare Comm'n*, 25 Cal.3d 200, 211 (1979).

II. THE WEIGHT OF THE EVIDENCE PROVIDES NO BASIS FOR DETERMINING THAT BBP CLEARLY CAUSES CANCER

Ferro provided materials regarding BBP carcinogenicity data to OEHHA in January 2013, provided here as Attachment D. Those materials included comments Ferro submitted in 2011 as part of the CIC prioritization process. Ferro incorporates those materials by reference and, in these comments, addresses additional issues raised by the HID for BBP. It is Ferro's position that the evidence is not sufficient to list BBP as "known to the State to cause cancer."

Ferro's prior submissions focus on the weight of evidence that BBP was not genotoxic and not reliably shown to be tumorigenic in animal bioassays. The evidence on these endpoints has not changed: animal bioassay evidence of BBP carcinogenicity is limited or negative and the evidence for genotoxicity supports the conclusion that BBP is not genotoxic.

The HID introduces mechanistic studies, mainly *in vitro* work, as evidence of potential mechanisms for BBP carcinogenicity, and postulates that tumors of the breast, uterus, ovaries or testes might result from the mechanism. All of this is highly speculative and lacking in scientific rigor. There is no empirical evidence in the gold standard life-time rodent bioassays that any of these mechanisms result in mammary, endometrial, ovarian, or testicular cancer. In addition, there is no support from two epidemiology studies of BBP and breast cancer. The HID also discusses the peroxisome proliferation activation receptor alpha (PPARα) mechanism as potentially causing pancreatic acinar cell cancer. But the sine qua non of the PPARα mechanism is development of liver tumors in rodents, and none have been detected in rodents. The HID further speculates on a PPARγ mechanism for urinary bladder tumors, but makes serious errors in its review and interpretation of the data.

The information in the OEHAA document addresses five areas:

- Human cancer experience
- Animal bioassays
- Genetic toxicity testing
- Mode-of-Action/Mechanism of tumorigenicity (cellular receptor activation and gene expression, perturbation of steroidogenesis, and generalized cell proliferation)
- Structure activity comparison with other phthalates.

We address each of these areas below.

A. Human cancer experience: Epidemiology studies show no association between breast cancer incidence and BBP exposure

Two breast cancer epidemiology studies are available which looked at an association between BBP-exposure and an increased incidence of breast cancer (Aschengrau, et al., 1998; Lopez-Carillo, et al., 2010). Both studies were negative for an association of breast cancer and BBP exposure. The HID cites both studies but minimizes or otherwise dismisses the significance of the results of these published works. Both studies had an adequately large population to allow

for statistical significance in data analysis. Subjects in both studies had documented exposure to BBP. One of the studies (Lopez-Carillo, et al., 2010) showed a *negative* association between breast cancer risk and exposure to BBP.

B. Animal bioassays: Evidence from long-term animal bioassays does not provide clear evidence that BBP causes cancer

Animal bioassay studies are discussed in some detail in Section I of our 2011 submission (in Attachment D of this submission) and are briefly summarized below. In addition, Ferro asked Dr. Ernest E. McConnell, an expert in pathology who was many years at NTP, to review the data. His expert opinion is provided as Attachment B to these comments and is summarized below.

NTP conducted lifetime bioassays on BBP in rats in mice in 1982 and twice in 1997. NTP is the premier animal testing laboratory for carcinogenicity. The studies produced a mixed response of extremely common tumors in the strain of rats used at that time by the NTP (Fischer 344 rats) and some less common tumors as well. There is considerable inconsistency across these studies with respect to the tumor types observed in rats. No tumors were found in mice.

Table 1 summarizes the findings of the NTP studies, along with two relevant, short-term carcinogenicity bioassays with BBP that we located in the open literature. See also Tables 1 and 2 in Dr. McConnell's opinion (Attachment B).

Table 1. Summary of Animal Bioassays with BBP	
Study	Results
NTP, 1982	2-year study in F344 rats and B6C3F1 mice. No tumor response in mice; an increase of mononuclear cell leukemia (MNCL) in female rats of questionable relevance.
NTP, 1997a	2-year study in F344 male and female rats; no MNCL increase in female rats (i.e., 1982 result not repeated; low incidence of increased pancreatic tumors in male rats possibly related to diet; bladder tumors seen but incidence not statistically-significantly elevated.
NTP, 1997b	24, 30 or 32–month studies with and without dietary restriction. Evidence for role of diet in pancreatic tumors in male rats; bladder tumors not elevated above control incidence, No increase in MNCL.
Theiss, et al. (1977)	24-week intraperitoneal injection study in Strain A mice (injected 3 times/week for 8 weeks). No evidence for carcinogenicity.
Singletary, et al. (1997)	16-week co-carcinogenicity study in female Sprague-Dawley rats (gavaged 7 times/week for one week, followed by a single dose of DMBA (dimethylbenz[a]anthracene). No co-carcinogenic effect observed. BBP reduced the incidence of DMBA-induced mammary tumors.

As stated, Ferro asked Dr. Ernest E. McConnell to review the extant cancer bioassay data on BBP. Dr. McConnell has over 40 years' experience in the field of experimental carcinogenesis, particularly in the design, conduct and interpretation of rodent bioassays. His positions have included head of the Pathology Branch of the National Toxicology Program (NTP) and Director of the NTP's Toxicology Research and Testing Program. Dr. McConnell's conclusions are listed below; his full report is Attachment B to these comments.

- "the MCL response cannot be attributed to exposure of BBP"
- "the pancreatic adenoma response cannot be attributed to exposure of BBP"
- "the pheochromocytoma response cannot be attributed to exposure of BBP"
- "the urinary bladder lesions may be attributable to the animal's exposure of BBP even though the increase in tumors was not statistically significant and failed to pass the Bradford-Hill criterion for "consistency. … If I am correct that the mode-of-action (for bladder tumors) is probably due to "crystal formation", these tumors are not relevant to humans"

In summarizing his findings, Dr. McConnell states:

> The only tumor finding that I felt might be associated with BBP in these studies were the tumors of the urinary bladder. And, even this finding does not rise to the level that would support listing BBP under Proposition 65 because of the unusually high exposure and unusually long exposure period (32 months), likely resulting in chronic irritation via physical damage to the epithelium from crystaluria. I would add that the NTP has not considered BBP for listing in its' Report on Carcinogens as would be expected if NTP believed it caused the tumor response that OEHHA proposes. In summary, in my opinion if one uses a "Weight of Evidence" approach, as per CIC guidance criteria for the evaluation of this robust set of data on BBP, the totality of the data do not rise to the level required for listing under Proposition 65.

C. Genetic toxicity testing: The weight of the evidence is that BBP is not genotoxic

BBP has been tested in a variety of *in vitro* and *in vivo* genetic toxicity assays for genetic toxicity endpoints and for the ability to induce morphologic transformation. The *in* vitro assays were conducted with bacterial, yeast and mammalian cell systems. *In vivo* assays were conducted in mice, rats and Drosophila. In most assays, BBP was negative; in the remainder results were equivocal. The great weight of the evidence indicates that BBP is not genotoxic. See Section II and Appendix B of our 2011 submission (included in Attachment D to these comments).

Ferro asked Dr. Errol Zeiger to review and comment on the genetic toxicity studies completed on BBP. Dr. Zeiger has worked as a genetic toxicologist for over 40 years. Among his laboratory positions, Dr. Zeiger is the former Head of the Mutagenesis Group of the Environmental Mutagenesis and Carcinogenesis Branch, National Institutes Environmental Health Sciences (NIEHS). Dr. Zeiger served as Editor-in-Chief, Environmental and Molecular Mutagenesis from 1994 through 1998. In his evaluation of the BBP genetic toxicity data, Attachment A to these comments, Dr. Zeiger concludes:

> [T]he weight of evidence, taking into consideration the lack of structural alerts for DNA reactivity in BBP or its putative metabolites, the routes of administration and doses used in the tests that produced the reported positive responses, the types and relevance of responses reported, the level of confidence in the reproducibility and predictability of the tests showing positives verses those showing negatives, and the lack of availability of the test protocols and data for some of the positive reports, leads to the conclusion that BBP should not be considered genotoxic.

D. Mode-of-Action/Mechanism of tumorigenicity: The mechanisms proposed in the HID are purely speculative and not supported by the apical animal bioassays nor the existing epidemiology studies

The very long sections on "Other Relevant Data" (Section 3.3) and "Mechanisms" (Section 4) in the HID are rife with unsupported speculation of human cancer risk from BBP, raising serious questions about the objectivity of the authors. These sections are completely inadequate to support a finding that any of these mechanisms could result in cancer from BBP. They contain

many significant errors and demonstrate a disturbing lack of scientific rigor. They provide no information to show that the speculated mechanisms can be tied to a BBP toxic effect. In fact, the opposite is the case – none of the tumors postulated to result from these mechanisms have been seen in the animal bioassays, and the existing human epidemiology studies show no evidence of one postulated tumor type – breast cancer.

The following highlights a number of the deficiencies of Sections 3.3 and 4. We then address the tumor outcomes which, though not seen in animal bioassays, the HID speculates might result from the mechanisms it discusses.

1. Section 3.3.2 Genotoxicity

The HID discussion of genotoxicity in Section 3.3.2 is incomplete. It does not consider strengths and weaknesses of each study, and there is no analysis of what the overall weight of evidence indicates. As discussed in Section II.C, above, the weight of the evidence is that BBP is not genotoxic.

2. Section 3.3.5.1 In vivo differential cDNA microarray analysis

This entire section is irrelevant, because there are no functional, mechanistic studies that demonstrate or support a link between the described changes and a toxic endpoint. The data are meaningless without performing experiments designed to show how these changes, if at all, can influence toxicity. The section catalogs effects observed but provides no interpretation, because the latter is not possible. In addition, these data are at the mRNA level and were not confirmed by quantitative polymerase chain reaction (qPCR) or quantitative western blotting.

See also the discussion of Moral (2007; 2011) in the opinion of Dr. Zacharewski, Attachment C to these comments.

3. Section 3.3.5.2 In vitro differential cDNA microarray analysis

As above for the in vivo studies, there is no confirmation by qPCR for all genes, no showing of functional changes in protein, and no mechanistic studies to link any change in expression with a toxic readout. In many cases, the HID implies mechanisms but provides no supporting evidence from the study cited. In other cases, a study is cited to support a statement. Altogether, this section is long on speculation and short on science.

4. Section 3.3.5.3 Proteomic analysis

As above for the descriptive arrays, this section is speculative and does not demonstrate that any of the reported changes are related to any toxic effect of BBP. In addition, a limitation to the Choi et al. (2010) study is that HepG2 cells are aneuploid and contain multiple mutations.

5. Section 3.3.11 Structure Activity Comparisons

The HID provides no basis for assuming a structure activity relationship. See Section II.E, below. It does not describe structure activity relationships – it simply lists a handful of phthalates and discusses various observations for some of them, but provides no insight as to

how the structure is related to any activity. Indicating that BBP, DEHP and DINP activate different receptors is not showing any structure activity comparison of any type. Table 26 is extremely misleading. It does not mention the negative genotoxicity studies, yet implies there is some relationship with receptors. Without citation to studies that link BBP with genotoxicity studies and show this may or may not be linked with receptor activity, this Table is not germane.

6. Section 4.1 Genotoxicity

The HID discussion of genotoxicity in Section 4.1 is incomplete, biased and highly speculative. It does not mention the studies showing that BBP is not genotoxic, but uses the few positive studies to suggest that BBP is genotoxic and contributes to mechanisms of cancer. The HID is purely speculative in linking the in vivo study showing changes in gene expression and the study done in a human liver cancer cell line with genotoxicity. No peer-reviewed studies are cited to support this link, and the HID fails to describe the limitations of these models.

7. Section 4.2 Activation of PPARs

The section on PPARs under "Mechanisms" is packed with errors and misleading statements. The entire section is misleading, because it does not demonstrate any mechanistic link between BBP activation of a PPAR and any toxic effect. And in fact, such data does not exist.

The prevailing data do not support a role of PPARα in causing cancer in humans (Corton et al., 2013). Further, the role PPARα has in rodents is induction of liver tumors. Since BBP does not cause liver cancer in rats or mice, and PPARα ligands do, there is no sound basis for discussing PPARα in relation to BBP. The hypothesis that PPARα might have a role in causing pancreatic acinar cells tumors was only described in a review. There are no studies to demonstrate this entirely speculative role for PPARα . Inclusion of this speculation is therefore misleading and incorrect. Further, Section 4.2.1.2 of the HID concludes, "Thus, there are no data to suggest that PPARα is involved in the induction of pancreatic acinar cell tumors by BBP, or in BBP tumorigenesis in general." It therefore is not clear why PPARα is discussed at all.

The HID has serious errors in its interpretation of PPARγ activity and bladder cancer. Not all PPARγ agonists cause transitional cell bladder tumors (troglitazone, ciglitazone, rosiglitazone) and pioglitazone only causes this tumor type in male rats, not female rats and not in male or female mice (Sato K et al, 2011). Since both rats and mice express PPARγ in the urothelium, a PPARγ-dependent mechanism seems highly unlikely. Failure to disclose these facts makes the HID unbalanced. The authors also cite two studies showing an association of bladder cancer with humans treated with pioglitazone, but fail to cite the more recent Kaiser trial showing no such relationship (Lewis et al., 2012), or Tseng (2012) which did not show the relationship, or Erdmann (2013), showing no relationship between administration of rosiglitazone and bladder cancer.

8. Section 4.4 Estrogen Receptor (ER)

Section 4.4 of the HID discusses the estrogen receptor and *in vitro* studies of BBP interaction with the estrogen receptor. This section completely ignores the opinion of Dr. Timothy R. Zacharewski and its citations. The opinion was included in Ferro's January 2013 submission and

is duplicated here as Attachment C.[17] Dr. Zacharewski is Distinguished Professor of Biochemistry & Molecular Biology at the College of Natural Sciences and member of the Center for Integrative Toxicology, at Michigan State University. He is an expert in the investigation of estrogenic endocrine disrupters and has served on advisory committees reviewing the health risks of endocrine disruptors, including the U.S. EPA Endocrine Disruptor Screening Program (EDSP) review committee.

As detailed in Dr. Zacharewski's opinion, *in vitro* studies of BBP estrogenicity are not reflective of what happens in the live organism, because in animals, BBP is rapidly metabolized to a monoester. Definitive *in vivo* studies demonstrate that BBP is not estrogenic. His overall conclusion is that, "the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action."

9. Section 4.5 Androgen receptor (AR) and steroidogenesis

The last sentence in the first paragraph of this section is incorrect. The study by Ward examined the effect of DEHP, not BBP.

10. Postulated outcomes of the speculative mechanisms are not supported by apical animal studies or epidemiology studies

The focus of the HID speculation based on these mechanistic arguments is that risk of breast cancer and possibly other cancers of female reproductive organs, and testicular cancer arising from the hypothetical Testicular Dysgenesis Syndrome (TDS) – all presumably as a result of BBP interaction with the estrogen receptor, the AhR receptor, or acting as an anti-androgen. There is no empirical evidence in the gold standard life-time rodent bioassays that any of these mechanisms result in mammary, endometrial, ovarian, or testicular cancer. In addition, there is no support from two epidemiology studies of BBP and breast cancer. In effect, the authors are saying, "If BBP were to cause cancer, here are some kinds of cancer it might cause and here is how it might cause them." Such baseless speculation does nothing to inform the question of whether BBP has been clearly shown to cause cancer and is therefore of no utility to the determination the CIC must make.

Breast and other female reproductive organ cancers. Mammary cancer, endometriosis and ovarian cancer are presented as potential outcomes of exposure to BBP.[18] There is no mention that each of the bioassays completed on BBP does not support consideration of the breast, uterus or ovaries as target organs for BBP toxicity, nor do the many rodent reproduction studies (which routinely include pathological examination of the uterus and ovaries) (Tyl et al., 2004; NTP, 2003). The HID provides no explanation for why, if the postulated estrogen receptor mechanisms are actually operational in organisms, tumors do not form in these estrogen-sensitive tissues as a result of lifetime exposure to high doses of BBP. Section 4.4 also is highly misleading because it does not juxtapose the positive results of the screening tests discussed

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[17] See also Section III of Ferro's 2011 submission, included with the 2013 submission, Attachment D to these comments.

[18] HID Sections 3.3 and 4.

therein with the negative results of breast cancer epidemiology studies and all of the apical rodent bioassays which uniformly showed no evidence of BBP-induced mammary tumors.

We also note the HID's statement: "Endometriosis behaves similarly in many ways to invasive cancer" is misleading.[19] It suggests that endometriosis is a form of cancer, which is no more valid than suggesting that gingivitis behaves similarly to oral cancer.

Testicular tumors. The HID speculates in both the Executive Summary and the Summary of Evidence that BBP poses "a potential increased risk of testicular tumors through induction of testicular dysgenesis syndrome in humans."[20] First, "testicular dysgenesis syndrome" (TDS) is no more than a hypothesis; there is not a consensus in the scientific/medical community that there actually is such a syndrome. Second, while rats are a sensitive model for disruption of testicular development *in utero* through steroidogenesis perturbation, there is no evidence of increased incidence of testicular cancer in rats as a result of treatment with BBP, including in utero treatment. Therefore, the assertion that there may be a potential increased risk of testicular tumors is unfounded.

Pancreatic acinar cell and bladder tumors. As explained above, there is no basis for the HID speculation that the PPARα mechanism might lead to pancreatic tumors, especially where it does not lead to any liver tumors in rodents. Likewise, there is no reasonable basis for the hypothesis that PPARγ activation might cause bladder tumors. Further, as explained in Section II.B, above, and in Dr. McConnell's opinion (Attachment B), animal bioassays do not provide clear evidence that BBP causes either type of tumor.

E. Structure activity comparisons: The HID provides no basis for finding a structural activity among phthalates

HID Section 3.11, titled "Structure Activity Comparisons," provides no information on what structure would account for a structure activity relationship (SAR) among phthalates, much less how that structure would function or how it might relate to any cancer concern. The section boils down to saying no more than, "Because di(2-ethylhexyl)phthalate (DEHP) has been listed for cancer under Proposition 65, and diisononyl phthalate (DINP) is being considered for listing for cancer, BBP also should be listed for cancer." Such superficial attempts to prejudice the reader have no place in a rigorous scientific document. Moreover, But the information in the section actually highlights the differences among the phthalates more so than their similarities.

On the molecular level, what phthalates have in common is having a phthalate moiety (benzene ring with two ortho carboxylates) and two alkyl "arms." What differentiates phthalates on the molecular level is the nature of the alkyl "arms." BBP is unique among the high production volume phthalates in that, properly speaking, it is not an alkyl phthalate. The "butyl" arm is an alkyl, but the "benzyl" arm is an aromatic.

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[19] HID at 65.

[20] HID at 3 and 91; see also HID Section 4.5.

On the physical, chemical and toxicological levels, the phthalates vary widely (see, e.g., David et al., 2001, the NTP monographs on seven phthalates,[21] and the toxicity reviews for 23 phthalates by the US Consumer Product Safety Commission).[22] Table 25 of the HID actually illustrates this with respect to sites of tumor increases. The only type of tumor seen in all three of BBP, DEHP and DINP is MNCL, which primarily has to do with the fact that they all were tested in the F344 rats, in which MNCL is known to occur spontaneously at such a very high incidence thatNTP has stopped using the rat strain. Note that, although pancreatic tumors are listed for all three of the phthalates, this column improperly combines pancreatic acinar cell tumors and pancreatic islet tumors. These are different types of tumors (see, e.g., Haseman et al., 1998). Also note that the table improperly lists tumors for which there was no statistically significant increase, and/or no reproducibility, and/or for which the tumor is known to not be relevant to humans. . The overall impression is very misleading, and such manipulative and selective comparisons should be disregarded entirely.

Phthalates are rapidly metabolized to monoesters in mammalian species, and it is the monoester or other metabolites that are distributed via the circulation to all tissues. In most mammalian species the primary metabolites of BBP are excreted in the urine as unconjugated monobutyl and monobenzyl esters and their derivatives (ECB, 2007).[23] Of all other commercial phthalates, only dibutyl phthalate can be metabolized to form a monobutylphthalate. Other than BBP, no commercial phthalate forms monobenzyl phthalate. Thus, there is no obvious rationale for a structure activity relationship and, as discussed in Section E, above, the HID provides utterly no discussion of a structure and what its activity would be.

This section of the HID rises to the level of speculative advocacy that might be expected of a stakeholder seeking to support listing, a political argument, but has no place in the kind of objective, rigorous not to the level of scientific discourse that an agency is expected to provide to an outside expert panel. It should be completely ignored by the CIC.

CONCLUSION

These comments express strong criticisms, which Ferro does not do lightly. The HID is an important document, and is intended to support very important deliberations by the CIC. But the HID is severely deficient, and inadequate to serve that purpose. For the reasons presented herein, the CIC should consider deferring evaluation of BBP until a proper HID has been prepared, and the CIC has adequate time to consider the revised HID and any public comment.

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[21] Available at http://ntp.niehs.nih.gov/?objectid=4980AA81-E919-4E85-60B789CA36E59FA5#archives.

[22] Available at http://www.cpsc.gov/en/Regulations-Laws--Standards/CPSIA/Phthalates/Chronic-Hazard-Advisory-Panel-CHAP-on-Phthalates/April-2010-Meeting/ and http://www.cpsc.gov//PageFiles/126213/toxreview.pdf .

[23] The predominant monester differs with mammalian species. The rat preferentially hydrolyzes BBP to form the monobutyl ester (Eigenberg, et al., 1986; Mikuriya, et al., 1988; Monsanto, 1996a; Monsanto, 1996b). In the rat BBP yields approximately 16% MBzP and 44% MBuP on a molar hydrolyzed to form MBzP (73% on a molar basis) over MBuP (6% on a molar basis (Anderson, et al., 2001)). This is in contrast to the rat.

If the CIC proceeds with its deliberations on December 5, Ferro respectfully submits that the CIC should find that the weight of the evidence does not support listing BBP.

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EXHIBIT A

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ATTACHMENT A
OPINION OF DR. ERROL ZEIGER

Evaluation of the Genetic Toxicity Test Data for Butyl Benzyl Phthalate

(CASRN 85-68-7)

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Table of Contents

1.0. Introduction 3
2.0. Genetic toxicity tests in vitro 4
2.1. Microbial test systems 4
2.1.1. DNA damage in vitro 4
2.1.2. Mutation in microbial tests in vitro 4
2.2. Mammalian cell test systems 5
2.2.1. DNA damage in mammalian cells in vitro 5
2.2.2. Mutation in mammalian cells in vitro 6
2.2.3. Chromosome damage in mammalian cells in vitro 6

3.0. Genetic Toxicity Tests in Somatic Cells in Vivo 7
3.1. DNA damage in vivo 7
3.2. Chromosome damage in somatic cells in vivo 7

4.0. Chromosome damage in germ cells in vivo 7
4.1. Rodents 7
4.2. Other organisms 8

5.0. Mammalian cell transformation in vitro 8

6.0. Other effects related to genetic damage 8

7.0. Overall evaluation of test results 9

8.0 Conclusions 10

9.0. Evaluation of the weight of evidence 12

10.0. References cited 13

Table 1. DNA damage/repair studies of BBP in bacteria 16
Table 2. Mutagenicity studies of BBP in bacteria 16
Table 3. DNA damage studies of BBP in mammalian cells in vitro 17
Table 4. Mutagenicity studies of BBP in cultured mammalian cells 17
Table 5. Chromosome damage studies of BBP in cultured mammalian cells 18
Table 6. DNA damage study of BBP in mice in vivo 18
Table 7. Chromosome damage studies of BBP in rodents in vivo 19
Table 8. Genetic toxicity studies of BBP in non-mammalian organisms 19
Table 9. In vitro cell transformation studies with BBP 20
Table 10. Summary of genetic toxicity test results 20

1.0 Introduction

My evaluations and opinions, which are described below, are based on my more than 40 years experience in the field of genetic toxicity testing.

I have M.S. and Ph.D. degrees in microbiology from George Washington University in Washington, D.C., a J.D. from North Carolina Central University School of Law. My career has been spent in biology and toxicology as a laboratory researcher, project director, and scientific program manager. I performed and directed genetic toxicology testing and research at the Food & Drug Administration (FDA) for 7 years and then at the National Institute of Environmental Health Sciences (NIEHS), NIH, for 24 years, until retiring in December 2000 to work as an independent consultant in Chapel Hill, NC.

I began doing research in genetic toxicology in 1969, and much of my scientific career has been in the design and direction of laboratory validation studies to determine the effectiveness of short-term genetic toxicity tests and develop standardized test protocols; the use of genetic toxicity tests to measure mutagenicity and predict carcinogenicity; and the evaluation, interpretation, and integration of toxicological test data; among other interests. Much of my work was performed under the auspices of the U.S. National Toxicology Program (NTP) to test chemicals of interest for toxic effects, including mutagenicity and carcinogenicity. I was responsible for developing, implementing, and managing the genetic toxicology testing program for the NTP and evaluating the test data, most of which was produced by contract laboratories under my direction. During my tenure in this program, from its inception in 1979 through 2000, I was responsible for the testing of more than 2,000 individual chemicals for mutagenicity in bacterial and mammalian test systems, including many of those described below. My own laboratory at NIEHS was also responsible for the testing of chemicals in the Ames test, and research relevant to the evaluation and further understanding of the bacterial test procedures. The data and results from all these tests are publicly available.

I am a member of a number of scientific societies, including the Environmental Mutagenesis and Genomics Society; Genetics and Environmental Mutagen Society, and the Society of Toxicology, and have more than 200 publications in the scientific literature, including more than 170 in peer-reviewed journals. I serve or have served on a number of editorial boards, was Editor-in-Chief of the scientific journal, *Environmental and Molecular Mutagenesis*, and was co-editor and contributor to the 1997 *Handbook of Carcinogenic Potency and Genotoxicity Databases*, and co-editor of *Jet Fuel Toxicology* which was published in 2011. Since 1978, I have been a volunteer lecturer in the graduate programs in the Pharmacology Department of Duke University or the Toxicology Program at N.C. State University on topics that have included genetic toxicology, the uses of short-term toxicology tests in carcinogen screening, and scientific publication and peer review. I continue to receive invitations to present lectures on genetic toxicity testing and data evaluation at scientific conferences and to various organizations, and to serve on expert committees.

Since January 2001, I have been working from a home office as an independent toxicology consultant, with clients in the U.S., Canada, and Europe. My clients have included U.S. and other governmental organizations; chemical, pharmaceutical, and cosmetic companies; contract research organizations; database development companies; the OECD; and law firms.

In sum, I have more than 40 years experience – as a government research scientist, mutagenicity test program manager, journal editor, peer-reviewer, and independent consultant – interpreting

and integrating chemical, mutagenicity, carcinogenicity, and other toxicological data to evaluate potential risk to humans based on a weight-of-evidence approach.

I evaluated all the available genetic toxicity data reports from the testing of BBP, to determine whether the various study conclusions were supported by the data, and the significance and reliability of the individual reports. The data were obtained from the peer-reviewed scientific literature, from unpublished, but publicly available, NTP reports, and from publicly available, industry testing reports. Much of the NTP genetic toxicity data that are available in the NTP carcinogenicity report on BBP and on-line have been published in peer-reviewed journals. For these studies, both the journal reports and the NTP sources are referenced.

The following constitutes my scientific opinion on whether the weight of evidence leads to the conclusion that butyl benzyl phthalate (BBP; CASRN 85-68-7) is genotoxic, or whether it supports a conclusion that BBP should not be considered genotoxic.

2.0 Genetic Toxicity Tests in Vitro

2.1. Microbial Test Systems

2.1.1. DNA damage in vitro (Table 1)

Omori [1976] summarized results of Kurata [1975] who reported that BBP did not induce DNA damage in *E. coli* or *B. subtilis* as measured by differential survival of DNA repair-deficient and repair-proficient cells. The results were presented as "–" (negative) with no accompanying data and no information on the author's criteria for a positive response.

2.1.2. Mutation in microbial tests in vitro (Table 2)

There are a number of reported studies of BBP mutagenicity in bacteria.

Omori [1976] summarized results of Kurata [1975] who reported that BBP was not mutagenic in *E. coli* wild-type or DNA repair-deficient cells. The author concluded that BBP was not mutagenic. The results were presented as "–" (negative) with no accompanying data and no information on the author's criteria for a positive response.

BBP (as Santicizer 160) was tested for mutagenicity in Salmonella strains TA 98, TA100, TA1535, TA1538, and TA1538 with and without rat liver S9 at concentrations from 0.1 – 10 µl/plate [Litton Bionetics, Inc., 1976]. No induction of mutations was seen.

BBP was tested for mutagenicity in Salmonella strains TA98, TA100, TA1535, TA1537, and TA1538 with and without Aroclor-induced rat liver S9 at concentrations from 0.001 – 10 µl/plate [Monsanto, 1976]. No induction of mutations was seen.

Kozumbo et al. [1982] tested BBP for mutagenicity in Salmonella strains TA98 and TA100 with and without rat liver S9. The authors noted that BBP was not mutagenic when tested up to a concentration of 1000 µg/plate, but no data were presented.

BBP was tested in two independent laboratories in Salmonella strains TA 98, TA100, TA1535, and TA1537, at concentrations from 333 – 11,550 µg/plate (lab. 1) and 100 – 10,000 µg/plate

(lab. 2), in the presence and absence of Aroclor-induced rat and hamster liver S9. No induction of mutations were seen [Zeiger et al., 1985; NTP, 1997*].

BBP (as Santicizer 160) was tested for mutagenicity in the yeast, *Saccharomyces cerevisiae* strain D4 with and without rat liver S9 at concentrations from 0.1 – 10 µl/plate. No induction of mutations were seen [Litton Bionetics, Inc., 1976].

2.2. Mammalian cell test systems

2.2.1. DNA damage in mammalian cells in vitro (Table 3)

DNA damage may be produced in mammalian cells by the interaction of the test substance with the DNA, but may also be a secondary effect of cell toxicity. Not all such damage, i.e., DNA breaks or crosslinks, or apurinic sites, translate to mutations or chromosome damage of the type that could lead to cancer. With the possible exception of the comet assay, methods for the detection of apurinic sites and DNA crosslinks are not standardized, and their reproducibility or inter-laboratory transferability have not been demonstrated.

A mouse osteoblast cell line (MT3T3-E1), mouse primary calvarial osteoblasts (COBs), and Py1a rat osteoblasts, were treated with BBP in vitro at concentrations of 10^{-5} – 10^{-8} M. The authors concluded that there was evidence for apurinic sites as indicated by the induction of p53, phospho-p53, p21, phospho-ATM protein kinase. The induction of these factors was coincident with the induction of apoptosis, a naturally occurring process, that could have been a causative factor of the apurinic sites. Similar treatment of Py1a rat osteoblasts did not induce apoptosis or apurinic sites [Sabbieti et al., 2009].

Apurinic sites are the most common DNA lesion in mammalian cells and are estimated to constitute approximately 60% of the total endogenous DNA lesions in the cells [Swenberg et al., 2011]. They are formed as a side effect of normal metabolism and are readily repaired by the cell. The procedure used [Sabbieti et al., 2009] does not distinguish between apurinic sites induced by direct action of BBP with DNA, or by an indirect mechanism involving cell-cycle progression leading to p53 activation, or by effects on the cell's metabolic processes.

DNA-protein crosslinks were induced in rat hepatocytes treated in vitro with BBP, as reported in a conference abstract by Qin et al. [2010]. Procedural details and data on the incidence of the presumed crosslinks were not provided, and the determination of their presence appeared to be via an indirect chemical response. The reported positive effect was seen at a concentration of 80 µmol/L, but not at lower concentrations. The chemical makeup of the presumed crosslinks was not determined, but their induction was coincident with the induction of oxidative damage, suggesting that active oxygen species may have produced the crosslinks.

Choi et al. [2010] performed the comet assay on human hepatoma (HepG2) cells exposed to 2.5 – 250 µM of BBP in vitro. A dose-related increase in DNA single-strand breaks was seen at all doses when the cells were treated with BBP for 24 and 48 hrs. They also assayed a number of specific proteins whose cellular levels were affected by the same BBP treatment. One of the proteins that was decreased by BBP treatment was DEK protein. Because DEK protein is

* Some of the NTP studies were published in peer-reviewed journals in addition to being included in the NTP carcinogenesis technical report [NTP, 1997] and being available on the NTP public web-site. Where this occurs, both the journal and NTP report references are included.

involved in repair of DNA strand breaks, the authors proposed that its reduction may have been associated with the DNA damage.

2.2.2. Mutation in mammalian cells in vitro (Table 4)

BBP (as Santicizer 160) was tested in the mouse lymphoma cell assay for mutagenicity in the absence and presence of mouse liver S9 metabolic activation at concentrations from 0.08 – 5.00 µl/ml [Litton Bionetics, Inc., 1977]. The highest scorable concentration without and with S9, 1.25 µl/ml resulted in relative total growth of 19.0% and 161.4%, respectively. No increases in mutation frequency were seen.

Barber et al. [2000] tested BBP for mutation in mouse lymphoma L5187Y cells in the absence and presence of rat liver S9. Without S9, there were increases in mutation at concentrations up to 0.040 µl/ml (11.3% relative growth) but there was no concentration-related response. Significant increases in mutant frequency were seen the presence of S9 but only the lowest test concentration (0.20 µl/ml) had an acceptable level of relative cell growth (survival), i.e., >10%. A non-significant increase in mutant frequency was seen in one of two flasks at this concentration. The other test concentrations, i.e., those from 0.6 – 1.20 µl/ml, all showed significant increases; however, the percent relative growth at positive concentrations ranged from 10.1% to 1% survival at 1.20 µg/ml [Barber et al., 2000]. The current OECD and ICH Test Guidelines for the mouse lymphoma assay [OECD, 1997; ICH, 2011], and recommendations by Moore et al. [2002], require testing to not less than 10% relative survival because increases in mutant frequency at highly toxic test chemical concentrations are considered to be an effect of the toxicity, rather than to DNA-damaging effects of the test chemical. The authors concluded that BBP was not mutagenic in mouse lymphoma L5178Y cells.

BBP was tested by the National Toxicology Program for mutagenicity in mouse lymphoma L5178Y cells [Myhr and Caspary, 1991; NTP 1997]. In the absence of S9, an initial positive response in the range of 5 – 60 nl/ml was not reproduced in two successive experiments, and BBP was concluded to be nonmutagenic. BBP was not mutagenic in the presence of Aroclor-induced rat liver S9 when tested in a dose range of 30 – 100 nl/ml. The authors concluded that BBP was not mutagenic in mouse lymphoma L5178Y cells.

2.2.3. Chromosome damage in mammalian cells vitro (Table 5)

BBP was tested for induction of sister chromatid exchanges (SCE) in CHO cells [Galloway et al., 1987; NTP, 1997] up to concentrations producing toxicity as measured by decreases in the proportions of mitotic cells and cell cycle delay. The initial trial, without metabolic activation using a dose range from 0.4 to 4.0 µg/ml was concluded to be questionable based on nonsignificant increases at all three concentration levels. In a repeat trial using the same protocol, but a concentration range from 0.4 to 12.5 µg/ml, there were no increases over the control value. A test with rat liver S9 metabolic activation in the concentration range of 125 – 1,250 µg/ml was concluded to be negative.

BBP was tested for induction of chromosome aberrations in CHO cells [Galloway et al., 1987; NTP, 1997] with and without rat liver S9 metabolic activation at concentrations ranging from 125 – 1,250 µg/ml. The top concentration was limited by toxicity as measured by cell growth after treatment. No increases in chromosome aberrations were seen.

3.0. Genetic Toxicity Tests in Somatic Cells in Vivo

3.1. DNA Damage in vivo (Table 6)

Intraperitoneal injections of 500 and 1,000 mg BBP/kg/d for 14 days led to what was reported to be a statistically significant increase in DNA-protein cross-links in the livers of mice [Guo et al., 2009]. The procedure for measuring the increase, the levels of the increase, and a chemical identification of the composition of the crosslinks was not provided. The authors attributed the response to a secondary effect of the oxidative damage that was induced at doses between 250 and 1,000 mg/kg/d, which suggests that the crosslinks reported were formed by interaction of the DNA with active oxygen species or malondialdehyde which is an oxidative stress product, rather than by interaction with BBP or a BBP metabolite.

3.2. Chromosome damage in somatic cells in vivo (Table 7)

BBP was tested for induction of SCE in male B6C3F1 mouse bone marrow cells following a single i.p. injection of 3 doses from 1,250 – 5,000 mg/kg [NTP, 1997]. When the bone marrow cells were sampled at 23 hrs, only the middle dose (2,500 mg/kg) was significantly increased, but there was no significant trend. In a subsequent 42 hr sampling, the low (1,250 mg/kg) and high 5,000 mg/kg) doses showed statistically significant increases in the percent cells with SCE over the control, with a weak, but significant, trend. The NTP [1997] concluded that BBP induced SCE in the bone marrow cells.

BBP was tested for induction of chromosome aberrations in male B6C3F1 mouse bone marrow cells following a single i.p. injection of 3 doses from 1,250 – 5,000 mg/kg [NTP, 1997]. A statistically significant increase in structural aberrations was seen at 5,000 mg/kg in cells harvested at 17 hrs after injection. The response was positive at the same dose in a repeat test by the laboratory. No increases in aberrations was seen in cells harvested 36 hrs after treatment. The NTP [1997] concluded that BBP induced chromosome aberrations in the bone marrow cells.

In a micronucleus (MN) evaluation that was appended to a BBP reproductive study, Ashby et al. [1997] administered BBP in the drinking water to pregnant Alpk:APfSD(AP) rats from day 1 of pregnancy until through pnd 20 at an average daily dose of 182.6 µg/kg/d. Peripheral blood was examined at sacrifice for the presence of micronucleated polychromatic erythrocytes. No increase over the control value was seen.

BBP was administered to mice by i.p. injection at doses from 312.5 – 2,500 mg/kg/d for 3 days and sampled 24 hrs following the final injection. There was no induction of MN in bone marrow polychromatic or normochromatic erythrocytes at any of the doses [NTP, 2013].

4.0. Chromosome damage in germ cells in vivo

4.1. Rodents (Table 7)

In a conference abstract, Bishop et al. [1987] reported that BBP was injected s.c. into male B6C3F1 and CD-1 mice on 3 days with doses ranging from 400 – 4,560 mg/kg/d prior to mating to untreated females. They concluded that BBP did not significantly increase fetal death rates or induce dominant lethal mutations. The actual numbers of implants, resorptions, and dead and viable fetuses were not reported.

Ashby et al. [1997] administered BBP in the drinking water to pregnant Alpk:APfSD(AP) rats from day 1 of pregnancy until littering. The estimated average daily dose was 183.6 μg/kg/d. No effect on litter size was seen. Although this was summarized as a dominant lethal test in one of the reports, it was not an adequate dominant lethal test protocol because there was no measurement of total implants, resorptions, or dead fetuses.

4.2. Other organisms (Table 8)

BBP was tested by the NTP in the fruit fly, *Drosophila melanogaster*. There was no induction of sex-linked recessive lethal mutations when adult male flies were fed with 10,000 and 50,000 ppm BBP in their food, or injected with 500 ppm [Valencia et al., 1985; NTP, 1997]. The authors concluded that BBP did not induce sex-linked recessive lethal mutations in Drosophila.

5.0. Mammalian cell transformation in vitro (Table 9)

In vitro mammalian cell transformation, although typically listed with genetic toxicity endpoints, is not considered to be indicative of a genotoxic effect because it can be induced by DNA-reactive and non-DNA reactive chemicals, and by non-genetic changes in the cells. The assays used measure the progression of cells in culture to a malignant state, which is indicated by morphological changes and/or growth patterns of the transformed cells. Exogenous metabolic activation (i.e., liver S9) has not been used in the Balb/3T3 or SHE cell transformation assays; the cells are considered metabolically competent because they are responsive to chemicals that require metabolic activation for their biological activity.

BBP (as 1D) was tested for cell transformation in Balb/3T3 cells in the absence of exogenous metabolic activation at concentrations from 10.0 – 160.0 nl/ml, with cell survival ranging from 90% to 8%. No induction of cell transformation was seen [Litton Bionetics, Inc., 1985].

Barber et al. [2000] tested BBP for cell transformation in Balb/3T3 cells in the absence of exogenous metabolic activation to a concentration estimated to produce 80 – 90% cell toxicity. The specific concentrations tested, and the test data, were not presented. The authors concluded that BBP did not induce cell transformation.

BBP was tested in the pH 6.7 Syrian Hamster Embryo (SHE) cell transformation system in the absence of exogenous metabolic activation [LeBeouf et al., 1996]. Treatment of the cells for 24 hrs with concentrations of 25 – 250 μg/ml BBP produced non-significant increases in cell transformation at all doses. When the cells were treated for 7 days with concentrations of 2.5 – 20 μg/ml BBP, significant increases in cell transformation were seen at the three lower concentrations. Concentrations above 20 μg/ml produced precipitate in the medium, but no cell transformation.

6.0. Other effects related to genetic damage

BPP, when tested up to a concentration of 200 μM, did not disrupt microtubulin assembly in cultured V79 Chinese hamster cells [Nakagomi et al., 2001]. Disruption of microtubulin assembly can cause chromosome nondisjunction which can lead to aneuploidy.

7.0. Overall evaluation of test results

The responses in the various test systems are summarized in Table 10.

In vitro effects of BBP

My conclusions that there were no in vitro genetic effects of BBP in microbial cells, and no induction of mutation, chromosome aberrations, or SCE in cultured mammalian cells, agree with those presented by OEHHA [2013]. I am also in agreement with the OEHHA evaluation of the cell transformation studies.

We differ, however, in the weight given to the DNA damage effects reported in mammalian cells in vitro (summarized in Tables 3 and 10). Only the study reporting a positive comet assay test, which indicated the induction of single-strand DNA breaks in HepG2 cells, could be adequately evaluated.

The report of the induction of apurinic sites provided only indirect evidence for their formation, i.e., the induction of enzymes that are associated with DNA damage, and they were associated with apoptosis, which is a cytotoxic pathway, one effect of which is the production of DNA damage. The reported positive response for DNA-protein crosslinks in rat hepatocytes was in a conference abstract without sufficient information on the test protocol used, and there were no data presented or available for analysis. These latter two studies, while suggestive of DNA damage induced by BBP, cannot be considered definitive evidence of a BBP – DNA interaction or a direct genotoxic effect.

In vivo effects of BBP

My conclusions with respect to the genetic effects of BBP in rodent bone marrow cells agree with those presented by OEHHA [2013]. That is, there were positive responses in the induction of SCE and chromosome aberrations in the mouse bone marrow following i.p. injection, but no induction of micronuclei in rats or mice when BBP was administered in the drinking water. The chromosome aberration studies showed no effect at the low and intermediate doses, with the positive response seen only at the highest (limit) dose [NTP, 1997]. This pattern, i.e., chromosome breakage only at the top dose strongly suggests that the damage was a secondary effect of bone marrow cell toxicity rather than of the chemical acting on the chromosome. There was no embryolethality in treated pregnant female rats, or dominant lethal effects reported in male mice administered BBP, although there were insufficient data provided to allow an evaluation of the dominant lethal test results.

I differ with OEHHA, however, with regard to the weight given to the reported DNA-protein crosslinks in mouse liver cells exposed in vivo. As noted above for this endpoint which is reported in a conference abstract, there are insufficient protocol details, and no data available for analysis. The authors also noted that the DNA-protein crosslinks were associated with oxidative damage. Chemical analysis would be required to determine whether the crosslinks are with the phthalate molecule or with an active oxygen species.

The NTP [1997] report on the Drosophila sex-linked recessive lethal test, and the prior publication of these data [Valencia et al., 1985], concluded that the assay was negative. However, OEHHA/Table 7 [2013] summarizes the 10,000 ppm dose as positive. Although the result is identified as negative in the OEHHA discussion, its designation as positive in the table is misleading, and could add to the weight of evidence for the casual reader.

The following genotoxicity effects were reported to be induced by BBP:

- Single strand DNA-breaks in HepG2 cells in vitro
- SCEs and chromosome aberrations in mice in vivo

The following reported positive effects are suggestive of genotoxic activity, but too little information was presented to allow an objective evaluation of the studies and the authors' conclusions:

- Apurinic sites in mouse osteoblasts in vitro
- DNA-protein crosslinks in rat hepatocytes in vitro
- DNA-protein crosslinks in mouse liver cells in vivo

In summary, no positive results were reported in the mutagenicity studies. The clastogenicity (chromosome damage) studies reporting positive responses were the bone marrow SCE and chromosome aberration studies, both of which were obtained by i.p. injection of high doses of BBP.

8.0. Conclusions

A generally accepted definition of 'genotoxic' is that the substance or its metabolic forms must directly interact with DNA or chromosomes. This definition is consistent with the definition in the US EPA's Framework for Determining a Mutagenic Mode of Action for Carcinogenicity [EPA, 2007] which requires the chemical "to interact with DNA early in the [carcinogenic] process and produce changes in the DNA that are heritable."

In 1987, the International Agency for Research on Cancer compiled approx. 173 tests and test endpoints that were being used to identify genotoxic substances. These included mutagenicity and clastogenicity tests that were well defined as to their predictivity and reproducibility, but the majority of the tests in the compilation were in use in relatively few labs and there was little or no information on their effectiveness or accuracy for identifying genotoxins, or their reproducibility. The measurement of crosslinks or induction of apurinic sites were not included in that compilation, and many additional new tests have been developed and proposed since 1987. Because of the large number of tests that putatively measure some aspect of the DNA's response to insult, it has been difficult to clearly identify the tests and biological or chemical endpoints that should be used to define a genotoxic chemical. The major consideration when defining a substance as genotoxic is whether the molecule, or a breakdown product, is capable of interacting with, and damaging, DNA. As a part of an analysis to determine whether a substance acts via a genotoxic mode of action, the question of whether the effect seen is the result of an interaction between the test substance with DNA, or if it is a downstream effect of some other cellular response, such as cytotoxicity, oxidative stress, as an effect of the apoptosis cascade, or whether it can be induced as an artifact of the test system used, e.g., high osmolality or decreased pH [see, e.g., Tice et al., 2000].

DNA damage is not equivalent to mutation or chromosome aberrations. The term "DNA damage" encompasses a number of effects, from DNA strand breaks to chemical adducts on DNA bases, to other modifications or loss of bases. Such damage is usually repairable by the cell, and is often lethal to the cell if not repaired. These effects can be caused by a direct interaction of the test substance with the DNA or with chromosome material, or as a secondary effect of treatment-induced cellular stress or toxicity. Such types of damage can be identified and

quantified using a number of different techniques, some of which, like the comet assay, are considered accurate measures of damage, whereas others, such as reactions with specific reagents, such as was used by Qin [2010], may be less specific. Therefore, it is important for a determination of genotoxicity to distinguish between DNA damage produced by the chemical itself, or its metabolite, from DNA damage that is a secondary, incidental, effect of the treatment.

Not all DNA-damaging effects will result in a mutated cell; the DNA adduct or other lesion may be lethal, so that the affected cell will be removed from the population, or be successfully repaired so as not to produce a mutation. Therefore, such DNA damage, in the absence of studies to ascertain the specific cause of the damage, or the survival of the affected cell, should be considered less of a concern than effects such as mutation, chromosome breakage and rearrangement, or aneuploidy.

The weight-of-evidence approach is appropriate for evaluating a dataset such as exists for BBP. It requires not only consideration of the positive/negative response, but also a consideration of a number of other factors, including the mechanism(s) leading to the damage, the doses used and route of administration, the relevance of the effect for human health, the relationship of the effect to mutagenicity and carcinogenicity, and the quality of the data.

Neither the phthalate moiety nor its anticipated monophthalate metabolites [NTP, 1997], have structural alerts for DNA reactivity, and the positive effects reported for BBP can all be explained by secondary effects of toxicity, rather than DNA reactivity. The comet assay has been reported to be highly sensitive to cells undergoing apoptotic or necrotic death [e.g., Henderson et al., 1988; Morley et al., 2006]. The report of DNA strand breaks in vitro, which were measured using the comet assay also reported that apoptosis, which includes DNA degradation as part of the programmed cell death process, was induced [Choi et al., 2010], and the appearance of the apoptosis-related proteins paralleled the induction of DNA strand breaks. The single-strand breaks measured by the comet assay can be the result of overt or nascent strand breakage by the chemical insult, DNA damage by reactive chemical species generated by the test chemical, or a secondary effect of a non-DNA-directed cytotoxic or apoptotic response.

Both SCE and chromosome aberrations were induced in mouse bone marrow cells following i.p. injection, a route of administration not relevant to human exposure. The use of i.p. exposure, although fairly common in the past, is rarely used now. It's primary advantages were that it was more easy to administer by i.p. injection than by other routes, and it allowed high doses to be administered. Depending on the substance tested, the doses achieved by i.p. injection may be significantly higher than could be achieved by other routes (oral; inhalation; dermal absorption). The high dose administered for the SCE and chromosome aberration tests, 5,000 mg/kg [NTP, 1997] was above the reported i.p. LD_{50} for mice of 3,160 mg/kg [Calley et al., 1966], and the oral LD_{50} was reported to be 4,000 – 6,000 mg/kg for mice [NTP, 1982].

The positive response for SCE induction in mice was seen only at 42 hrs after exposure, but not at 23 hrs. SCE can be induced by direct damage to the chromosome or by interference with mitosis or cell division. For this reason, this endpoint is no longer recommended by regulatory authorities because it does not provide clear evidence of DNA interaction, and can be induced by perturbations in the cell cycle. The increase in chromosome aberrations in mouse blood cells was weak, but reproducible at the 17 hr harvest, but not after 36 hrs, and was seen only at the high dose of 5,000 mg/kg/d in a pattern consistent with a secondary effect of toxicity. The induction of MN in bone marrow cells is considered a more sensitive endpoint than chromosome aberrations, yet administration of BBP in the drinking water in rats for an extended period of

time, or by multiple i.p. injections in mice at doses up to 2,500 mg/kg/d, a dose that approached the LD_{50}, did not induce MN.

The other reported positive responses are apurinic sites in mouse osteoblast cells, but not in rat cells, treated in vitro, or DNA-protein crosslinks in cultured liver cells and mouse liver cells exposed in vivo. These effects are considered genotoxic but there is insufficient evidence presented to allow an objective evaluation of the studies or their conclusions. Also, there was no evidence presented to demonstrate that the DNA-protein crosslinks reported, if valid, were with BBP or a metabolite, rather than with an oxygen or other radical that resulted from the treatment. Similarly, the conclusion that apurinic sites were produced in mouse osteoblasts by BBP was based on the induction of enzymes and other cellular factors that were associated with cell toxicity as evidenced by the induction of apoptosis. The responses to BBP in the mouse and rat cells did not predict BBP's carcinogenicity in those species [NTP, 1982, 1997], i.e., BBP was associated with apurinic sites in the mouse cells but not in the rat cells, whereas tumors were induced in rats but not in mice.

9.0. Evaluation of the weight of evidence

The weight of the evidence, taking into consideration the lack of structural alerts for DNA reactivity in BBP or its putative metabolites, the routes of administration and doses used in the tests that produced the reported positive responses, the types and relevance of responses reported, the level of confidence in the reproducibility and predictivity of the tests showing positives versus those showing negatives, and the lack of availability of the test protocols and data for some of the positive reports, leads to the conclusion that BBP should not be considered genotoxic.

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Table 1. DNA Damage/Repair Studies of Butyl Benzyl Phthalate in Bacteria

Assay System	Protocol	Tester Strains	S9	Response	HTD/LED	Comment	Reference
B. subtilis	not described	recA$^-$	np	–	30 mg/plate	Single dose tested. No experimental details or data presented.	Omori, 1976 (Kurata, 1975)
E. coli	not described	(uvrA$^-$) (PolA$^-$) (recA$^-$) not otherwise identified	np	–	30 mg/plate	Single dose tested. No experimental details or data presented.	Omori, 1976 (Kurata, 1975)

np: information not provided
HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 2. Mutagenicity Studies of Butyl Benzyl Phthalate in Bacteria

Assay System	Protocol	Tester Strains	S9	Response	HTD/LED	Comment	Reference
E. coli	not described	wild-type (not otherwise identified)	–	–	30 mg/plate	Single dose tested. No experimental details or data presented.	Omori, 1976 (Kurata, 1975)
		recA$^-$ (not otherwise identified)	–	–			
S. typhimurium	plate test	TA98, TA100, TA1535, TA1538, TA1538	+/–	–	10 µl/plate		Litton, 1976
S. typhimurium	not specified	TA98, TA100, TA1535, TA1538, TA1538	+/–	–	10 µl/plate	S9 source not identified	Monsanto, 1976
S. typhimurium	preincubation	Lab. 1: TA98, TA100, TA1535, TA1537	+/–	–	11,550 µg/plate	S9 from rats and hamsters	Zeiger et al., 1985; NTP, 1997.
		Lab. 2: TA98, TA100, TA1535, TA1537	+/–	–	10,000 µg/plate		
S. typhimurium	plate test	TA99, TA100	+/–	–	np	No experimental details or data presented.	Kozumbo et al., 1982
S. cerevisiae	plate test	D4	+/–	–	10 µl/plate		Litton, 1976

np: information not provided
HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 3. DNA Damage Studies of Butyl Benzyl Phthalate in Mammalian Cells In Vitro

Assay system	Endpoint	S9	Response	HTD/LED	Comments	Reference
Mouse osteoblasts	Apurinic sites	–	+	10^{-6} M	Indirect measure of DNA damage using enzyme induction as a surrogate marker	Sabbieti et al., 2009
Rat osteoblasts		–	–	10^{-6} M		
Rat hepatocytes	DNA-protein crosslinks	np	+	80 µm/l	Conference abstract; no experimental details or data presented.	Qin et al., 2010
Human HepG2 cells	DNA single-strand breaks (Comet assay)	–	+	5.00 µM		Choi et al., 2010

np: information not provided
HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 4. Mutagenicity Studies of Butyl Benzyl Phthalate in Mammalian Cells In Vitro

Cell line	Target gene	S9	Response	HTD/LED	Comments	Reference
Mouse lymphoma L5178Y	tk	–/+	–	5.00 µl/ml	The highest scorable dose was 1.25 µl/ml; higher doses were too toxic to score	Litton, 1977
Mouse lymphoma L5178Y	tk	–	–	1.015 µl/ml	Increases at all dose levels, but no dose response.	Barber et al., 2000
		+	–	0.20 µl/ml	Only the lowest dose (0.20 µl/ml) was within the acceptable survival range. Significant increases only in the presence of high toxicity.	
Mouse lymphoma L5178Y	tk	–	–	60 nl/ml	Initial dose-response not repeatable in two successive experiments	Myhr & Caspary, 1991; NTP, 1997
		+	–	100 nl/ml		

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 5. Chromosome Damage Studies of Butyl Benzyl Phthalate in Mammalian Cells In Vitro

Cell line	Endpoint	S9	Response	HTD/LED	Comment	Reference
CHO	SCE	–/+	–	1,250 µg/ml		Galloway et al., 1987; NTP, 1997
CHO	Abs	–/+	–	1,250 µg/ml		Galloway et al., 1987; NTP, 1997

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 6. DNA Damage Study of Butyl Benzyl Phthalate in Mice In Vivo

Species/strain	Tissue	Endpoint	Route	Protocol	Response	HTD/LED	Comments	Reference
Mouse	liver	DNA-protein crosslinks	i.p.	14 d. treatment	+	500 mg/kg/d	Conference abstract; no experimental details or data presented.	Guo et al., 2009

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 7. Chromosome Damage Studies of Butyl Benzyl Phthalate in Rodents In Vivo

Species/strain	Tissue	Endpoint	Route	Protocol	Response	HTD/LED	Comments	Reference
Somatic cells								
Male B6C3F1 mice	Bone marrow	SCE	i.p.	single inj. in corn oil	+	1,250 mg/kg		NTP, 1997
Male B6C3F1 mice	Bone marrow	Chromosome aberrations	i.p.	single inj. in corn oil	+	5,000 mg/kg		NTP, 1997
Female Alpk:APfSD(AP) rats	Bone marrow (peripheral blood)	MN-PCE	drinking water	day 1 of pregnancy to pnd 20	–	182.6 µg/kg/d		Ashby et al., 1997
Male B6C3F1 mice	Bone marrow	MN-PCE; MN-NCE	i.p.	3 daily inj. in corn oil	–	2,500 mg/kg/d		NTP, 2013
Germ cells								
Female Alpk:APfSD(AP) rats	Zygote and developing embryo	Embryo lethality	drinking water	throughout pregnancy	–	182.6 µg/kg/d	Not an adequate test; number of implants not determined	Ashby et al., 1997
Male B6C3F1 mice	Male germ cells	Dominant lethal effects	s.c.	3x prior to mating	–	4,560 mg/kg/d	Conference abstract; no test data presented	Bishop et al., 1987
Male CD-1 mice	Male germ cells	Dominant lethal effects	s.c.	3x prior to mating	–	4,560 mg/kg/d	Conference abstract; no test data presented	Bishop et al., 1987

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 8. Genetic Toxicity Studies of Butyl Benzyl Phthalate in Non-Mammalian Organisms

Test organism	Endpoint	Treatment	Result	LED/HDT	Comments	Reference
Drosophila melanogaster	Sex-linked recessive lethal mutations	injection of males	–	500 ppm		Valencia et al., 1985; NTP, 1997
		larval feeding	–	50,000 ppm		

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 9. In Vitro Cell Transformation Studies with Butyl Benzyl Phthalate

Cell line	Endpoint	S9	Response	HTD/LED	Comment	Reference
Balb/3T3	morphological cell transformation	–	–	160 nl/ml		Litton, 1985
Balb/3T3	morphological cell transformation	–	–	not given	Concentrations tested and test data not presented	Barber et al., 2000
Syrian hamster embryo (SHE)	morphological cell transformation	–	+	2 µg/ml	Significant increases in 7-day treatment protocol	LeBeouf et al., 1996

HTD/LED: Highest Tested Dose (negative responses)/Lowest Effective Dose (positive responses)

Table 10. Summary of genetic toxicity test results

Biological system	Genotoxic effect reported	Result	Section	Table
Microorganisms				
B. subtilis	non-specific DNA damage	–	2.1.1	1
E. coli	non-specific DNA damage	–	2.1.1	1
S. typhimurium	Gene mutation	–	2.1.2	2
E. coli	Gene mutation	–	2.1.2	2
S. cerevisiae	Gene mutation	–	2.1.2	2
Mammalian cells in vitro				
Mouse osteoblasts	Apurinic sites	+	2.2.1	3
HepG2 cells	DNA single-strand breaks	+	2.2.1	3
Mouse hepatocytes	DNA-protein crosslinks	+	2.2.1	3
Mouse lymphoma L5178Y cells	Gene mutation	–	2.2.2	4
CHO cells	SCE	–	2.2.3	5
CHO cells	Chromosome aberrations	–	2.2.3	5
Rodent somatic cells in vivo				
Rat liver	DNA-protein crosslinks	+	3.1	6
Mouse bone marrow cells	SCE	+	3.3	7
Mouse bone marrow cells	Abs	+	3.3	7
Rat bone marrow cells	MN	–	3.3	7
Germ cells in vivo				
Developing mouse zygote	Embryo lethality	–	4.1	7
Male mouse germ cells	Dominant lethal mutations	–	4.1	7
Male Drosophila germ cells	Sex-linked recessive lethal mutations	–	4.2	8

ATTACHMENT B
OPINION OF DR. GENE MCCONNELL

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15 November 2013

To: Ferro Corporation
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Subject: OEHHA Hazard Identification Document on Butyl Benzyl Phthalate (BBP)

Per your request, following is my evaluation of the subject document. I come to this evaluation with over 40 years' experience in the field of experimental carcinogenesis, particularly in the design, conduct and interpretation of rodent bioassays (acute, subacute and chronic). During that time I was head of the Pathology Branch of the National Toxicology Program (NTP) and later Director of the NTP's Toxicology Research and Testing Program. In those positions I was directly involved with over 200 rodent bioassays involving mice and rats. And, subsequent to this I have continued to evaluate these types of studies for various bodies, e.g. NIOSH, FDA, EPA, IARC, NAS, IOM and private concerns. In total I have evaluated well over 500 of the type of studies being reported by OEHHA.

While I have read the entire OEHHA document, I will focus on the following sections per your request:

Section 3.2 Carcinogenicity Studies in Animals

Section 3.3.4 Animal Tumor Pathology

Section 6. Summary and Conclusion (as appropriate).

As context for evaluating tumor findings from bioassays, one thing I learned decades ago from my statistical colleagues is that you can/will have what appear to be positive findings by chance alone. Consider the fact that in a typical chronic cancer bioassay that one examines histopathologically 32 different tissues/organs. And, that each of these organs on average probably has the chance to develop at least three different types of neoplasms. That means that in a given bioassay one has the chance of finding five different kinds of tumors that may be statistically increased or decreased. Add to this that since there are 8 studies under consideration (see tables) there is a chance of 40+ possibilities of having a positive or negative response. So, how does one determine if the response is "real" (related to the chemical) or not? While this can

be very problematic, I've found that the larger the data set the easier it becomes to make a conclusion in this regard.

I have found the reasoning espoused by Sir Austin Bradford-Hill's on "Criteria for Causation" to be extremely useful for differentiating treatment-related effects from those that occur by chance. While his nine criteria were specifically developed to evaluate epidemiology data I have found them extremely valuable when looking at a robust data set like we have for BBP. And, the criteria are ideally suited for use in a "weight-of-the-evidence approach for the evaluation of a given data set as is recommended by OEHHA guidance. The Bradford-Hill (BH) criteria for determining causality are as follows:

1. **Strength**: A small association does not mean that there is not a causal effect, though the larger the association, the more likely that it is causal.
2. **Consistency**: Consistent findings observed by different persons in different places with different samples strengthens the likelihood of an effect.
3. **Specificity**: Causation is likely if a very specific population at a specific site and disease with no other likely explanation. The more specific an association between a factor and an effect is, the bigger the probability of a causal relationship.
4. **Temporality**: The effect has to occur after the cause (and if there is an expected delay between the cause and expected effect, then the effect must occur after that delay).
5. **Biological gradient**: Greater exposure should generally lead to greater incidence of the effect. However, in some cases, the mere presence of the factor can trigger the effect. In other cases, an inverse proportion is observed: greater exposure leads to lower incidence.
6. **Plausibility**: A plausible mechanism between cause and effect is helpful (but Hill noted that knowledge of the mechanism is limited by current knowledge).
7. **Coherence**: Coherence between epidemiological and laboratory findings increases the likelihood of an effect.
8. **Experiment**: "Occasionally it is possible to appeal to experimental evidence".
9. **Analogy**: The effect of similar factors may be considered.

For toxicology studies, particularly those involving whole animal data, I've found during my 40+ years as a veterinary pathologist and my evaluation of over 500 rodent cancer bioassays the following criteria especially useful when analyzing robust sets of toxicology data:

#2 – "Consistency" (The same or similar response across studies of similar design)

#3 – "Specificity" (Does the response make toxicological sense, i.e. strength of evidence?)

#5 – "Biological gradient" (With toxicology data this equates to dose-response)

#6 – "Plausibility" (Would one expect a given finding based on the type of chemical?)

Section 3.2 Carcinogenicity Studies in Animals

There were at least 14 carcinogenicity bioassays in rodents (4 in male and 4 in female rats, 2 in male and 2 in female mice) and two "special" studies (Strain A male mouse and gavage in

female rat). I will first speak to the types of tumors highlighted by OEHHA that they felt were caused by BBP and discuss them in terms of the BH criteria for causation. See Tables 1 and 2 for a summary of the tumor observations.

Mononuclear cell leukemia (MCL) – While the data from the National Toxicology Program (NTP 1982, Technical Report 213) show a statistical increase in MCL in high-dose (12,000 ppm) female rats only, there was no increase in MCL in male rats in the same study. Moreover, there was no treatment-related increase in MCL in a subsequent NTP study (NTP 1997a, Technical Report 458) in male or female rats at the same doses and no increase in further feed restriction NTP studies (NTP 1997b, Technical Report 460). Note that the latter study in females also included a dose twice as high (24,000 ppm) as that that produced MCL in the first study (Technical Report 213) with no evidence of an increase in MCL. In all of these studies the rats were the same strain (F344N) and were exposed the same way *via* feed. An additional fact that needs to be considered in deciding if the reported increase in MCL is related to BBP exposure is that MCL is the most common spontaneous neoplasm in F344N female rats and the second most common in male rats. If fact, MCL was such a common finding during this period that the NTP stopped using this strain of rat in their carcinogenicity studies.

Finally, there was no increase in lymphoid neoplasms in the two co-carcinogenicity studies in rats and mice, albeit an increase in these types of tumors might not be expected in these models. Nor was there an increase in lymphoid neoplasms in either sex of mice, although this negative finding would not impact the significance of the MCL response in rats. In summary, only one of eight studies in rats showed an increase in lymphoid neoplasms.

For these reasons, I do not think that the MCL response can be attributed to exposure of BBP. It fails the BH criteria for both "consistency" and "biological gradient" (dose-response).

Pancreas acinar cell adenoma/carcinoma (ACA) – I found the OEHHA text on this tumor very confusing. They concluded that BBP caused ACA in male rats. They based this conclusion on a statistical increase in high-dose male rats (NTP 1997a, Technical Report 458). The way the findings are reported in the OEHHA report suggests that there is an additional study (NTP 1997b, Technical Report 460) that shows an increase in ACAs in male rats. But, when I examined the supporting table (Table 9 in the OEHHA report) the incidence is exactly the same suggesting that the animals were the same male rats reported in Table 6. If so, this means that there was only a single study showing this response. Also, there was no significant increase in ACA in female rats at twice the dose in the same study or either sex in the original NTP study (NTP 1982, Technical Report 213) that used the same dose levels. It should also be noted that the increase in pancreas ACA was based almost totally on an increase in benign tumors (adenomas); there was only one carcinoma in the study.

Finally, there was no increase in pancreas tumors noted in the mice studies or co-carcinogenicity studies.

For these reasons, I do not think that the pancreatic adenoma response can be attributed to exposure of BBP. The data fail to pass the BH tests for "consistency", "plausibility" and "biological gradient" (dose-response).

Adrenal medulla, pheochromocytoma – OEHHA suggests that BBP caused an increase in these neoplasms based on a single observation in male rats; a comparison of the high-dose to weight-matched controls in the diet restriction study. However, the incidence was exactly the same as in the *ad libitum* controls in the same study. Note that the NTP did not identify these tumors as being related to BBP exposure. Also, they do not report if the increase is due to predominately benign or malignant neoplasms

For these reasons, I do not think that the pheochromocytoma response can be attributed to exposure of BBP. Again, it fails both the BH "consistency" and "biological gradient" (dose-response) criteria.

Transitional cell tumors of the urinary bladder – While there was a nonsignificant ($p > 0.05$) increase in one study (32 month exposure), the increase was only at the 24,000 ppm dose and did not occur at 24-months under the same conditions. It would be interesting to know if BBP or its' metabolites might precipitate in the urine at these high levels but not the lower level (12,000 ppm) where no tumors were observed. This is important because these types of tumors are often the result of chronic irritation *via* physical irritation to the transitional epithelium due to crystals being formed during concentration of the urine, e.g. Na saccharine. In either case one would expect a steep dose-response for this finding and threshold for the response. This hypothesis is supported by the lack of this finding in the other studies where the highest dose was 12,000 ppm.

For these reasons, I think that the urinary bladder lesions may be attributable to the animal's exposure of BBP, even though the increase in tumors was not statistically significant and failed to pass the BH criterion for "consistency". If I am correct that the mode-of-action is probably due to "crystal formation", these tumors are not relevant to humans.

Summary of tumor findings – The only tumor finding that I felt might be associated with BBP in these studies were the tumors of the urinary bladder. And, even this finding does not rise to the level that would support listing BBP under Proposition 65 because of the unusually high exposure and unusually long exposure period (32 months), likely resulting in chronic irritation via physical damage to the epithelium from crystaluria. I would add that the NTP has not considered BBP for listing in its' Report on Carcinogens as would be expected if NTP believed it caused the tumor response that OEHHA proposes. In summary, in my opinion if one uses a "Weight of Evidence" approach, as per CIC guidance criteria for the evaluation of this robust set of data on BBP, the totality of the data do not rise to the level required for listing under Proposition 65.

If you have questions concerning this report please feel free to contact me.

Sincerely,

Ernest E. McConnell

Ernest E. McConnell

TABLE 1 - NATIONAL TOXICOLOGY PROGRAM BIOASSAY RESULTS WITH BBP

MONONUCLEAR CELL LEUKEMIA				
STUDY	**TEST SPECIES**	**MALE FINDINGS**	**FEMALE FINDINGS**	**COMMENT**
NTP – 213	RAT	INADEQUATE FOR CLASSIFICATION	POSITIVE – OEHHA NEGATIVE _NTP	HIGH DOSE ONLY
NTP – 458	RAT	NEGATIVE	NEGATIVE	
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED - 2 YEARS
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED- LIFETIME
NTP – 213	MOUSE	NEGATIVE	NEGATIVE	

ACINAR PANCREAS TUMORS				
STUDY	**TEST SPECIES**	**MALE FINDINGS**	**FEMALE FINDINGS**	**COMMENT**
NTP – 213	RAT	INADEQUATE FOR CLASSIFICATION	NEGATIVE	
NTP – 458	RAT	POSITIVE	NEGATIVE	HIGH DOSE ONLY
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED - 2 YEARS
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED- LIFETIME
NTP – 213	MOUSE	NEGATIVE	NEGATIVE	

ADRENAL MEDULLA PHEOCROMOCYTOMA				
STUDY	**TEST SPECIES**	**MALE FINDINGS**	**FEMALE FINDINGS**	**COMMENT**
NTP – 213	RAT	INADEQUATE FOR CLASSIFICATION	POSITIVE-OEHHA NEGATIVE -NTP	HIGH DOSE ONLY
NTP – 458	RAT	NEGATIVE	NEGATIVE	
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED - 2 YEARS
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED- LIFETIME
NTP - 213	MOUSE	NEGATIVE	NEGATIVE	

URINARY BLADDER – TRANSITIONAL CELL TUMOR				
STUDY	**TEST SPECIES**	**MALE FINDINGS**	**FEMALE FINDINGS**	**COMMENT**
NTP – 213	RAT	INADEQUATE SURVIVAL FOR CLASSIFICATION	NEGATIVE	
NTP – 458	RAT	NEGATIVE	EQUIVOCAL (stats not reported)	24,000PPM
NTP – 460	RAT	NEGATIVE	NEGATIVE	RESTRICTED FEED - 2 YEARS
NTP – 460	RAT	NEGATIVE	POSITIVE OEHHA NEGATIVE NTP P>0.5	RESTRICTED FEED- LIFETIME 24,000PPM
NTP – 213	MOUSE	NEGATIVE	NEGATIVE	

TABLE – 2 FEMALE RAT URINARY BLADDER TRANSITIONAL CELL TUMOR INCIDENCE IN NTP STUDIES

NTP STUDY REPORT	24 MONTH STUDY		32 MONTH STUDY	
	0 ppm	**24,000 ppm**	**0 ppm**	**24,000 ppm**
NTP 213	0	0	NA	NA
NTP 458	1/50 Papilloma	2/50 Papilloma	NA	
	Statistical significance not included in Report Table B3, Statistical Analysis of Primary Neoplastic Lesions in female rats		NA	
NTP 460	0/50 Carcinoma 0/50 Combined papilloma or carcinoma	0/50 Carcinoma 2/50 Combined papilloma or carcinoma P=0.061	0/49 Carcinoma 1/49 Combined papilloma or carcinoma	4/50 Carcinoma 6/50 Combined papilloma or carcinoma P=0.059

ATTACHMENT C
OPINION OF DR. TIM ZACHAREWSKI

Expert Report on the Estrogenic Activities of Butylbenzyl Phthalate (BBP)

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September 7, 2011

Introduction

I have been asked by the Ferro Corporation to comment on the "Other relevant data - Estrogenicity activity" studies in the Office of Environmental Health Hazard Assessment (OEHHA)'s Butyl Benzyl Phthalate (BBP) background document that the Carcinogen Identification Committee (CIC) will consider at its Oct. 12-13, 2011 meeting. I am a Distinguished College of Natural Sciences Professor of Biochemistry & Molecular Biology and member of the Center for Integrative Toxicology at Michigan State University. I obtained my PhD in toxicology at Texas A&M University in the area of *in vitro* toxicology and obtained additional training in molecular biology and nuclear receptors in the laboratory of Professor Pierre Chambon (LGME-CNRS, Strasbourg, France) as a Medical Research Council of Canada Post Doctoral Fellow. I have 25+ years of research experience investigating the mechanisms of toxicity of environmental contaminants and industrial chemicals including the examination of estrogenic endocrine disruptors using *in vitro* and *in vivo* models. To date, my laboratory has published more than 100 peer-reviewed primary papers. I have also participated in numerous invited national and international workshops on *in vitro* screening assays and served on advisory committees reviewing the health risks of endocrine disruptors. This includes serving on the U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) review committee and on the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction committee that reviewed 6 high production volume phthalate esters including butylbenzyl phthalate (BBP). I am currently on leave from Michigan State University and placed on special assignment at EPA's National Center for Environmental Assessment (NCEA) investigating the use of high throughput *in vitro* assay and omics data in risk assessment as an Oak Ridge Institute for Science and Education (ORISE) Faculty Fellow. The following comments represent my own opinions and do not necessarily reflect the views or policies of the US EPA. For the purposes of this report, I am acting independently of Michigan State University and EPA.

Summary

This report outlines my opinions on the value of *in vitro* assays in assessing the estrogenic activities of chemicals. I describe their strengths and weaknesses as well as discuss specific technical issues that must be considered when critically assessing data from *in vitro* assays. Most importantly, the predictability of *in vivo* responses and the use of *in vitro* data in risk assessment are described. These principles are applied when evaluating the *in vitro* estrogenicity of BBP and its potential human carcinogenicity.

Although *in vitro* assays can be useful to identify chemicals that interact with the estrogen receptor and to elucidate mechanisms of action, they do not replicate *in vivo* conditions. Overall, *in vitro* assays have a poor record of predicting *in vivo* responses, especially for complex diseases such as breast cancer. Consequently, it is my opinion that *in vitro* assays are not useful for evaluating the potential carcinogenicity of BBP.

Endocrine Disruption Screening

Endocrine hormones, including steroids, regulate diverse physiological processes such as reproductive tract development, fertility, energy balance and behavior. Imbalances in hormones can also contribute to complex diseases such as cancer and diabetes as well as compromise reproductive fitness and development (e.g., reduced sperm counts, cyrptorchidism). In response to public concern regarding the possible disruption of the endocrine system following exposure to drugs, chemicals, natural products and environmental contaminants, the U.S. Environmental Protection Agency (EPA) established the Endocrine Disruptor Screening Program (EDSP) in compliance with the Food Quality Protection Act and the Safe Drinking Water Act Amendments of 1996. This requires EPA to test all food contact chemicals

and any chemical found in drinking water for effects similar to those elicited by female hormones (estrogens) and gives EPA the authority to screen for other endocrine effects (e.g., androgen, thyroid) in humans and wildlife (www.epa.gov/endo).

EDSP uses a two-tiered approach to determine the potential for chemicals to cause endocrine disruption in humans and wildlife. The Tier 1 Screening battery, adopted by EPA based on recommendations from the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), consists of a battery of complementary *in vitro* and short-term *in vivo* assays designed to identify chemicals affecting the estrogen, androgen and/or thyroid hormone systems through any of several recognized modes of action. This includes using assays that measure *in vitro* receptor binding and/or transactivation (estrogen and androgen receptor), inhibition of aromatase activity (estrogen biosynthesis), frog metamorphosis (thyroid activity), effects on fish (estrogen and androgen effects), male development (Hersberger assay, androgen and anti-androgen effects), pubertal female development (estrogen and thyroid activity), pubertal male development (androgen/anti-androgen and thyroid), uterine response (estrogen effects) and 15-day adult intact male responses (anti-androgen and thyroid activity). Data from these assays is then used in a weight of evidence (WoE) approach to determine which chemicals in Tier 1 Screening warrant further examination in Tier 2 Testing to generate the data needed to support a risk assessment (www.epa.gov/endo).

Tier 2 Testing *in vivo* assays assess whole organism effects and provide apical, as well as mechanistic, information from one or multiple endpoints within the assay. Tier 2 testing consists of *in vivo* tests in males and females with an intact hypothalamic-pituitary-gonadal axis, using multiple pathways of exposure, exposures at different life-stages and exposure to various taxa to further identify and characterize chemical induced interactions with the endocrine system that can be used in a risk assessment. Note that Tier 2 tests are designed to quantify dose-response relationships in the larger context of toxicity and potential adversity that may involve interactions with other biological systems (e.g., neurological, immunological, hepatic, renal, and cardiovascular). Therefore, regulatory action is based on Tier 2 testing (www.epa.gov/endo).

For the EDSP, weight-of-evidence evaluation (WoE) is a prescribed process where potentially relevant studies are critically assessed for data quality (www.epa.gov/endo). More specifically, potential compound effects, mode of action (MOA), and assay performance are evaluated. This goes beyond assessing positive and negative results within and between studies. Critical scientific assessment of the entire body of available data is considered to account for consistency, coherence, and biological plausibility. EPA continues to refine its WoE approach for use in risk assessments of chemicals suspected of causing toxicity, and especially cancer. Most important is the use of expert judgment formed through the scientific process, a current understanding of toxicity mechanisms, and knowledge of complementary fields (*e.g.*, developmental, reproductive, neurological and immunological toxicology, pharmacokinetics/ pharacodynamics) (Borgert *et al.*, 2011a; Borgert *et al.*, 2011b). The concept of using complementary *in vitro* and *in vivo* assays to inform risk assessment and regulatory decision making also appears to gaining acceptance among stakeholders (Hartung and Daston, 2009). Consequently, these same principles should be applied when evaluating the potential carcinogenicity of BBP in humans.

Limitations of *In Vitro* Assays

The endocrine system involves the integration of signals across multiple nodes (i.e., organs, tissues) throughout an organism (e.g., hypothalamic-pituitary-gonadal axis), which can be profoundly affected by the absorption, distribution, metabolism and excretion (ADME) of an endocrine disruptor. Interactions between different cell types and organs at different developmental stages can also affect a chemical's behavior. Therefore, in order to assess potential impacts on the endocrine system, the chemical must be tested in an intact *in vivo* model (Ankley *et al.*, 1998; Gray *et al.*, 1997; Spielmann *et al.*, 1998; Zacharewski, 1998; Zacharewski, 1997). Nevertheless, data from *in vitro* assays, when appropriately qualified, can also be informative regarding potential sites of action and mechanism of

action. Ideally, a comprehensive and complementary battery of assays should be used to avoid false positives and negatives, since estrogens and estrogenic endocrine disruptors, can elicit species-, tissue-, cell-, and response-specific effects. Moreover, despite the conservation of function and modes of action of endocrine systems between species, there are significant differences that can dramatically alter the activity of a chemical. Therefore, *in vitro* assays used to assess endocrine disrupting activity should be human-based, and reflect human ADME characteristics, when possible. For example, rodent models and *in vitro* assays lack sex hormone binding globulins (SHBG) that are present in human serum (Hammond, 2011; Hammond and Bocchinfuso, 1996). SHBG is an estrogen inducible protein present in serum that binds estrogens and estrogenic chemicals to regulate their bioavailability and metabolic clearance (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997).

In vitro assays also lack metabolic capabilities to bioactivate a proestrogenic chemical to its estrogenic metabolite or neutralize it through metabolism and eventual excretion. It is extraordinarily difficult to replicate the pharmacokinetic (e.g., metabolism) and pharmacodynamic (e.g. SHBG) interactions that are important for proper endocrine function, especially during development (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997). Differences between species further confound data interpretation. For instance, humans preferentially metabolize BBP to mBzP (73% on a molar basis) over mBp (6% on a molar basis) (Anderson *et al.*, 2001). In contrast, rat metabolism of BBP yields 16% mBzP and 44% mBP (Anderson, et al., 2001). Consequently, due to their poor record of predicting *in vivo* activity, *in vitro* assay results for endocrine disrupting activities can only be used in a WoE approach to rank and prioritize chemicals that warrant additional *in vivo* Tier 2 testing. It should also be noted that *in vitro* assay results are not used by the EPA for risk assessment. EPA also does not use data from other *in vitro* based screening programs such as ToxCast (Dix *et al.*, 2007) and Tox21 (Shukla *et al.*, 2010), for the purposes of risk assessment.

Assessment of *In Vitro* Studies Examining the Estrogenicity of BBP

The Office of Environmental Health Hazard Assessment (OEHHA) cites several papers under "Other relevant data – Estrogenic activity" that suggest BBP exhibits estrogenic activity based on *in vitro* data. For example, several papers are cited that report BBP binds to the estrogen receptor and induces ER-mediated effects such as the proliferation of human MCF-7 breast cancer cells. BBP is reported to be weakly estrogenic (>10^6 times weaker than 17β-estradiol, the predominant female sex steroid) in several *in vitro* assays (e.g., competitive estrogen receptor (ER) binding assays, ER-mediated gene expression in mammalian cells, ER-mediated reporter gene assays in mammalian cells, ER-mediated activity in yeast cells). However, BBP is not estrogenic *in vivo* based on the uterotropic assay, the gold standard for assessing the estrogenicity of a chemical (Brady *et al.*, 2000; Ryu and Kim, 2006; Zacharewski, 1997; Zacharewski *et al.*, 1998). These studies included doses that far exceed human exposure levels (e.g., daily doses ranging from 20 mg/kg to 2000 mg/kg per day for three consecutive days). In addition, estimated average drinking water exposures of 182.6 ug/kg/d to pregnant dams during gestation (gestational days 1-20) and from postnatal day 15 onward, had no effect on pup uterine weights (Ashby *et al.*, 1997b). Other sensitive *in vivo* markers of estrogen exposure including vaginal epithelial cell cornification (Zacharewski, et al., 1998), induction of uterine vascular permeability (Milligan *et al.*, 1998), and the differential expression of estrogen responsive uterine genes (Hong *et al.*, 2005), were also not affected by BBP.

Several factors must be considered when evaluating these *in vitro* studies. The first is that BBP is readily metabolized by non-specific esterases to monobutyl (mBP) and monobenzyl (mBzP) phthalate metabolites (Kayano *et al.*, 1997; Mentlein and Butte, 1989; Mentlein *et al.*, 1980). Consequently, mBP and mBzP should be the test chemicals used for *in vitro* assays. Interestingly, several of the cited reports indicate that mBP and mBzP are negative for estrogenic activity in the E-Screen and yeast-based assays (Harris *et al.*, 1997; Hashimoto *et al.*, 2003; Okubo *et al.*, 2003; Picard *et al.*, 2001). The concentrations used in the studies cited by OEHHA are also extremely high. Results obtained with doses exceeding 10

uM should be viewed with skepticism due to the insolubility of BBP in aqueous solutions (Moore, 2000). Precipitates would result in cells experiencing much higher concentrations than those nominally applied, which would confound data interpretation, and possibility cause cell toxicity. The Fernandez and Russo (Fernandez and Russo, 2010), Kang et al., (Kang and Lee, 2005), Kim et al., (Kim *et al.*, 2004), and Hashimoto et al., (Hashimoto, et al., 2003) studies all used BBP concentrations in excess of 10 uM, and even concentrations as high as 1 mM.

Of greatest concern are results derived from the E-Screen assay (Jones *et al.*, 1998; Welshons *et al.*, 1992; Zacharewski, 1997). Numerous studies have demonstrated that MCF-7 cell proliferation is highly variably and elicits a modest response (Picard, et al., 2001). This assay is also prone to false positives as a wide variety of chemicals and other treatments induce proliferation (Jones, et al., 1998; Welshons, et al., 1992). Assay reproducibility is problematic since several factors such as clone selection, culture conditions, serum lots and cell density influence proliferation (Jones, et al., 1998; Welshons, et al., 1992). As a result of these concerns, a panel of experts (Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC)) did not recommend the MCF-7 cell proliferation assay (E-Screen) as part of US EPA's EDSP Tier 1 Screening battery (www.epa.gov/endo).

Potentially more relevant and interesting studies have examined the *in vitro* transforming effects of BBP on MCF-10F cells using a collagen assay (Fernandez and Russo, 2010; Huang *et al.*, 2007). In this assay, MCF-10F cells are induced to form duct-like structures and solid masses in response to estrogenic substances. Although BBP at high concentrations (i.e., 1 and 10 uM) induced modest increases in MCF-10F cell invasion capacity relative to controls, it did not induce the formation of duct-like or solid mass structures. In contrast, another weak estrogenic endocrine disruptor, Bisphenol A (BPA), did induce duct-like structures and the formation of solid masses (Fernandez and Russo, 2010). The authors concluded that only "BPA as well as 17β-estradiol are able to induce the neoplastic tranformation of human breast epithelial cells" (Fernandez and Russo, 2010). To date, there have been no other peer reviewed publications using this assay. Additional studies investigating other estrogenic endocrine disruptors are required to evaluate the overall reliability of this assay.

Diet and environmental factors are potential contributors to breast cancer risk. More recently, attention has focused on changes in the hormonal environment during critical stages of development that may modify the architecture and biological characteristics of the developing mammary gland, increasing its future susceptibility to cancer. Two cited studies examining morphological and gene expression changes in the rat mammary gland following developmental exposure (post-natal days 2-20 and day 10 post-conception to delivery) exposure to 500 mg/kg/d BBP provide compelling data that BBP does not contribute to mammary carcinogenesis (Moral *et al.*, 2011; Moral *et al.*, 2007). They report that "BBP did not induce significant changes in the morphology of the gland, but changed the proliferation index" of several mammary gland structures (i.e., terminal end bud (TEB) at 35 days but not at 21, 50 and 100 days and lobule type 1 structures (Lob1) at 35, 50 and 100 days) in the post-natal study (Moral, et al., 2007). They further describe the TEB changes as "subtle" and "slight" at doses that far exceed human exposures. The *in utero* study (day 10 post-conception to delivery) reports trends in changes in epithelial structures (e.g., terminal end buds, terminal ducts, alveolar buds, lobules type 1) that are not statistically significant, and increases in the proliferation index that are modest, transient and only statistically significant at select times (TEB only at 35 days, terminal ducts only at 100 days, Lob1 only at 100 days). Although increases in the proliferation index suggest these structures are more susceptible to carcinogenesis, Singletary et at., report that 500 mg/kg BBP significantly reduced *in vivo* formation of mammary DNA adducts by 95%, and mammary adenocarcinomas by 70% induced by 1,12-dimethylbenz[a]anthracene (DMBA) (Singletary *et al.*, 1997) .

Mammary gland differential gene expression is reported in both studies with functions associated with proliferation, differentiation, immune function, cell signaling and metabolism. However, these changes are also modest, and not anchored to a phenotypic response (Boverhof and Zacharewski, 2006; Paules, 2003; Waters *et al.*, 2008). More specifically, these studies did not confirm that changes in gene

expression resulted in a phenotypic or apical response such as an increase in protein expression or enzyme activity. Exposure to any substance at the appropriate dose will cause changes in gene expression, but this may not lead to changes in protein expression, enzyme activity or an adverse affect. For example, despite BBP induced changes in mammary gland gene expression, there is no reported evidence of BBP causing mammary carcinogenesis in high dose multigenerational reproductive and developmental toxicity studies (Kamrin, 2009; Kavlock *et al.*, 2002; Tyl *et al.*, 2004).

Conclusion

BBP is an excellent example of a chemical that exhibits very weak *in vitro* estrogenic activity but does not induce significant estrogenic activity *in vivo* (Ashby *et al.*, 1997a; Moore, 2000). Although *in vitro* assay results suggest effects on MCF-7 cell proliferation (E-Screen), estrogen receptor mRNA expression, 17β-estradiol-induced MCF-7 apoptosis, and recombinant yeast reporter gene activity, these responses are not predictive of *in vivo* responses. Furthermore, the modest changes in mammary gland proliferation and gene expression that did not result in statistically significant morphological or phenotypic effects, consistent with the lack of mammary gland carcinogeneisis in multigenerational reproductive and developmental toxicity studies at doses that far exceed human exposures. In conclusion, the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action.

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ATTACHMENT D
FERRO JANUARY 22, 2013 SUBMISSION

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January 22, 2013

VIA EMAIL

P65Public.Comments@oehha.ca.gov

Ms. Cynthia Oshita
Office of Environmental Health Hazard Assessment
1001 I Street
Sacramento, California 95814

Dear Ms. Oshita:

On behalf of Ferro Corporation, we are submitting the enclosed information for consideration by OEHHA as it prepares cancer hazard identification materials for butyl benzyl phthalate (BBP) (CASRN 85-68-7). Ferro is a major producer of BBP.

There is a robust set of data for BBP. For the reasons given in our September 20, 2011 comments regarding prioritization, as well as the additional information herein, Ferro believes the evidence does not support listing of BBP under Proposition 65 as a carcinogen. NTP, which conducted the bioassays on BBP, has not considered BBP for listing in the Report on Carcinogens. IARC and the EU have not classified BBP as a possible or probable carcinogen. There are no human carcinogenicity data for BBP. The great weight of evidence is that BBP is not genotoxic. Tumor formation has been inconsistent and marginal in cancer bioassays, and other animal data do not support a concern for carcinogenicity. Further, biomonitoring data demonstrate that human exposure levels for BBP are extremely low.

If OEHHA has any questions or wishes additional information, please contact the undersigned at the telephone or email given above..

Sincerely,

Ann Claassen
of LATHAM & WATKINS LLP

Enclosure

cc: Alan Olson, Ferro Corporation

Submission of

Ferro Corporation

on

SCIENTIFIC EVIDENCE PERTAINING TO BUTYL BENZYL PHTHALATE (BBP)

for consideration by the

State of California
Office of Environmental Health Hazard Assessment

for Preparation of

Hazard Identification Materials under Proposition 65

January 22, 2013

TABLE OF CONTENTS

Page

Introduction 1

I. Uses of BBP 2

II. Epidemiology Data 2

III. Animal Carcinogenicity Bioassays 2

A. Long-term Bioassays 2
B. Short-term Bioassays 3

IV. Genotoxicity Testing 3

V. Mechanistic Data 4

A. Estrogenicity 4
B. Other Mechanistic Data 5
C. Metabolism 5

VI. Human Exposure 6

Conclusion 6

References 7

Appendix A: Pertinent Articles on BBP Identified in PubMed Search 9

Attachment 1: Ferro Submission in Advance of CIC Prioritization Meeting 12

INTRODUCTION

Ferro Corporation (Ferro) is submitting the information herein to the California Office of Environmental Health Hazard Assessment (OEHHA) regarding scientific evidence pertaining to preparation of hazard identification materials for butyl benzyl phthalate (BBP) (CASRN 85-68-7).[1] Ferro is a major producer of BBP.

OEHHA's preliminary toxicological review describes three long-term cancer bioassays and two short-term carcinogenicity studies conducted on BBP, all conducted by the National Toxicology Program.[2] The review also identified *in vivo* and *in vitro* genotoxicity data and mechanistic studies, including estrogenic activity assays. For prioritization of BBP by the Carcinogenic Identification Committee,[3] Ferro provided comments examining the significance of these studies for evaluating the potential carcinogenicity of BBP, plus other relevant information. A copy of those comments is provided as Attachment 1 to these comments.[4] This present document provides additional information.

In early January, 2013, we conducted a literature search for Butyl Benzyl Phthalate (CAS RN 85-68-7) on the National Center for Biomedical Information (NCBI) database using the PubMed search tools. Our search identified 129 titles dating back to 1980. Most of these relate to ecotoxicity or developmental toxicity. We selected 18 articles regarding higher-order mammalian toxicity studies relevant to assessing carcinogenic potential; these are listed in Appendix A. Only the first two have been published since our prior submission.

Our search found a bioassay that was not discussed in OEHHA's preliminary assessment or our prior submission (Kohno et al., 2004); it is discussed below. Nearly all other titles in Appendix A describe *in vitro* studies which examined genomic or endocrine disruption markers, which, as discussed below, are of limited usefulness for predicting the carcinogenicity of BBP.

Ferro believes that, in agreement with the implied or explicit assessments of NTP, IARC, Canada, and the European Union,[5] the evidence is insufficient to list BBP as "known to the State of California to cause cancer."

The following describes first the uses of BBP, then discusses the epidemiological, toxicological, genotoxicity and mechanistic data, and then discusses exposure data.

---

1 *See* OEHHA, Announcement of Chemical Selected by OEHHA for Consideration for Listing by the Carcinogen Identification Committee and Request for Relevant Information on the Carcinogenic Hazards of Butyl Benzyl Phthalate (Nov. 26, 2012), *available at* http://www.oehha.ca.gov/prop65/CRNR_notices/state_listing/data_callin/note112312.html.

2 OEHHA, Butyl Benzyl Phthalate (undated), available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ButBenzPhthalate_CIC.pdf.

3 *See* OEHHA, Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee (July 22, 2011 Notice), available at http://www.oehha.org/prop65/public_meetings/prior072211.html; OEHHA, October 12 and 13, 2011 Meeting of the Carcinogen Identification Committee (Sept. 30, 2011; posted Sept. 23, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic092311.html; OEHHA, Meeting Agenda and presentations of the October 12, 2011 Carcinogen Identification Committee (Oct. 13, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic101211.html; OEHHA, Meeting Synopsis and Slide Presentations Carcinogen Identification Committee Meeting Held on October 12, 2011 (Nov. 02, 2011), *available at* http://www.oehha.org/prop65/public_meetings/cic101211synop.html.

4 Some typographic errors that were in the 2011 submission are corrected in this version.

5 NTP (2011); IARC (1982; 1999); IPCS (1999); ECB (2007). *See also* Section IV of Attachment 1.

I. Uses of BBP

The primary use of BBP in the US is as a plasticizer in vinyl flooring (sheet and tile), carpet tile, and other building materials such as wallpaper and weather stripping. It is also used in caulks, adhesives, artificial leather, tarps, and automotive trim.

A number of reviews of BBP and articles in the literature list two types of uses which are no longer applicable – toys and cosmetics.

- Prior to this century, there was some limited use of BBP in toys; however, BBP has been prohibited in toys and child care articles since 1999 in the European Union (EU)[6] and since 2009 in the United States.[7] The EU prohibition had led to deselection of BBP in toys even before the ban in the US.

- The US Cosmetic Ingredient Review Expert Panel reviewed BBP in 1992 and found it safe for use in cosmetics (CIR, 1992), and there has been some limited use of BBP in cosmetics. However, BBP is now banned from cosmetics in the EU[8] and for many years has been used minimally, if at all, in cosmetics in the US.

If OEHHA has any specific questions regarding uses of BBP in the United States, Ferro would be pleased to provide answers to the best of its ability.

II. Epidemiology Data

There are no human studies on BBP carcinogenicity.

III. Animal Carcinogenicity Bioassays

A. Long-term Bioassays

As discussed in our prior submission (Section I of Attachment 1), NTP has examined the carcinogenicity of BBP in three rodent bioassays employing two species: rats and mice (NTP, 1982; 1997a; 1997b). The results are briefly summarized in Table 1; see also Appendix A of Attachment 1. There were no tumors observed in mice. Comparison of the results in rats from these three assays reveals a lack of consistency in tumor sites among the studies, even when tested in the same strain, so that the weight of evidence for a given tumor type in rats is at most equivocal.

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[6] A temporary ban in 1999 was made permanent in 2005. Directive 2005/84/EC of the European Parliament and of the Council of 14 December 2005, amending for the 22nd time Council Directive 76/769/EEC on the approximation of the laws, regulations and administrative provisions of the Member States relating to restrictions on the marketing and use of certain dangerous substances and preparations (phthalates in toys and childcare articles), O.J. L344:40 (Dec. 27, 2005).

[7] Consumer Product Safety Improvement Act of 2008, Section 108(a), P.L. 110-314 (Aug. 14, 2008).

[8] Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC), Annex II (consolidated version), *available at* http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1976L0768:20100301:en:PDF.

Table 1. Cancer Bioassay Results

Lesion	Male Mice	Female Mice	Male Rats	Female Rats
MNCL	**No**	**No**	**No** NTP 97a – No NTP 97b – w/in historical range	**Equivocal** NTP 82 – Yes NTP 97a – No NTP 97b – w/in historical range
Pancreatic Tumor	**No**	**No**	**Equivocal** NTP 97a – Yes NTP 97b -- Diet link; No tumor ↑ in weight-controlled animals	**No**
Bladder	**No**	**No**	**No**	**No** NTP 82 – No NTP 97a & 97b, – Not statistically significant
Mammary	**No**	**No**	**No** NTP 97b – reduced	**No** NTP 97b – reduced
Tumor Promotion	**N/A**	**N/A**	**No** – negative in all 3 studies	**No** – negative in all 3 studies

B. Short-term Bioassays

Two short-term bioassays were discussed in our previous submission (Section I.D of Attachment 1). In a short-term intraperitoneal injection study in mice, there was no increase in pulmonary tumors (Theiss, et al., 1977). In a short-term co-carcinogenicity study in rats, BBP administration by gavage *inhibited* tumor formation by dimethylbenz[a]anthracene (DMBA), and reduced mammary DMBA-DNA adduct formation (Singletary, et al., 1997).

We have identified another study that used a standard initiation-promotion tumor model (Kohno, et al., 2004). Forty weeks of treatment with BBP did not promote dimethyl aminobiphenyl prostate tumor formation and also did not induce tumor formation.

***

Thus, *in vivo* animal bioassays indicate that BBP has low potential to cause cancer in humans.

IV. <u>Genotoxicity Testing</u>

As discussed in our prior comments (Section II of Attachment 1), BBP has been tested in a variety of *in vitro* and *in vivo* genetic toxicity assays for genetic toxicity endpoints and for the

ability to induce morphologic transformation. The *in vitro* assays were conducted with bacterial, yeast and mammalian cell systems. *In vivo* assays were conducted in mice, rats and *Drosophila*. In most assays, BBP was negative; in the remainder, results were equivocal.

The results of the BBP genotoxicity assays are summarized in Table 2 below and in Appendix B (Tables 1-4) of Attachment 1. As the table shows, the great weight of the evidence indicates that BBP is not genotoxic.

Table 2. Genotoxicity Assay Results

Type	Positive	Negative	Equivocal
In vitro mutation, prokariotic cells	0	6	0
In vitro mutation, mammalian cells	0	2	1
In vitro chromosome, cell morphology, mammalian	0	3	1
In vivo	1	3	1
TOTAL	1	14	3

V. Mechanistic Data

A. Estrogenicity

In its preliminary toxicology review, OEHHA cited data concerning estrogenic activity. Appendix C of our prior submission (Attachment 1) was an opinion provided by Dr. Timothy R. Zacharewski of Michigan State University. Dr. Zacharewski explained that the weight of the evidence is that BBP is not estrogenic. In particular, it is negative for estrogenicity in the definitive *in vivo* studies. Dr. Zacharewski's opinion explains that *in vitro* data are not useful for evaluating the carcinogenicity of BBP, stating:

> Although *in vitro* assays can be useful to identify chemicals that interact with the estrogen receptor and to elucidate mechanisms of action, they do not replicate *in vivo* conditions. Overall, *in vitro* assays have a poor record of predicting *in vivo* responses, especially for complex diseases such as breast cancer. Consequently, it is my opinion that *in vitro* assays are not useful for evaluating the potential carcinogenicity of BBP.

Dr. Zacharewski concluded that the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode.

Dr. Zacharewski has recently completed a sabbatical at the US Environmental Protection Agency (USEPA) in North Carolina, where he evaluated the use of data from High Throughput Screening Assays and genomics-based assays for predicting toxicological outcomes. He was specifically tasked with identifying challenges likely to be encountered with the use of *in vitro* assays in risk assessment. Dr. Zacharewski has prepared a manuscript presenting his findings. The manuscript is currently in USEPA review; clearance by USEPA is anticipated soon. We will provide the manuscript to OEHHA as soon as it is available for distribution.

B. Other Mechanistic Data

Our prior submission discussed two other publications OEHHA identified as "mechanistic considerations" and explained that the studies do not provide substantial support for a concern of potential carcinogenicity of BBP in humans. See Section III.C of Attachment 1.

C. Metabolism

In most mammalian species the primary metabolites of BBP are excreted in the urine as unconjugated monobutyl and monobenzyl esters. Examination of urinary metabolites of rats following oral administration of 3.6 mmol BBP/kg/d for 3 days indicated that approximately 70% of the metabolites were unconjugated monoesters, while the remainder was conjugated (Eigenberg, et al., 1986). Eigenberg, et al. also showed that urinary metabolites of BBP account for about 50% of a range of oral doses to F-344 rats. Nativelle, et al. (1999) showed essentially the same in female Wistar rats. In dogs, however, only about 10% of an oral dose was metabolized (Erikson, 1965). Each of these studies also produced evidence of changes in phthalate metabolism occurring with increasing oral dose. The half-life of BBP in blood of rats is 10 minutes, while the blood half-life of monoester metabolites of BBP is 5.9 h (Eigenberg, et al. 1986).

The predominant monester differs with mammalian species. The rat preferentially hydrolyzes BBP to form the monobutyl ester (Eigenberg, et al., 1986; Mikuriya, et al., 1988; Monsanto, 1996a; Monsanto, 1996b. In the rat BBP yields approximately 16% MBzP and 44% MBuP on a molar basis (Eigenberg, et al. 1986).

Anderson, et al. (2001) exposed human volunteers to low doses of isotope-labeled BBP and measured the metabolites in urine. A single oral dose of 253 or 506 µg of BBP was administered and 24-hour urine samples were collected for analysis. On average, 140 µg and 323 µg of monobenzyl phthalate (MBzP) and 20 µg of monobutyl phthalate (MBuP) was eliminated. The MBuP was measureable in the high exposure group only. Anderson's data show that in humans, in contrast to the rat, BBP is preferentially hydrolyzed to form MBzP (73% on a molar basis) over MBuP (6% on a molar basis).

VI. Human Exposure

In our prior comments (Section V of Attachment 1), we discussed the biomonitoring data published by the Centers for Disease Control and Prevention (CDC)[9] and showed that the lowest BBP dose levels in rats that produce (equivocal) evidence of tumors are 250,000 to 4,000,000-fold above the levels for adult human exposure and 73,000 to 1,100,000-fold above the levels for children's exposure.

Other sources of exposure data support these findings. Guo and Kannan (2011) measured concentrations of BBP in indoor dust in China and the US (Albany NY) and calculated estimates of daily exposure via dust ingestion and dermal dust exposure. For the US age-group estimated to have the highest exposure (toddlers), exposure from ingestion of dust was estimated to be 0.1 micrograms BBP per kilogram per day (ug/kg/day) and from dust dermal absorption was 0.002 ug/kg/day. Wittassek, et al. (2010) back-calculated BBP exposures from US biomonitoring studies of 0.73-0.5 ug/kg/day at the median and 2.5-3.3 ug/kg/day at the 95th percentile. USEPA (2005) estimated exposures to BBP, among other chemicals, via ingestion (including dust ingestion) and inhalation. The estimated median potential exposure for preschool children was 10.0 ug/kg/day and the potential absorbed dose was estimated to be 0.29 ug/kg/day.[10] These values align well with those derived from the CDC biomonitoring, showing geometric mean aggregate exposure to BBP to be 1.1 ug/kg/day for children aged 6-11.[11]

At a January 13, 2013 meeting of OEHHA and Ferro representatives, OEHHA asked whether there is biomonitoring data using metabolites other than the monoester. We assume this question follows from the finding for some other phthalates that metabolites other than the monoester are more readily detected than is the monoester. We are not aware of biomonitoring for other BBP metabolites. However, we would note that, while another metabolite might effectively lower the detection limit, and thus increase the percentage of the population showing detectable BBP metabolite, the relative ratios of metabolites to the BBP dose will remain the same. Thus, the median and 95th percentile values derived from other metabolites will be similar to those derived from the monoester.

CONCLUSION

Ferro believes that the totality of data for BBP demonstrates that concern for BBP induced carcinogenicity is very low. The evidence is not sufficient to list BBP as a human carcinogen.

Ferro would be pleased upon request to provide, to the extent possible, additional information to assist OEHHA in its preparation of Hazard Identification Materials.

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[9] The prior submission cited CDC (2011). Updated tables are available as CDC (2012), but the values for BBP are the same as in the 2011 tables.

[10] These are the values for children observed in Ohio. Levels for children in North Carolina were slightly lower.

[11] See Section V of Attachment 1.

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APPENDIX A

Pertinent Articles on BBP Identified in PubMed Search

1. Benzyl butyl phthalate induces necrosis by AhR mediation of CYP1B1 expression in human granulosa cells.

   Chen HS, Chiang PH, Wang YC, Kao MC, Shieh TH, Tsai CF, Tsai EM.

   Reprod Toxicol. 2012 Jan;33(1):67-75. doi: 10.1016/j.reprotox.2011.11.004. Epub 2011 Nov 25.

   PMID: 22138065 [PubMed - indexed for MEDLINE]

2. Xenoestrogens down-regulate aryl-hydrocarbon receptor nuclear translocator 2 mRNA expression in human breast cancer cells via an estrogen receptor alpha-dependent mechanism.

   Qin XY, Zaha H, Nagano R, Yoshinaga J, Yonemoto J, Sone H.

   Toxicol Lett. 2011 Oct 10;206(2):152-7. doi: 10.1016/j.toxlet.2011.07.007. Epub 2011 Jul 12.

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ATTACHMENT 1

Ferro Submission in Advance of CIC Prioritization Meeting

September 20, 2011

Comments of

Ferro Corporation

on

SCIENTIFIC EVIDENCE PERTAINING TO BUTYL BENZYL PHTHALATE (BBP)

for consideration by the

State of California
Office of Environmental Health Hazard Assessment
Science Advisory Board Carcinogen Identification Committee

with respect to

Prioritization of Chemicals for Possible Preparation of Hazard Identification Materials under Proposition 65

September 20, 2011

Typographic errors corrected, January 22, 2013

EXECUTIVE SUMMARY

Ferro Corporation (Ferro) is submitting these comments to the California Office of Environmental Health Hazard Assessment (OEHHA) on the extent of the scientific evidence pertaining to the selection of butyl benzyl phthalate (BBP) (CASRN 85-68-7) for possible preparation of hazard identification materials. BBP is one of 39 chemicals to be discussed at the October 12-13, 2011 meeting of the Proposition 65 Carcinogen Identification Committee (CIC). These comments are submitted to assist the CIC in providing advice to OEHHA on the prioritization of BBP. Ferro is a major producer of BBP.

The evidence strongly supports a low prioritization for BBP. This is shown by review of the studies cited by OEHHA in its compilation of the preliminary toxicological review of BBP (summarized in Appendix A) and by other relevant information.

The body of these comments provides more detailed analysis of the BBP data. The complete database for BBP, including all studies completed by the National Toxicology Program (NTP), reveals that, at most, BBP has low potential to produce tumors in laboratory animals and does not produce rare tumors or induce tumors at an early onset. Findings from the BBP database include:

- the great weight of the evidence is that BBP is not genotoxic;
- no increase in tumors was observed in mice treated with BBP (NTP, 1982);
- a statistically significant increase in mononuclear cell leukemia (MNCL), a common tumor in F344 rats of questionable relevance to humans, was observed in female rats in one study (NTP, 1982), but this finding was not reproducible (NTP, 1997a);
- there was no other statistically significant increase in tumors in female rats;
- the marginal increase in pancreatic tumors in female rats in one study (NTP, 1997a) was not repeated in a subsequent 2-year bioassay (NTP, 1997b);
- there was a marginal increase in urinary bladder tumors (NTP, 1997a), but tumors were significantly increased in the subsequent bioassay only after 32 months (NTP,1997b);
- a statistically significant increase in pancreatic tumors was observed in male rats fed *ad libitum* (NTP, 1997a; NTP, 1997b); however, no such increase was seen in male rats kept on a weight-restricted diet for 2 years, indicating that diet may play an important role;
- in an 8-week intraperitoneal injection study (a standard tumor induction model), BBP caused no pulmonary tumors after 24 weeks (Theiss, et al., 1977); and
- in a short-term co-carcinogenicity study, BBP inhibited tumor formation by DMBA (Singletary, et al., 1997).

Thus, taken as a whole, the bioassay data for BBP do not reveal any strong tumor responses. This can reasonably be concluded from the lack of response in mice; inability to reproduce the leukemia response in female rats; absence of rare or early onset tumors; and the

low incidences and influence of the diet or length of study on the pancreatic tumors in male rats or the urinary bladder tumors in female rats.

The genotoxicity information on BBP, which includes a large number of *in vitro* and *in vivo* assays, is overwhelmingly negative. A summary of these studies is provided in Appendix B; a more detailed discussion is in the text.

OEHHA cites *in vitro* studies on estrogenicity and other mechanistic data. Timothy Zacharewski, Ph.D., a leading expert on *in vitro* studies and endocrine modulation studies, has reviewed the studies cited by OEHHA and provided an opinion, attached here as Appendix C, on the usefulness of that data for assessing BBP carcinogenicity. *In vitro* data have a poor record of predicting *in vivo* responses; in the definitive *in vivo* study, BBP is negative. Dr. Zacharewski concludes that the weight of evidence is that BBP is not estrogenic, and that *in vitro* estrogenicity studies are not useful for evaluating the potential carcinogenicity of BBP. The other mechanistic data cited by OEHHA do not provide a basis for concern for BBP carcinogenicity, because they involve a mechanism not relevant to humans, or are grounded in an erroneous interpretation of the data.

Tellingly, NTP, which conducted the three bioassays and many of the genotoxicity tests on BBP, has never formally considered BBP for listing in the Report on Carcinogens. Evaluations by other expert bodies have concluded that the potential for BBP to cause carcinogenicity in humans is, at most, marginal. The International Agency for Research on Cancer (IARC) has classified BBP as Group 3 (not classifiable as to its carcinogenicity to humans) (IARC, 1999). The International Programme on Chemical Safety, based on a 1998 assessment by Health Canada and Environment Canada, concluded that BBP can be considered, at most, possibly carcinogenic to humans (IPCS, 1999). OEHHA itself has previously reviewed the data for BBP and given it a low priority for consideration as a carcinogen (OEHHA, 1997). Most recently, the European Commission made a determination not to classify BBP as a carcinogen (ECB, 2007).

Biomonitoring data provide further reason to give a low priority to BBP. The substantial data provided by the Centers for Disease Control and Prevention (CDC) yields estimates of human exposure that, at the 95th percentile, are four to five orders of magnitude below the rat dose that produced equivocal evidence of tumors and, at the geometric mean, are six orders of magnitude below that dose. The CDC data also show that exposures to BBP are decreasing.

In summary, as has been concluded by several reviewing expert bodies, the animal data for carcinogenicity from BBP is at most marginal, and exposure to BBP is extremely low. Therefore, BBP should be given a low priority for preparation of hazard identification materials.

TABLE OF CONTENTS

Page

EXECUTIVE SUMMARY i

INTRODUCTION 1

I. Animal Bioassay Evidence of BBP Carcinogenicity Is Limited or Negative; therefore BBP Should Be Given a Low Priority.....2

A. NTP, 1982: No tumor response in mice; an increase of MNCL in female rats of questionable significance.....2
B. NTP, 1997a: Increase in MNCL not repeatable; low incidence of increased pancreatic tumors in male rats possibly related to diet.....3
C. NTP, 1997b: Evidence for role of diet in pancreatic tumors in male rats 5
D. Short-term animal studies for carcinogenic response: Theiss, et al. (1977); Singletary, et al. (1997).....6

II. The Weight of the Evidence Strongly Indicates that BBP Is Not Genotoxic 7

A. In Vitro Assays 7
B. In Vivo Assays.....8

III. Other Animal Data Do Not Support a Concern that BBP is a Potential Carcinogen 9

A. The Weight of Evidence is That BBP is Not Estrogenic.....9
B. In Vitro Studies of Potential Estrogenicity Are Not Useful for Evaluating the Potential Carcinogenicity of BBP 10
C. Other Mechanistic Data Do Not Support a Concern of Potential Carcinogenicity of BBP 10

IV. IARC and Other Authoritative Reviews of BBP Carcinogenicity Data Have Concluded There Is Low Concern for Human Carcinogenicity 11

V. Biomonitoring Data for BBP Show that Human Exposures Are Extremely Low.....13

CONCLUSION.....14

REFERENCES 15

Appendix A: Summary Comments on OEHHA Preliminary Toxicological Evaluation Compilation.....20

Appendix B: Genetic Toxicity Tables 23

Appendix C: Opinion of Timothy R. Zacharewski, Ph.D.....27

INTRODUCTION

Ferro Corporation (Ferro) is submitting these comments to the California Office of Environmental Health Hazard Assessment (OEHHA) on the extent of the scientific evidence pertaining to the selection of butyl benzyl phthalate (BBP) (CASRN 85-68-7) for possible preparation of hazard identification materials. BBP is one of 39 chemicals to be discussed at the October 12-13, 2011 meeting of the Proposition 65 Carcinogen Identification Committee (CIC).[1] These comments are submitted to assist the CIC in providing advice to OEHHA on the prioritization of BBP. Ferro is a major producer of BBP.

OEHHA has applied the human and animal data screens of its prioritization process to BBP and other chemicals,[2] and then conducted a preliminary toxicological evaluation for each chemical that met the screening criteria.[3] No cancer epidemiology studies were identified for BBP. In the animal data screen, OEHHA identified chemicals for which any of the following criteria are met: two or more positive animal cancer bioassays; one positive animal cancer bioassay with findings of tumors at multiple sites or with malignant (or combined malignant and benign) tumors occurring to an unusual degree with regard to incidence, site, type of tumor or age at onset; or one positive animal cancer bioassay and evidence from a second animal cancer bioassay of benign tumors of a type known to progress to malignancy.[4] OEHHA indicates that a positive bioassay is one in which a statistically-significant increase in tumor formation occurs as a result of treatment with test material, or any increase in a biologically-significant tumor (rare tumor) is seen.[5]

The preliminary toxicological review describes three long-term cancer bioassays and two short-term carcinogenicity studies conducted on BBP. The cancer bioassays were all conducted by the National Toxicology Program. The review also identified *in vivo* and *in vitro* genotoxicity data and mechanistic studies, including estrogenic activity assays.

These comments examine the significance of these studies for evaluating the potential carcinogenicity of BBP, plus other relevant information. Review of the database demonstrates that concern for BBP-induced carcinogenicity is low and that BBP should be given a low priority for preparation of hazard identification materials.

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1 OEHHA, Prioritization: Chemicals for Consultation by the Carcinogen Identification Committee (July 22, 2011 Notice), available at http://www.oehha.org/prop65/public_meetings/prior072211.html.

2 OEHHA, Process for Prioritizing Chemicals for Consideration under Proposition 65 by the "State's Qualified Experts." (December 2004), available at http://www.oehha.ca.gov/prop65/CRNR_notices/state_listing/pdf/finalPriordoc.pdf.

3 A compilation of studies OEHHA identified as relevant during the preliminary toxicological evaluation for BBP are provided in OEHHA, Butyl Benzyl Phthalate (undated), available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ButBenzPhthalate_CIC.pdf.

4 OEHHA, Prioritization: Chemicals Identified for Consultation with the Carcinogen Identification Committee (July 2011), Reproductive and Cancer Hazard Assessment Branch, p. 3, available at http://www.oehha.ca.gov/prop65/public_meetings/CIC101211/101211ChemPriorCIC2011.pdf.

5 *Id.*

I. Animal Bioassay Evidence of BBP Carcinogenicity Is Limited or Negative; therefore BBP Should Be Given a Low Priority

There are no human studies on BBP carcinogenicity. NTP has examined the carcinogenicity of BBP in three rodent bioassays employing two species: rats and mice (NTP, 1982; 1997a; 1997b). There were no tumors observed in mice. Comparison of the results in rats from these three assays reveals a lack of consistency in tumor sites among the studies. At most, these studies indicate that BBP has a low potential to produce tumors in laboratory rats, as observations of tumors are either not repeated, or are dependent on dietary status or length of study and limited to a single species. In a short-term intraperitoneal injection study in mice, there was no increase in pulmonary tumors (Theiss, et al., 1977). In a short-term co-carcinogenicity study in rats, BBP administration by gavage *inhibited* tumor formation by dimethylbenz[a]anthracene (DMBA), and reduced mammary DMBA-DNA adduct formation (Singletary, et al., 1997). Thus, the evidence is that BBP has low potential to cause cancer in humans and it accordingly should be given a low priority for development of hazard identification materials.

The results of these animal carcinogenicity studies are briefly summarized in Appendix A. More detailed discussion follows here.

A. NTP, 1982: No tumor response in mice; an increase of MNCL in female rats of questionable significance

NTP first examined the carcinogenicity of BBP in rats and mice in 1982 (NTP, 1982). Groups of 50 male or female rats and fifty male or female mice were exposed to BBP via the diet, at levels of 0, 6000 or 12000 ppm (0, 300 and 600 mg/kg/day, rats and 0, 780, or 1560 mg/kg/day, mice). Male and female mice and female rats were exposed for 103 weeks. Due to poor survival, all male rats were sacrificed at weeks 29-30; this part of the study was later repeated (NTP, 1997a).

No treatment-related neoplasms were observed in mice. Survival was not affected. A dose dependent reduction in body weight in both sexes was the only treatment related effect in mice observed in this study. Further, non-neoplastic changes were all within the normal limits of incidence for B6C3F1 mice. The NTP concluded that, under the conditions of the bioassay, BBP was not carcinogenic for B6C3F1 mice of either sex.

As male rats were terminated early, only female rats were evaluated at study completion. The incidence of mononuclear cell leukemia (MNCL) in control, low and high dose animals was 7/49, 7/49 and 18/50, respectively. The increased incidence in the high dose group was significant ($p=0.011$) by pair-wise comparison and a trend analysis was significant as well ($p=0.006$). No other treatment related findings were observed. The incidence in the high dose group and the overall trend remained significant ($p=0.008$ and $p=0.019$, respectively) when compared with historical control data at the time. The NTP concluded that BBP was "probably carcinogenic for female F344/N rats, causing an increased incidence of mononuclear cell leukemias" (NTP, 1982).

However, in a separate publication, the authors of the NTP report discussed the significance of the MNCL observed from this study. The authors concluded, "Although of statistical significance, the increased incidence of myelomonocytic leukemia in the female rats receiving the high dose of BBP was considered to be of equivocal biological significance due to the considerable variation in the background incidence of myelomonocytic leukemia in Fischer 344 rats" (Kluwe, et al., 1982). This view was amplified in Caldwell (1999), which points out that MNCL is a common neoplasm in F-344 rats that occurs at a high but variable rate,[6] that MNCL is uncommon or does not occur in other mammalian species, and that it is a lesion for which there is no human correlate neoplastic lesion. Thomas, et al. (2007) assert there is a human correlate, but note that the mechanism for development of the tumor may be different between species. Thomas, et al. advocate a weight of the evidence approach to assessing MNCL observations.

Caldwell (1999) notes that repeated chronic studies of BBP by the NTP have failed to produce consistent findings of an increased incidence of MNCL in F-344 rats. Caldwell provides other examples of inconsistency in the incidence of MNCL in F-344 rats (in repeat studies) and concludes that MNCL in the F-344 rat, alone, is not a useful basis for determining that a substance presents a carcinogenic hazard to humans.

Furthermore, as discussed below, the finding of increased MNCL in female rats was not reproducible in a second bioassay. Therefore, the weight of the evidence is that the MNCL observations in the 1982 study cannot be considered indicative of potential carcinogenicity for BBP.

B. NTP, 1997a: Increase in MNCL not repeatable; low incidence of increased pancreatic tumors in male rats possibly related to diet

NTP conducted a second bioassay on BBP (NTP, 1997a). This study was conducted in groups of fifty male and female Fischer 344 rats. Rats were fed diets containing 3000, 6000, and 12000 ppm BBP for male rats (delivering approximately 0, 120, 240 or 500 mg/kg/day) and 6000, 12000, and 24000 ppm BBP for female rats (approximately 0, 300, 600 or 1200 mg/kg/day). In addition to a terminal sacrifice at 103 weeks, the protocol included periodic hematological evaluation and hormonal assays, and a 15-month interim sacrifice.

There were no differences in survival between exposed groups and their control. The mean body weight of high-dose male rats was 10% lower than control males, indicating that the maximum tolerated dose had been reached. No increase in the incidence of mononuclear cell leukemia in female rats was observed. This is in direct contrast to the results of the initial bioassay, although both studies contained a similar dosing group (600 mg/kg/day) at which the increased incidence was observed in the earlier bioassay. The 1997 repeat study results lend further weight to the conclusion of the initial study authors that the increase in incidence of MNCL was of equivocal biological significance.

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[6] NTP historical control data for F-344 rats show that MNCL occurs in 14 to 74 percent of control animals (Haseman, et al., 1998). The background incidence is highly variable and has more than doubled from about 1985 to about 2005 (Haseman,, et al., 1985; Thomas, et al., 2007). NTP decided to stop use of the F-344 strain, in part because of the high spontaneous incidence of MNCL in that strain (King-Herbert and Thayer, 2006; NTP BSC, 2007).

The incidences of transitional epithelial papilloma of the urinary bladder in female rats at 2 years were 1/50, 0/50, 0/50, and 2/50 in control, 300, 600, and 1200 mg/kg/day groups, respectively. The incidence of this lesion in the high-dose group, 2/50, is not statistically different from the incidence in the control group, 1/50; however, it is outside the reported range of 0-2% for untreated controls in NTP studies. The nonstatistically-significant increase in a benign neoplasia combined with the elevated (statistically-significant) hyperplasia of the urinary bladder epithelium was one of two elements forming the basis for NTP to conclude that BBP produced equivocal evidence of carcinogenic activity in female F-344 rats in this bioassay.

Unfortunately, NTP did not report whether urinary calculi were present in any of the female rats treated with BBP, especially those in the high-dose group developing bladder hyperplasia or tumors. Urinary bladder neoplasia in rodents and proliferative responses of the urinary bladder epithelium (like the hyperplasia reported in this study) have been observed in rats and mice following mechanical irritation by foreign bodies or calculi (Alison, et al., 1994). According to Alison, et al., "when administration of a chemical results in calculi and tumor formation, it is necessary to establish whether the (bladder) tumors are indeed induced by the chemical or occur as a secondary effect of the calculi. Low doses of compound that do not produce calculi do not produce tumors. This provides a simple example of a threshold effect for secondary carcinogenesis."

Pancreatic acinar cell adenomas were reported for the high-dose females (2/50 vs. 0/50 in controls). This incidence in high-dose female rats was not statistically different from the incidence in control animals and was within the range of NTP historical controls values. Nonetheless, because a pancreatic acinar cell tumorigenic effect occurred in male rats, NTP cited the finding of pancreatic acinar cell adenomas in female rats as the other reason (in addition to bladder effects described above) to conclude that there is equivocal evidence of carcinogenic activity of BBP in female rats in the assay. NTP's definition of "equivocal evidence of carcinogenic activity" is a study interpretation that shows a marginal increase of neoplasms that may be chemical related. Given that there was no real increase in neoplasms in female rats in this study, the designation of "equivocal evidence" appears to be questionable – "no evidence" is likely more appropriate.

In male rats, an increased incidence of pancreatic acinar cell adenoma (3/50, 2/49, 3/50 and 10/50 in control, 120, 240 and 500 mg/kg /day groups, respectively) and pancreatic acinar cell adenoma or carcinoma (combined) (3/50, 2/49, 3/50 and 11/50 in control, 120, 240 and 500 mg/kg /day groups, respectively) was observed in the high-dose group. These incidences were statistically significant. No difference in food consumption was reported for the high-dose male rats compared to control; however, the high-dose male rats in this study weighed less, on average, than control animals, suggesting that the caloric intake for the high-dose animals was greater (on a body weight basis) than for concurrent control animals. A possible dietary role in pancreatic carcinogenesis, as suggested in this study, was, in fact, demonstrated in the third NTP bioassay of BBP (NTP, 1997b). The findings of that study, a dietary restriction study, are described below in the next section.

It is known from published analyses of the NTP carcinogenicity database (Haseman, et al., 1985; Haseman and Rao, 1992) that for male F-344 rats a relationship can be shown between pancreatic acinar cell tumors and corn oil gavage treatment. The effect is not related to gavage

technique and appears to be sex-specific. The BBP cancer bioassay reported in 1997 (NTP, 1997a) was not a gavage study and did not involve the use of corn oil as a vehicle, but did provide an indication that dietary factors may play an important role in male rat pancreatic tumorigenesis, a role confirmed in the 1997 dietary restriction study with BBP (NTP, 1997b).

Moreover, a series of papers published beginning in 1997 identified a number of factors in addition to diet which influence the development of pancreatic lesions in the rat (Obourn, et al., 1997a; 1997b; Biegel, et al., 2001). These lesions included acinal cell hyperplasia and adenocarcinoma formation. The authors of those studies stated that any factor which can affect circulating steroid levels or cholecystokinin (CCK) levels, or cause overexpression of the CCK(A) receptor may increase the development of pancreatic acinar cell changes and lead to tumor formation in the rat. Accordingly, these factors, even if induced secondarily as a result of toxicity in another organ, can stimulate the development of pancreatic acinar cell pathology. In fact, Obourn (1997a) and Biegel, et al. (2001) concluded in a study of a peroxisome proliferating agent that a mild but sustained increase in CCK production secondary to liver changes (cholestasis) may be responsible for pancreatic acinar cell pathology, including tumors. These reports may have applicability to BBP and the findings of pancreatic acinar cell tumors in the NTP studies because of the ability of BBP to induce hepatic changes in the Fischer rat at dietary doses equivalent to the mid- and high-dose concentrations employed in the NTP studies, and the role of diet in the observation of pancreatic tumors (Monsanto, 1994).

The significance of secondary toxic effects and the role these may play in rat pancreatic pathogenesis should be part of the interpretation of the pancreatic acinal cell tumorigenic effects observed in male rats in the BBP studies. This is particularly the case since no other elevation in tumor incidence was observed in male rats in this study and since the pancreatic effect thought to be secondary to liver damage is considered to be species specific, *i.e.*, limited to the rat (Obourn, 1997a).

Finally, since focal abnormalities of acinar pancreatic cells (atypical acinar cell foci or nodules) are reported to occur spontaneously in rats at an incidence of zero to 75% in 24 month old rats (Woutersen, et al., 1991), the elevated incidence of focal hyperplasia of the pancreatic acinar cell in the high-dose males of the 1997 NTP bioassay should have been put into a broader perspective than provided by NTP. The authors concluded that there was "some evidence of carcinogenic activity" in male rats, based upon the increased incidences of pancreatic acinar cell adenoma and of acinar cell adenoma or carcinoma (combined). Given that the only neoplastic lesions or even preneoplastic lesions observed in male rats were pancreatic and occurred at very low incidences, and considering the mitigating factor of a dietary role in pancreatic tumor formation, NTP would have more properly categorized the results of this study as “equivocal evidence” of carcinogenic activity, *i.e.*, a study showing a marginal increase of neoplasms that may be chemical related.

C. NTP, 1997b: Evidence for role of diet in pancreatic tumors in male rats

The third NTP bioassay on BBP was conducted as part of an effort to compare the effect of *ad libitum* feeding versus dietary restriction on the outcome of chronic bioassays (NTP, 1997b). Male rats were dosed with 12,000 ppm BBP in their diet for 24 or 30 months; females

were dosed with 24,000 ppm for 24 or 32 months. Test groups included *ad libitum* fed control and treated rats, control and treated rats that received an amount of food so that mean body weight matched the mean body weight of the *ad libitum* dosed group (weight-matched), and control and treated rats that were maintained at about 85% of the body weight of the untreated controls in the *ad libitum* study (weight restricted).

An increase in the incidence of pancreatic acinar cell neoplasms was observed in BBP-treated *ad libitum* fed male rats compared to *ad libitum* fed and weight-matched controls. Interestingly, no increase was observed in restricted diet treated group after 2 years but acinar cell adenomas were observed in 3 animals at 30 months reinforcing the role of diet in the expression of this tumor following administration of BBP.

In female rats, a slight increase in urinary bladder neoplasms was observed, but only at a 32-month time point, in the restricted feed treated group. The increase was not statistically significant.

The incidences of MNCL in exposed males were statistically-significantly greater than those in the weight-matched controls but similar to the incidence in the controls fed *ad libitum* and within the historical control ranges for leukemia (all types) in untreated rats. The incidences of MNCL in exposed females were greater than those in the weight-matched controls but less than the incidence in the controls fed *ad libitum* and within the historical control ranges for leukemia (all types) in untreated rats. The incidences in weight-restricted male rats at 24 months were slightly higher than weight-restricted controls (54% v. 42%) and slightly lower (92% v. 94%) than weight-restricted controls at 30 months, but the differences were not statistically significant. The incidences in weight-restricted female rats at 24 months was slightly higher than weight-restricted controls (36% v. 32%) and significantly higher (78% v. 58%) than weight-restricted controls at 32 months. In evaluating the study results, NTP noted that all MNCL findings were within historical control incidence ranges for untreated rats, and NTP did not include MNCL in their pathology and statistical analyses of "significant or biologically noteworthy changes."

Mammary gland tumor incidences, both fibroadenoma and adenomas and carcinomas combined, were statistically-significantly reduced by BBP treatment compared to the *ad libitum* fed group and to the 24 and 30-month restricted feed control groups.

D. Short-term animal studies for carcinogenic response: Theiss, et al. (1977); Singletary, et al. (1997)

Theiss's group (Theiss, et al., 1977) showed that intraperitoneal injections to Strain A mice of BBP three times per week for 8 weeks (a standard tumor induction model) failed to produce a treatment-related increase in pulmonary tumors, the end point of the bioassay. Doses used in the study were high: 160, 400 and 800 mg/kg/injection, yielding a total dose of 3,840, 9,600 and 19,200 mg per animal, respectively. Twenty animals were used per dose level. Mice were held for 24 weeks following the final injection. All animals survived to study termination. No increase in pulmonary tumors occurred as a result of BBP treatment.

Singletary, et al. (1997) employed an initiation-promotion model for assessment of BBP tumorigenicity and DNA adduct formation. Mammary tumors were initiated with dimethylbenz[a]anthracene (DMBA) in groups of 27 female rats. DMBA was given by oral gavage at a dose of 31 mg/kg. BBP was administered orally via gavage at doses of 250 or 500 mg/kg. BBP was administered for 7 consecutive days prior to administration of DMBA. BBP inhibited total mammary tumor formation by 37% at each dose level. Adenocarcinoma formation was inhibited 60% and 70% at the low and high-dose of BBP, respectively.

Singletary, et al. (1997) also reports that BBP administration for 5 days via intraperitoneal injection (ip) at 100 and 500 mg/kg/day or oral gavage at 100 or 500 mg/kg/day reduced mammary DMBA-DNA adduct formation by 2% and 92% (ip) and 48% at 500 mg/kg/day by gavage.

Thus, these short-term studies provide no evidence that BBP is a carcinogen or co-carcinogen.

II. The Weight of the Evidence Strongly Indicates that BBP Is Not Genotoxic

BBP has been tested in a variety of *in vitro* and *in vivo* genetic toxicity assays for genetic toxicity endpoints and for the ability to induce morphologic transformation. The *in vitro* assays were conducted with bacterial, yeast and mammalian cell systems. *In vivo* assays were conducted in mice, rats and *Drosophila*. In most assays, BBP was negative; in the remainder results were equivocal. The great weight of the evidence indicates that BBP is not genotoxic.

The results of the BBP genotoxicity assays are summarized in Appendix B (Tables 1-4). More detailed discussion follows here.

A. In Vitro Assays

BBP was not mutagenic in the Ames *Salmonella* assay with and without activation (Litton Bionetics Inc., 1976; Rubin, et al., 1979; Kozumbo, et al., 1982; Zeiger, et al., 1985). It was also shown to be without genotoxic activity (mutation) in *E. coli* and was negative for DNA damage in *Bacillus* bacteria (Omari, 1976). When tested in eurokaryotic cells, BBP was negative for mutation in D4 yeast cells (Litton Bionetics Inc., 1976).

In the mouse lymphoma assay, BBP produced either negative (Litton Bionetics Inc., 1977; Hazleton Biotechnologies Company, 1986; Barber, et al., 2000), or equivocal responses (Myhr, et al., 1986; Myhr and Caspary, 1991). Testing in that assay system, the L5178Y Mouse Lymphoma cell line, was performed in multiple trials with and without exogenous metabolic activation and was conducted at BBP concentrations at or greater than the limit of BBP solubility in cell culture medium.

BBP did not produce *in vitro* transformation of Balb/c-3T3 cells (Litton Bionetics Inc., 1985; Barber, et al., 2000), nor did it produce transformation in Syrian hamster embryo cells (Le Boeuf, 1996). Primary cultures of Syrian hamster embryo cells retain significant innate

metabolic capability and transformation assays using these cells are typically performed without exogenous metabolic activation (Ashby, et al., 1985), as was the case with BBP testing.

In an assay for chromosomal aberrations and sister chromatid exchanges (SCE) in Chinese hamster ovary cells (Galloway, et al., 1987), there was slight evidence for a trend in an increase in SCE formation in one of two trials without activation, but no evidence for SCE formation in a trial with activation. There was no evidence for induction of chromosome aberration by BBP. The authors concluded that the study was negative for the induction of SCE and chromosome aberration.

B. In Vivo Assays

In vivo assays have also been negative or equivocal for evidence of genetic toxicity. A negative response was reported in assays for the induction of sex-linked recessive lethals in *Drosophila melanogaster* dosed by feed and also dosed by injection (Valencia, et al., 1985).

Results from mouse bone marrow tests examining induction of either sister chromatid exchanges (SCE) or chromosomal aberrations indicated weak responses (NTP, 1997a). A close look at the data shows that the results of these tests must be viewed with caution. Groups of 5 B6C3F1 male mice received a single intraperitoneal (ip) injection of 1250, 2500 or 5000 mg/kg BBP for evaluation of SCE in a single trial. The ip LD50 for mice (Swiss Webster) is 3160 mg/kg (Calley, et al., 1966). Information on test animal survival, weight gain or signs of systemic toxicity during the test period was not included in the NTP report of this study. However, SCE test data from the top dose (5000 mg/kg) were excluded by the investigators from analysis because of “a reduction in response,” presumably due to excessive systemic toxicity. Two marrow cell harvest times were used, 23 and 42 hours post-dose. The number of cells scored per dose group was low: twenty-five marrow cells per animal (4 animals per group) were scored. There was no dose at either harvest time reported to have induced a statistically significant elevation in SCE formation. Despite, or perhaps because of, the extreme dose levels used (well into the systemically toxic range and in excess of the LD50) and the aggressive route of administration (intraperitoneal injection), there was no dose-response characteristic to the study data. The results were reported as positive for a trend in increased SCE formation without a significant increase in SCE levels in treated animals.

Similarly, mice treated with BBP as described above for the SCE study were evaluated for chromosomal aberration (NTP, 1997a). Ten male mice per group received an injection of 1,250, 2,500 or 5,000 mg/kg BBP and were evaluated at 17 or 36 hours post-dose for signs of chromosomal aberration in bone marrow cells. Fifty metaphase cells were examined for each animal; current guidelines for this type of study (OPPTS, 1998) require evaluation of 1000 cells per animal. No data were provided in the report concerning mortality, signs of systemic toxicity, etc., but a significant increase in chromosomal aberration was cited for the high dose group in each of the 17-hour harvests. There was no increase in aberrations in the 36-hour harvest. Although there was no dose-response relationship established for any of the three trials, a significant trend was reported for the 17-hour harvest trials. Without information on the condition of the high dose animals, and understanding that the dose employed in this group was nearly 60% above the LD50 for BBP by ip injection, interpretation of the results from the study is difficult and should be done with caution.

In contrast, negative results were reported by Ashby, et al. (1997) in a micronucleus assay in rats and by Bishop (1987) for a mouse (two species) dominant lethal mutation assay.

Thus, the weight of the evidence strongly indicates that BBP is not mutagenic or genotoxic. This evidence is summarized in Appendix B, Tables 1-4.

III. Other Animal Data Do Not Support a Concern that BBP is a Potential Carcinogen

Besides the data discussed above, OEHHA's compilation from its preliminary toxicology review cites data concerning estrogenic activity, plus two other mechanistic studies. These studies do not point to potential carcinogenicity for BBP. The weight of the evidence is that BBP is not estrogenic *in vivo*; further, *in vitro* estrogenicity studies such as those cited by OEHHA are not useful for evaluating the potential carcinogenicity of BBP. One of the mechanistic studies concerns activation of peroxisome proliferator-activated receptors (PPARs), a mechanism that is not relevant to human risk assessment. The other relies on an inaccurate interpretation of the BBP database.

A. The Weight of Evidence is That BBP is Not Estrogenic

OEHHA cites several *in vivo* studies on estrogenic activity and BBP. These papers and their relevance to human carcinogenicity assessment are the topic of an opinion written by Timothy Zacharewski, Ph.D., a leading expert on *in vitro* and *in vivo* models for estrogenicity and other endocrine modulating. Dr. Zacharewski's opinion is attached as Appendix C. It shows that the weight of the evidence is that BBP is not estrogenic.

Dr. Zacharewski discusses the need for a weight-of-evidence approach to assessing toxicity data and a critical assessment of data quality, all of which go beyond scoring positive and negative results of a group of studies. While properly qualified *in vitro* assays can be useful in identifying chemicals that interact with the estrogen receptor, they do not replicate *in vivo* conditions and have a poor record of predicting *in vivo* responses. *In vitro* studies are limited by the absence of pharmacokinetic and pharmacodynamic processes that occur in the intact organism. "Therefore, in order to assess potential impacts on the endocrine system, the chemical must be tested in an intact *in vivo* model" (Zacharewski, Appendix C, p. 3).

In several *in vitro* assays of estrogenicity, including proliferation of MCF-7 breast cancer cells, BBP has given very weak positive responses – more than a million times weaker than 17β-estradiol, the predominant female sex steroid. Dr. Zacharewski discusses factors that limit the usefulness or reliability of these studies for evaluation of BBP estrogenicity. Further, and most importantly, "BBP is not estrogenic *in vivo* based on the uterotropic assay, the gold standard for assessing the estrogenicity of a chemical" even at dose levels far above those of human exposures (Zacharewski, Appendix C, p. 4).

B. In Vitro Studies of Potential Estrogenicity Are Not Useful for Evaluating the Potential Carcinogenicity of BBP

Dr. Zacharewski's opinion also discusses the value of *in vitro* assays for evaluating the potential carcinogenicity of BBP. In addition to the inherent limitations on the value of *in vitro* assay for evaluating estrogenicity summarized above, his points include the following:

- In whole organisms, BBP is readily metabolized to monoester metabolites. These metabolites are negative for estrogenic activity in the E-Screen and yeast-based assays;
- BBP concentrations in the studies cited by OEHHA were extremely high – well beyond the aqueous solubility of BBP – confounding data interpretation;
- The E-Screen assay (MCL-7 cell proliferation) is prone to false positives;
- In a collagen assay, unlike 17β-estradiol, BBP did not cause formation of duct-like or solid mass structures;
- BBP significantly reduced *in vivo* formation of mammary DNA adducts and mammary adenocarcinomas induced by DMBA; and
- There is no reported evidence of BBP causing mammary carcinogenesis in high dose multigenerational reproductive and developmental toxicity studies.

Dr. Zacharewski concludes that "the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action" (Zacharewski, Appendix C, p. 6).

C. Other Mechanistic Data Do Not Support a Concern of Potential Carcinogenicity of BBP

OEHHA cites two publications it identifies as "other mechanistic considerations" – Hurst and Waxman (2003) and Agas, et al. (2007). The studies do not provide substantial support for a concern of potential carcinogenicity of BBP in humans.

Hurst and Waxman (2003) characterize activation of rodent and human peroxisome proliferator-activated receptors PPARα and PPARγ. These receptors are associated with a variety of cellular activities and phthalate monoesters are ligands for these receptors. Activation of PPAR receptors in rodents by some phthalate esters (but not BBP) is responsible for the development of liver cancer in those species. Based on rodent models, Hurst and Waxman argue that activation of PPAR receptors in humans, particularly PPARγ, may lead to adverse consequences including cancer. Other, data, however, do not support this theory.

A 2004 article by Bility, et al. investigated rodent and human PPARα, $PPAR_{\beta}$ and PPARγ activation by phthalate monoesters including monobutyl phthalate and monobenzyl phthalate. Bility, et al. showed that, among the common phthalate monoesters, those derived from BBP are the least or next-to-least potent activators of PPAR. More importantly, Bility, et al. showed major species differences in receptor activation between mouse, rat and human PPAR. Human PPAR receptors were sensitive to activating ligands including BBP metabolites compared to rodent PPAR. This lack of receptor sensitivity is mirrored in significant differences in the response of humans and rodents to phthalates and other peroxisome proliferators – humans and other primates are more refractory to phthalates than rodents (*see, e.g.*, Klaunig, et al., 2003). Bility, et al. also point out that in animal models activation of PPARγ can be both a potentiator of

carcinogenic effects and an inhibitor of carcinogenic effects. The findings reported by Bility, et al. suggest that any PPAR-based mechanistic consideration of BBP as a carcinogen, especially a human carcinogen, is premature.

Agas, et al. (2007) report on *in vitro* studies showing actin redistribution in a rat osteoblast cell line exposed to BBP. Standard techniques to assess gene activation and protein synthesis showed a decrease in actin synthesis in the presence of BBP and, following removal of BBP from the culture, an increase in actin synthesis. The authors interpret this as triggering an overall potentiation of cell growth. Intracellular localization of actin also was demonstrated. The authors reported that BBP increased osteoblast "viability" and culture growth (cell number) but relied on a single indirect technique – an increase in a metabolic breakdown product (formazan) – to assess both parameters. They also provided a qualitative indication that cyclin D3 is increased *in vitro* by BBP. Without presenting any additional empirical data the authors speculated that BBP could: affect translocation of fibroblast growth factor 2 (FGF) into the nucleus; alter DNA synthesis, cell proliferation and cell cycle progression since "some FGF's" are reported to regulate DNA synthesis; disrupt regulation of the D-cyclins since "D-type cyclins probably serve as integrators of growth factor-induced signals;" contribute to oncogenesis because of aberrant protein expression; and effect all of this through stimulation of cyclin D3 because "cyclins are molecules implicated in various cancers."

The authors support these hypotheticals by erroneously citing Zacharewski (1998) to claim that BBP mimics 17β-estradiol and then intimating that BBP is the physiologic equivalent of 17β-estradiol, a carcinogen. From this, Agas, et al. appear to apply findings on 17β-estradiol to BBP. As pointed out by Dr. Zacharewski in his opinion (Appendix C), summarized above, BBP exhibits only very weak *in vitro* estrogenicity and does not induce significant activity *in vivo*. Thus, the foundation for the supposition of Agas, et al. is undercut, and this paper does not provide a reliable basis for hypothesizing potential carcinogenicity of BBP.

IV. IARC and Other Authoritative Reviews of BBP Carcinogenicity Data Have Concluded There Is Low Concern for Human Carcinogenicity

The two-year cancer bioassays on BBP were conducted by the National Toxicology Program. NTP is responsible for publishing the Report on Carcinogens (ROC), listing chemicals NTP determines to be known or reasonably anticipated to cause cancer in humans. NTP often selects chemicals for evaluation for ROC listing from chemicals it has tested. NTP has never formally considered BBP for ROC listing,[7] indicating that it does not find the bioassay findings to raise significant concern about the potential carcinogenicity of BBP.

The BBP data have been reviewed by several other authoritative agencies. In each case, the conclusion indicates low concern for human carcinogenicity from BBP exposure. In

[7] Appendix C of the ROC (NTP, 2011) is "Substances Reviewed but Not Recommended for Listing in the Report on Carcinogens." BBP is not listed there, indicating that NTP has never considered BBP for listing.

accordance with the findings of these other careful agency reviews, BBP should be given a low priority for development of hazard identification materials.

The International Agency for Research on Cancer (IARC) classified BBP as Group 3 (not classifiable as to human carcinogenicity) in 1982 (IARC, 1982). In 1999, IARC reevaluated BBP (including consideration of the three NTP assays) and reconfirmed the Group 3 classification, finding the evidence in experimental animals to be limited (IARC, 1999).

OEHHA previously evaluated the carcinogenicity data for BBP (including the first two NTP bioassays) and determined there was a low level of carcinogenicity concern for BBP. OEHHA therefore gave BBP a low priority for further evaluation for listing under California Proposition 65 (OEHHA, 1997). The primary difference in the BBP database since that time is the addition of the third NTP bioassay, which indicates a role for diet in the pancreatic acinar cell tumors observed in male rats.

In 1998, Health Canada and Environment Canada produced an assessment of BBP toxicology, which then became the basis for an International Programme on Chemical Safety (IPCS)[8] Concise International Chemical Assessment Document (CICAD) (IPCS, 1999). These parties concluded:

> Therefore, BBP has induced an increase in pancreatic tumours primarily in one sex of one species, the full expression of which was prevented in a dietary restriction protocol, and a marginal increase in bladder tumours in the other sex, which was delayed upon dietary restriction. The weight of evidence of genotoxicity is negative, and, although weak clastogenic potential cannot be ruled out, available data are consistent with the compound not interacting directly with DNA. On this basis, BBP can be considered, at most, possibly carcinogenic to humans, likely inducing tumours through a non-genotoxic (although unknown) mechanism. (IPCS, 1999, Exec. Summ.)

Most recently, the European Commission of the European Union issued its European Union Risk Assessment Report for BBP (ECB, 2007). In that document the European Commission concluded that "BBP may be a borderline case between no classification (not a carcinogen) and Carcinogen Category 3 (available information is not adequate for making a satisfactory assessment). However, due to the lack of genotoxic effects no classification is proposed."

Thus, several separate assessments by agencies with expertise in carcinogenicity risk assessment have found BBP to pose low concern for carcinogenic risk to humans. Accordingly, BBP should be given a low priority for development of hazard identification materials.

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[8] IPCS is a joint venture of the United Nations Environment Programme, the International Labour Organisation, and the World Health Organization

V. Biomonitoring Data for BBP Show that Human Exposures Are Extremely Low

Substantial urinary metabolite data for BBP are available in the National Health and Nutrition Examination Survey (NHANES) database of the Centers of Disease Control and Prevention (CDC). Using the method of David (2000) and Kohn, et al. (2000), this data can be converted to provide an estimate of the exposure to BBP, using the following equation.

$$DI = [UC \times CE / (FUE \times 1000)] \times [MWd/MWm]$$

DI == daily intake of BBP (μg/kg/day)
UC = creatinine corrected urinary metabolite concentration (μg/kg) (from CDC, 2011)
CE = creatinine excretion rate (mg/kg/day) (for adults, 20 mg/kg/day from David, 2000 and Kohn, et al., 1999; 25 mg/kg/day for children ages 6-11, extrapolated from Wittassek, et al., 2011)
FUE = fractional urinary excretion rate of the metabolite (unitless) (0.73 from Anderson, et al., 2001)
MWd = molecular weight of BBP (312)
MWm = molecular weight of the monobenzyl phthalate (MBzP) (256)[9]

Using the most recent NHANES data reported by CDC (CDC, 2011, 2007-2008 samples), the geometric mean exposure of the total population is 0.3 ug/kg/day and the 95th percentile exposure is 2 ug/kg/day. Geometric mean and 95th percentile exposures for children aged 6-11 are 1.1 and 6.8 ug/kg/day, respectively. Of note, exposure levels have been trending downward.

In the 1997(a) NTP study the lowest BBP dose administered *ad libitum* that produced evidence of tumor formation (albeit equivocal evidence) was 12,000 ppm and 24,000 ppm for male and female rats, respectively. This equates to approximately 500 mg/kg/day in males and 1,200 mg/kg/day in females. As discussed in section I.B., above, treatment at these levels produced equivocal evidence of pancreatic acinar cell adenomas and carcinomas in males but did not produce statistically-significant tumors in females. These BBP dose levels in rats are 250,000 to 4,000,000-fold above the CDC NHANES levels for adult human exposure and 73,000 to 1,100,000-fold above the CDC NHANES levels for children's exposure.

Thus, the biomonitoring data for BBP indicate that there is little concern of health effects from human exposures to BBP. Again, this indicates that BBP should be a low priority for preparation of hazard identification materials.[10]

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[9] Note: It is inappropriate to use monobutyl ester concentrations to estimate BBP exposure, as they may be sourced in dibutyl phthalate exposures.

[10] We note that in 1997, OEHHA gave a low priority ranking to BBP even though its potential for human exposure was rated "high" (OEHHA, 1997). The subsequent biomonitoring data confirm that a low priority ranking for BBP is appropriate.

CONCLUSION

The foregoing demonstrates that the concern for BBP induced carcinogenicity is very low. Taken as a whole, the bioassay data for BBP do not reveal any strong tumor responses, as shown by the lack of response in mice; inability to reproduce the leukemia response in female rats; absence of rare, or early onset tumors; and the low incidences and influence of the diet or length of study on the pancreatic tumors in male rats or the urinary bladder tumors in female rats. The genotoxicity information on BBP is overwhelmingly negative. The weight of evidence is that BBP is not estrogenic, and that *in vitro* estrogenicity studies are not useful for evaluating the potential carcinogenicity of BBP. Other mechanistic data do not provide a basis for concern for BBP carcinogenicity, because they involve a mechanism not relevant to humans, or are grounded in an erroneous interpretation of the data.

The NTP, which conducted the cancer bioassays on BBP, has not considered BBP for listing in its Report on Carcinogens. Prior evaluations of the BBP database by other expert bodies have concluded that the potential for BBP to cause carcinogenicity in humans is, at most, marginal. Further, biomonitoring data demonstrate that human exposures to BBP are extremely low.

For all these reasons, BBP should be given a low priority for development of hazard identification materials under Proposition 65.

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APPENDIX A

Summary Comments on OEHHA Preliminary Toxicological Evaluation Compilation

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
Long-term feeding studies of 103-week studies in male and female B6C3F1 mice: NTP (1982) - No treatment-related tumor findings in males or females	No evidence for carcinogenicity
103-week studies in male and female *ad libitum* fed F344/N rats: NTP (1982) - Increase in mononuclear cell leukemia (by pairwise comparison and trend) in females	MNCL finding not reproduced in 1997(a) NTP bioassay. NTP authors (and others) state that MNCL is common in female rats, has a high spontaneous incidence rate, and is not a relevant lesion in humans.
103-week studies in male and female F344/N rats: NTP (1982) - No treatment-related tumor findings in males, but the study was judged inadequate due to high mortality in treated animals.	No evidence for carcinogenicity (early termination of males)
105-week studies in male and female F344/N rats: NTP (1997a) - Increase in pancreatic acinar cell adenoma, and adenoma and carcinoma (combined) (by pairwise comparison and trend) in males	A dietary role for pancreatic carcinogenesis was demonstrated in a third NTP study of BBP (NTP, 1997b).
105-week studies in male and female F344/N rats: NTP (1997a) - urinary bladder transitional epithelial papilloma (significant compared to historical controls) in females	Bladder tumors were not statistically-significantly elevated above controls in this study
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in urinary bladder carcinoma, and carcinoma and papilloma (combined) in females on restricted diet	Slight increase in urinary bladder neoplasm but only at 32 months on restricted diet and none were statistically-significantly elevated above controls at $P<0.05$
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in pancreatic adenoma in males fed *ad libitum* (by pairwise comparison, for either *ad libitum* or weight-matched controls	No increase in pancreatic tumors in weight-restricted rats at 2 years. This reinforces the role of diet in pancreatic tumorigenesis.

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - Increase in mononuclear cell leukemia in males and females fed *ad libitum* (pairwise comparison with weight-matched controls)	The incidence of MNCL in BBP-treated animals was within the range of historical controls for this strain and sex rat and not elevated above *ad libitum*-fed controls. NTP authors (and others) state that MNCL is common in female rats, has a high spontaneous incidence rate, and is not a relevant lesion in humans and did not consider MNCL in this study as biologically noteworthy.
24- or 30- or 32-month studies in male and female F344/N rats on restricted or *ad libitum* diets: NTP (1997b) - No treatment-related tumor findings in males on restricted diet	No evidence for carcinogenicity
Short-term intraperitoneal injection study in mice 24-week study in Strain A mice (injected 3 times/week for 8 weeks): Theiss, et al. (1977), as reviewed by NTP (1982) - No treatment-related increase in pulmonary tumors	No evidence for carcinogenicity
Short-term co-carcinogenicity 16-week study in female Sprague-Dawley rats (gavaged 7 times/week for one week, followed by a single dose of dimethylbenz[a]anthracene): Singletary, et al. (1997), as described in IARC (1999, p. 118) - No co-carcinogenic effects observed	BBP reduced the incidence of DMBA-induced breast tumors
Genotoxicity Review: NTP (1997a, pp. 7-8, 50); IARC (1999, pp. 123-124) - Salmonella reverse mutation assays (negative) - Drosophila melanogaster sex-linked recessive lethal mutation assays (negative) - Mouse lymphoma cell mutation assay (negative) - Sister chromatid exchange (SCE) in Chinese hamster ovary (CHO) cells (negative) chromosomal aberrations in CHO cells (negative) - SCE and chromosomal aberrations in mouse bone marrow cells *in vivo* (positive)	*See* Appendix B, Tables 1-4 for comprehensive summary of genetic toxicity studies completed on BBP. The results are overwhelmingly negative for genetic toxicity.
Estrogenic activity	*See* Opinion of Dr. Timothy Zacharewski, Appendix C, concluding that "the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action."
Mechanistic considerations; Hurst and Waxman (2003) - Showed weak peroxisome proliferation inducing activity	There are significant species differences in activation of PPARα and PPARγ by phthalate monoesters. Literature supports activation of PPARγ as being both carcinogenic and anti-carcinogenic. (Bility, et al., 2004)

OEHHA PRELIMINARY TOXICOLOGICAL REVIEW	EVIDENCE FOR POTENTIAL CARCINOGENICITY
Mechanistic considerations; Agas, et al. (2007) - Influenced actin distribution and cell proliferation in rat osteoblasts and increased levels of Cyclin D3 at G1 to S transition.	Authors offer support for BBP role in *in vitro* actin utilization and offer evidence for BBP *in vitro* induction of cyclin D but do not provide data to support a cancer mode-of-action for any tumor type.
IARC Reviews	IARC, Health Canada, OEHHA (1997) and the European Union have reviewed the carcinogenicity database for BBP and concluded that there should be no or low priority assigned

APPENDIX B

Genetic Toxicity Tables

Genetic Toxicity Table 1 *In Vitro* Gene Mutation Tests in Prokaryotic cells		
Study Design	Results	Reference
Salmonella typhimurium Strains TA98, TA100, TA1535, TA1537 and TA1538 With and without S-9 exogenous metabolic activation	Negative for mutation in all strains tested	Litton Bionetics, 1976
Salmonella typhimurium Strains TA98, TA100, TA1535, TA1537 and TA1538 With and without S-9 exogenous metabolic activation	Negative for mutation in all strains tested	Monsanto, 1976; Rubin, 1979; Kozumbo, 1982; Zeiger, 1985
Salmonella typhimurium Strains TA 98, TA 100, TA 1535, TA 1537 Four trials; with and without S-9 rat or hamster liver cell exogenous metabolic activation	Negative for mutation in all strains, all trials tested except one trial TA100 gave equivocal results without activation	NTP, 1997
Escherichia coli	Negative for gene mutation	Kurata, 1975 as cited in Omori, 1976
Bacillus subtilis	Negative for DNA repair	Kurata, 1975 as cited in Omori, 1976
Saccharomyces cerevisiae, Strain D4 With and without S-9 exogenous metabolic activation	Negative for mutation	Litton Bionetics, 1976

Genetic Toxicity Table 2 *In Vitro* Gene Mutation Tests in Mammalian Cells		
Study Design	Results	Reference
L5178Y Mouse lymphoma cells With and without mouse liver S-9 exogenous metabolic activation	Negative for mutation in all concentrations tested up to the limit of BBP solubility in cell culture medium	Litton Bionetics, 1977; Hazleton, 1986
L5178Y Mouse lymphoma cells With and without mouse liver S-9 exogenous metabolic activation	Equivocal for point mutation	Myhr, 1986; Myhr and Caspary, 1991
L5178Y Mouse lymphoma cells With (four trials) and without (two trials) rat liver S-9 exogenous metabolic activation	Negative for mutation in all concentrations tested up to the limit of BBP solubility in cell culture medium	NTP, 1997a

Genetic Toxicity Table 3 *In Vitro* Chromosome Damage and Morphologic Cell Transformation Tests in Mammalian Cells		
Study Design	Results	Reference
Chinese hamster ovary cell One trial with and two trials without rat liver S-9 exogenous metabolic activation for evaluation of sister chromatid exchange (SCE) figures	Negative -- Positive trend for SCE in first trial without activation but no significant increase in SCE at any test concentration; no increase in SCE at any test concentration or positive trend in second trial without activation or trial with activation. Author concluded study was negative for the induction of SCE.	Galloway, 1987
Chinese hamster ovary cell With and without rat liver S-9 exogenous metabolic activation	Negative -- No induction of chromosome aberration was detected with or without activation	Galloway, 1987
Syrian hamster embryo cells	Equivocal -- Morphologic cell transformation did not occur at 24 hours in any concentration tested within the range of BBP solubility in the cell culture medium; a positive response was observed following 7 days in culture.	Le Boeuf, 1996
BALB/3T3 cells	Negative -- Morphologic cell transformation did not occur at any concentration tested within the range of BBP solubility in the cell culture medium	Monsanto, 1985

Table 4 *In Vivo* Genetic Toxicity Tests		
Study Design	Results	Reference
Drosophila melanogaster dosed in feed at 10,000 or 50,000 ppm BBP or by injection at 500 ppm	Negative for sex-linked recessive lethal mutation	Valencia, 1985
Mouse, CD-1 and $B_6C_3F_1$ 400-4560 mg/kg/day subcutaneously for three days	Negative for dominant lethal mutation	Bishop, 1987
Male $B_6C_3F_1$ mice intraperitoneal injection of 1250, 2500 or 5000 mg/kg	Positive trend for SCE when top dose level was excluded from analysis of 23 hour cell harvest and for SCE for 48 hour cell harvest	NTP, 1997a
Male $B_6C_3F_1$ mice intraperitoneal injection of 1250, 2500 or 5000 mg/kg	Equivocal -- Positive trend for chromosomal aberration at 17 hour cell harvest; No positive trend (or significant elevation) in chromosome aberration at 36 hour harvest	NTP, 1997a
Female Alpk:AP_fSD (AP) rats receiving BBP in their drinking water at approx. 182.6 mg/kg/day during gestation and lactation (approx. 45 days)	Negative -- No induction of micronuclear bodies in bone marrow smears; Negative micronucleus test.	Ashby, 1997

APPENDIX C

Opinion of Timothy R. Zacharewski, Ph.D.

Expert Report on the Estrogenic Activities of Butylbenzyl Phthalate (BBP)

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Introduction

I have been asked by the Ferro Corporation to comment on the "Other relevant data - Estrogenicity activity" studies in the Office of Environmental Health Hazard Assessment (OEHHA)'s Butyl Benzyl Phthalate (BBP) background document that the Carcinogen Identification Committee (CIC) will consider at its Oct. 12-13, 2011 meeting. I am a Distinguished College of Natural Sciences Professor of Biochemistry & Molecular Biology and member of the Center for Integrative Toxicology at Michigan State University. I obtained my PhD in toxicology at Texas A&M University in the area of *in vitro* toxicology and obtained additional training in molecular biology and nuclear receptors in the laboratory of Professor Pierre Chambon (LGME-CNRS, Strasbourg, France) as a Medical Research Council of Canada Post Doctoral Fellow. I have 25+ years of research experience investigating the mechanisms of toxicity of environmental contaminants and industrial chemicals including the examination of estrogenic endocrine disruptors using *in vitro* and *in vivo* models. To date, my laboratory has published more than 100 peer-reviewed primary papers. I have also participated in numerous invited national and international workshops on *in vitro* screening assays and served on advisory committees reviewing the health risks of endocrine disruptors. This includes serving on the U.S. Environmental Protection Agency's (EPA) Endocrine Disruptor Screening Program (EDSP) review committee and on the National Toxicology Program (NTP) Center for the Evaluation of Risks to Human Reproduction committee that reviewed 6 high production volume phthalate esters including butylbenzyl phthalate (BBP). I am currently on leave from Michigan State University and placed on special assignment at EPA's National Center for Environmental Assessment (NCEA) investigating the use of high throughput *in vitro* assay and omics data in risk assessment as an Oak Ridge Institute for Science and Education (ORISE) Faculty Fellow. The following comments represent my own opinions and do not necessarily reflect the views or policies of the US EPA. For the purposes of this report, I am acting independently of Michigan State University and EPA.

Summary

This report outlines my opinions on the value of *in vitro* assays in assessing the estrogenic activities of chemicals. I describe their strengths and weaknesses as well as discuss specific technical issues that must be considered when critically assessing data from *in vitro* assays. Most importantly, the predictability of *in vivo* responses and the use of *in vitro* data in risk assessment are described. These principles are applied when evaluating the *in vitro* estrogenicity of BBP and its potential human carcinogenicity.

Although *in vitro* assays can be useful to identify chemicals that interact with the estrogen receptor and to elucidate mechanisms of action, they do not replicate *in vivo* conditions. Overall, *in vitro* assays have a poor record of predicting *in vivo* responses, especially for complex diseases such as breast cancer. Consequently, it is my opinion that *in vitro* assays are not useful for evaluating the potential carcinogenicity of BBP.

Endocrine Disruption Screening

Endocrine hormones, including steroids, regulate diverse physiological processes such as reproductive tract development, fertility, energy balance and behavior. Imbalances in hormones can also contribute to complex diseases such as cancer and diabetes as well as compromise reproductive fitness and development (e.g., reduced sperm counts, cyrptorchidism). In response to public concern regarding the possible disruption of the endocrine system following exposure to drugs, chemicals, natural products and environmental contaminants, the U.S. Environmental Protection Agency (EPA) established the Endocrine Disruptor Screening Program (EDSP) in compliance with the Food Quality Protection Act and the Safe Drinking Water Act Amendments of 1996. This requires EPA to test all food contact chemicals

and any chemical found in drinking water for effects similar to those elicited by female hormones (estrogens) and gives EPA the authority to screen for other endocrine effects (e.g., androgen, thyroid) in humans and wildlife (www.epa.gov/endo).

EDSP uses a two-tiered approach to determine the potential for chemicals to cause endocrine disruption in humans and wildlife. The Tier 1 Screening battery, adopted by EPA based on recommendations from the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), consists of a battery of complementary *in vitro* and short-term *in vivo* assays designed to identify chemicals affecting the estrogen, androgen and/or thyroid hormone systems through any of several recognized modes of action. This includes using assays that measure *in vitro* receptor binding and/or transactivation (estrogen and androgen receptor), inhibition of aromatase activity (estrogen biosynthesis), frog metamorphosis (thyroid activity), effects on fish (estrogen and androgen effects), male development (Hersberger assay, androgen and anti-androgen effects), pubertal female development (estrogen and thyroid activity), pubertal male development (androgen/anti-androgen and thyroid), uterine response (estrogen effects) and 15-day adult intact male responses (anti-androgen and thyroid activity). Data from these assays is then used in a weight of evidence (WoE) approach to determine which chemicals in Tier 1 Screening warrant further examination in Tier 2 Testing to generate the data needed to support a risk assessment (www.epa.gov/endo).

Tier 2 Testing *in vivo* assays assess whole organism effects and provide apical, as well as mechanistic, information from one or multiple endpoints within the assay. Tier 2 Testing consists of *in vivo* tests in males and females with an intact hypothalamic-pituitary-gonadal axis, using multiple pathways of exposure, exposures at different life-stages and exposure to various taxa to further identify and characterize chemical induced interactions with the endocrine system that can be used in a risk assessment. Note that Tier 2 tests are designed to quantify dose-response relationships in the larger context of toxicity and potential adversity that may involve interactions with other biological systems (e.g., neurological, immunological, hepatic, renal, and cardiovascular). Therefore, regulatory action is based on Tier 2 Testing (www.epa.gov/endo).

For the EDSP, weight-of-evidence evaluation (WoE) is a prescribed process where potentially relevant studies are critically assessed for data quality (www.epa.gov/endo). More specifically, potential compound effects, mode of action (MOA), and assay performance are evaluated. This goes beyond assessing positive and negative results within and between studies. Critical scientific assessment of the entire body of available data is considered to account for consistency, coherence, and biological plausibility. EPA continues to refine its WoE approach for use in risk assessments of chemicals suspected of causing toxicity, and especially cancer. Most important is the use of expert judgment formed through the scientific process, a current understanding of toxicity mechanisms, and knowledge of complementary fields (*e.g.*, developmental, reproductive, neurological and immunological toxicology, pharmacokinetics/ pharmacodynamics) (Borgert *et al.*, 2011a; Borgert *et al.*, 2011b). The concept of using complementary *in vitro* and *in vivo* assays to inform risk assessment and regulatory decision making also appears to gaining acceptance among stakeholders (Hartung and Daston, 2009). Consequently, these same principles should be applied when evaluating the potential carcinogenicity of BBP in humans.

Limitations of *In Vitro* Assays

The endocrine system involves the integration of signals across multiple nodes (i.e., organs, tissues) throughout an organism (e.g., hypothalamic-pituitary-gonadal axis), which can be profoundly affected by the absorption, distribution, metabolism and excretion (ADME) of an endocrine disruptor. Interactions between different cell types and organs at different developmental stages can also affect a chemical's behavior. Therefore, in order to assess potential impacts on the endocrine system, the chemical must be tested in an intact *in vivo* model (Ankley *et al.*, 1998; Gray *et al.*, 1997; Spielmann *et al.*, 1998; Zacharewski, 1998; Zacharewski, 1997). Nevertheless, data from *in vitro* assays, when appropriately qualified, can also be informative regarding potential sites of action and mechanism of

action. Ideally, a comprehensive and complementary battery of assays should be used to avoid false positives and negatives, since estrogens and estrogenic endocrine disruptors can elicit species-, tissue-, cell-, and response-specific effects. Moreover, despite the conservation of function and modes of action of endocrine systems between species, there are significant differences that can dramatically alter the activity of a chemical. Therefore, *in vitro* assays used to assess endocrine disrupting activity should be human-based, and reflect human ADME characteristics, when possible. For example, rodent models and *in vitro* assays lack sex hormone binding globulins (SHBG) that are present in human serum (Hammond, 2011; Hammond and Bocchinfuso, 1996). SHBG is an estrogen inducible protein present in serum that binds estrogens and estrogenic chemicals to regulate their bioavailability and metabolic clearance (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997).

In vitro assays also lack metabolic capabilities to bioactivate a proestrogenic chemical to its estrogenic metabolite or neutralize it through metabolism and eventual excretion. It is extraordinarily difficult to replicate the pharmacokinetic (e.g., metabolism) and pharmacodynamic (e.g. SHBG) interactions that are important for proper endocrine function, especially during development (Ankley, et al., 1998; Gray, et al., 1997; Spielmann, et al., 1998; Zacharewski, 1998; Zacharewski, 1997). Differences between species further confound data interpretation. For instance, humans preferentially metabolize BBP to mBzP (73% on a molar basis) over mBP (6% on a molar basis) (Anderson *et al.*, 2001). In contrast, rat metabolism of BBP yields 16% mBzP and 44% mBP (Anderson, et al., 2001). Consequently, due to their poor record of predicting *in vivo* activity, *in vitro* assay results for endocrine disrupting activities can only be used in a WoE approach to rank and prioritize chemicals that warrant additional *in vivo* Tier 2 Testing. It should also be noted that *in vitro* assay results are not used by the EPA for risk assessment. EPA also does not use data from other *in vitro* based screening programs such as ToxCast (Dix *et al.*, 2007) and Tox21 (Shukla *et al.*, 2010), for the purposes of risk assessment.

Assessment of *In Vitro* Studies Examining the Estrogenicity of BBP

The Office of Environmental Health Hazard Assessment (OEHHA) cites several papers under "Other relevant data – Estrogenic activity" that suggest BBP exhibits estrogenic activity based on *in vitro* data. For example, several papers are cited that report BBP binds to the estrogen receptor and induces ER-mediated effects such as the proliferation of human MCF-7 breast cancer cells. BBP is reported to be weakly estrogenic (>10^6 times weaker than 17β-estradiol, the predominant female sex steroid) in several *in vitro* assays (e.g., competitive estrogen receptor (ER) binding assays, ER-mediated gene expression in mammalian cells, ER-mediated reporter gene assays in mammalian cells, ER-mediated activity in yeast cells). However, BBP is not estrogenic *in vivo* based on the uterotropic assay, the gold standard for assessing the estrogenicity of a chemical (Brady *et al.*, 2000; Ryu and Kim, 2006; Zacharewski, 1997; Zacharewski *et al.*, 1998). These studies included doses that far exceed human exposure levels (e.g., daily doses ranging from 20 mg/kg to 2000 mg/kg per day for three consecutive days). In addition, estimated average drinking water exposures of 182.6 ug/kg/d to pregnant dams during gestation (gestational days 1-20) and from postnatal day 15 onward, had no effect on pup uterine weights (Ashby *et al.*, 1997b). Other sensitive *in vivo* markers of estrogen exposure including vaginal epithelial cell cornification (Zacharewski, et al., 1998), induction of uterine vascular permeability (Milligan *et al.*, 1998), and the differential expression of estrogen responsive uterine genes (Hong *et al.*, 2005), were also not affected by BBP.

Several factors must be considered when evaluating these *in vitro* studies. The first is that BBP is readily metabolized by non-specific esterases to monobutyl (mBP) and monobenzyl (mBzP) phthalate metabolites (Kayano *et al.*, 1997; Mentlein and Butte, 1989; Mentlein *et al.*, 1980). Consequently, mBP and mBzP should be the test chemicals used for *in vitro* assays. Interestingly, several of the cited reports indicate that mBP and mBzP are negative for estrogenic activity in the E-Screen and yeast-based assays (Harris *et al.*, 1997; Hashimoto *et al.*, 2003; Okubo *et al.*, 2003; Picard *et al.*, 2001). The concentrations used in the studies cited by OEHHA are also extremely high. Results obtained with doses exceeding 10

uM should be viewed with skepticism due to the insolubility of BBP in aqueous solutions (Moore, 2000). Precipitates would result in cells experiencing much higher concentrations than those nominally applied, which would confound data interpretation, and possibility cause cell toxicity. The Fernandez and Russo (Fernandez and Russo, 2010), Kang and Lee, (Kang and Lee, 2005), Kim et al., (Kim *et al.*, 2004), and Hashimoto et al., (Hashimoto, et al., 2003) studies all used BBP concentrations in excess of 10 uM, and even concentrations as high as 1 mM.

Of greatest concern are results derived from the E-Screen assay (Jones *et al.*, 1998; Welshons *et al.*, 1992; Zacharewski, 1997). Numerous studies have demonstrated that MCF-7 cell proliferation is highly variably and elicits a modest response (Picard, et al., 2001). This assay is also prone to false positives as a wide variety of chemicals and other treatments induce proliferation (Jones, et al., 1998; Welshons, et al., 1992). Assay reproducibility is problematic since several factors such as clone selection, culture conditions, serum lots and cell density influence proliferation (Jones, et al., 1998; Welshons, et al., 1992). As a result of these concerns, a panel of experts (Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC)) did not recommend the MCF-7 cell proliferation assay (E-Screen) as part of US EPA's EDSP Tier 1 Screening battery (www.epa.gov/endo).

Potentially more relevant and interesting studies have examined the *in vitro* transforming effects of BBP on MCF-10F cells using a collagen assay (Fernandez and Russo, 2010; Huang *et al.*, 2007). In this assay, MCF-10F cells are induced to form duct-like structures and solid masses in response to estrogenic substances. Although BBP at high concentrations (i.e., 1 and 10 uM) induced modest increases in MCF-10F cell invasion capacity relative to controls, it did not induce the formation of duct-like or solid mass structures. In contrast, another weak estrogenic endocrine disruptor, Bisphenol A (BPA), did induce duct-like structures and the formation of solid masses (Fernandez and Russo, 2010). The authors concluded that only "BPA as well as 17β-estradiol are able to induce the neoplastic tranformation of human breast epithelial cells" (Fernandez and Russo, 2010). To date, there have been no other peer reviewed publications using this assay. Additional studies investigating other estrogenic endocrine disruptors are required to evaluate the overall reliability of this assay.

Diet and environmental factors are potential contributors to breast cancer risk. More recently, attention has focused on changes in the hormonal environment during critical stages of development that may modify the architecture and biological characteristics of the developing mammary gland, increasing its future susceptibility to cancer. Two cited studies examining morphological and gene expression changes in the rat mammary gland following developmental exposure (post-natal days 2-20 and day 10 post-conception to delivery) exposure to 500 mg/kg/d BBP provide compelling data that BBP does not contribute to mammary carcinogenesis (Moral *et al.*, 2011; Moral *et al.*, 2007). They report that "BBP did not induce significant changes in the morphology of the gland, but changed the proliferation index" of several mammary gland structures (i.e., terminal end bud (TEB) at 35 days but not at 21, 50 and 100 days and lobule type 1 structures (Lob1) at 35, 50 and 100 days) in the post-natal study (Moral, et al., 2007). They further describe the TEB changes as "subtle" and "slight" at doses that far exceed human exposures. The *in utero* study (day 10 post-conception to delivery) reports trends in changes in epithelial structures (e.g., terminal end buds, terminal ducts, alveolar buds, lobules type 1) that are not statistically significant, and increases in the proliferation index that are modest, transient and only statistically significant at select times (TEB only at 35 days, terminal ducts only at 100 days, Lob1 only at 100 days). Although increases in the proliferation index suggest these structures are more susceptible to carcinogenesis, Singletary et at., report that 500 mg/kg BBP significantly reduced *in vivo* formation of mammary DNA adducts by 95%, and mammary adenocarcinomas by 70% induced by 1,12-dimethylbenz[a]anthracene (DMBA) (Singletary *et al.*, 1997) .

Mammary gland differential gene expression is reported in both studies with functions associated with proliferation, differentiation, immune function, cell signaling and metabolism. However, these changes are also modest, and not anchored to a phenotypic response (Boverhof and Zacharewski, 2006; Paules, 2003; Waters *et al.*, 2008). More specifically, these studies did not confirm that changes in gene

expression resulted in a phenotypic or apical response such as an increase in protein expression or enzyme activity. Exposure to any substance at the appropriate dose will cause changes in gene expression, but this may not lead to changes in protein expression, enzyme activity or an adverse affect. For example, despite BBP induced changes in mammary gland gene expression, there is no reported evidence of BBP causing mammary carcinogenesis in high dose multigenerational reproductive and developmental toxicity studies (Kamrin, 2009; Kavlock *et al.*, 2002; Tyl *et al.*, 2004).

Conclusion

BBP is an excellent example of a chemical that exhibits very weak *in vitro* estrogenic activity but does not induce significant estrogenic activity *in vivo* (Ashby *et al.*, 1997a; Moore, 2000). Although *in vitro* assay results suggest effects on MCF-7 cell proliferation (E-Screen), estrogen receptor mRNA expression, 17β-estradiol-induced MCF-7 apoptosis, and recombinant yeast reporter gene activity, these responses are not predictive of *in vivo* responses. Furthermore, the modest changes in mammary gland proliferation and gene expression did not result in statistically significant morphological or phenotypic effects, consistent with the lack of mammary gland carcinogenesis in multigenerational reproductive and developmental toxicity studies at doses that far exceed human exposures. In conclusion, the weight of evidence indicates that BBP is not carcinogenic via an estrogenic mode of action.

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