Texas Commission on Environmental Quality (TCEQ) Responses to Public Comments Received on the Proposed Development Support Document for Benzene October 15, 2007

The public comment period for the proposed Development Support Document (DSD) for benzene ended August 17, 2007. The Texas Chemical Council (TCC) submitted comments. The Toxicology Section (TS) of the TCEQ appreciates the effort put forth by TCC to provide technical comments on the proposed DSD for benzene. The goal of the Toxicology Section and TCEQ is to protect human health and welfare based on the most scientifically-defensible approaches possible (as documented in the DSD), and evaluation of these comments furthered that goal. TCC comments are provided below, followed by TCEQ responses. TCEQ responses indicate what changes, if any, were made to the DSD in response to the comment.

Texas Chemical Council (TCC) Comments Regarding the TCEQ Development Support Document for Benzene ESL Values

1.0 The list of studies considered for the Acute ESL should explicitly state the limitations of specific studies in mice.

The Development Support Document recommends using the results of Rozen et al (1984) as the key study. It is agreed this is an acceptable choice, however, the document includes a list of several studies in mice that were considered (Developmental Support Document table 3), and included on the list are studies that can be categorized as neonatal hematological studies. These studies have limitations and correctly were not used by TCEQ. There is a concern, however, the limitations of these studies have not been completely articulated, and by listing them unqualified on Table 3 might suggest they have comparable value for risk assessment as does the Rozen, et al study. In this regard, we are submitting a short report prepared by Dr. Richard Irons. This report was originally prepared on behalf of the Aromatic Producers Association of the Council of the European Chemical Industry (CEFIC) and was forwarded by CEFIC to the European Union (EU) with respect to the EU benzene risk assessment. The EU risk assessment correctly used more appropriate studies in their final risk assessment.

> From the EU Risk Assessment: '*Overall, investigations on the hematotoxic properties of benzene in the developing organism did not reveal signs of clinical hematotoxicity after indirect (in utero) exposure to benzene concentrations of up to 20 ppm. Evidence for some biological effect may be derived from the changes that had been observed in these studies for hematopoietic progenitor cells, however, there is large variance of the results in the CFU-assays of the studies, both within individual assays, but also when effects on different types of progenitors (CFU-E, BFU-E, CFU-GM) and*

between different developmental ages and from independent studies applying the same protocol are compared. Also, the reported statistical significance between groups remains doubtful, since the statistical methods applied in the earlier study of 1986 are not considerd appropriate and no justification is given. The overall results from these studies are not considered to provide indications for an increased sensitivity of the developing organism to the hematotoxic properties of benzene in comparison to the adult organism.'

TCEQ Response:

TS appreciates TCC's acknowledgment that Rozen et al. (1984) is acceptable as a key study for derivation of the acute ReV and ESL. Generally, TCC's comment indicates that several studies in Table 3 of the DSD can be categorized as neonatal hematological studies, and expresses concern that the limitations of these studies were not articulated in the proposed DSD. TCC is apparently referring to the Corti and Snyder (1996) and Keller and Snyder (1988) studies mentioned in Table 3 of the DSD.

As noted by TCC, TS did not use any of these studies as the key study for acute ReV/ESL development. As part of their comments, TCC submitted a report by Richard D. Irons, Ph.D. (attached), which was originally prepared on behalf of the Aromatic Producers Association of the Council of the European Chemical Industry (CEFIC) and was forwarded to the European Union (EU) with respect to the EU benzene risk assessment. The Irons report concludes that the Keller and Snyder (1986, 1988) studies regarding benzene-induced neonatal hematotoxicity do not meet minimum criteria for use in developmental risk assessment, the biological significance of the endpoints measured in these studies (e.g., colony forming units for erythroid (CFU-E) and granulocytic (CFU-GM-C) progenitor cells in the bone marrow and spleen in fetal, neonatal, and adult mice) has not been established, the reported findings in these studies do not correlate with significant adverse health effects, and no dose-response relationships were demonstrated.

The Irons report pertains to the use of neonatal hematology measurements in developmental risk assessment. TS did not use the Corti and Snyder (1996) and Keller and Snyder (1988) studies for risk assessment of developmental effects, and agrees with the quote from the EU risk assessment that, "The overall results from these studies are not considered to provide indications for an increased sensitivity of the developing organism to the hematotoxic properties of benzene in comparison to the adult organism." These studies were not included in the proposed DSD as evidence of potential increased sensitivity of the developing organism to the hematotoxic effects of benzene. They were included as part of the overall database which suggests that approximately 10 ppm is the lowest-observed-adverse-effect-level (LOAEL) which has been reported in short-term mouse studies and is often reported as the no-observed-adverse-effect-level (NOAEL), although the definition

of "adverse" may be debatable in some instances (e.g., Keller and Snyder 1988). Keller and Snyder (1988), one of the studies which was a subject of the Irons report, was removed from Table 3. The effects reported for *in utero*-exposed mice in the Corti and Snyder (1996) study were not the basis for inclusion in Table 3, as exposed adult male mice were reported to be affected. In response to this comment, a footnote was added to Table 3 for the Corti and Snyder (1996) study which indicates that the reported effects in adult mice (e.g., decreased CFU-E in male mice exposed as adults to 10.2 ppm) were the basis for inclusion in Table 3, and increased sensitivity was not demonstrated in the developing organism.

2.0 The Acute ESL is not based on a classically "acute" effect, and the overall uncertainty factor is excessive.

Acute effects for benzene are usually regarded as those that can arise after minutes or perhaps one hour of exposure. These values are usually measured in ppm rather than ppb (see EPA AEGL and ACGIH ERPG values). Since the pharmacokinetic factor is usually moot for these effects, uncertainty factors on the order of 3 rather than 10 are justified for intraspecies effects. Perhaps TCEQ should re-examine the rationale for an acute ESL.

TCEQ Response:

TS acknowledges that the acute ReV and ESL are not based on a classically acute effect. However, the subacute mouse studies used for ReV/ESL development and the others referred to in Table 3 of the DSD by far represent the most robust data upon which to base a short-term value, and have been used by other agencies to derive health-protective, short-term air concentrations (e.g., ATSDR acute MRL). Using these studies as a basis for a 1-hour value may be viewed by some to be conservative since animal exposure occurred intermittently over several days. While the data were used to derive a 1-hour ReV and ESL, public exposure to benzene does not occur just a few hours per year, one hour at a time. For example, it is likely if not certain that individuals living near significant point sources are frequently/ intermittently exposed to benzene peaks due to changes in meteorological conditions (e.g., wind direction). Therefore, based on the expected exposure for at least a portion of the public, TS believes the exposure regimens in these mouse studies and the resulting ReV/ESL to be relevant. The short-term ReV/ESL is protective of intermittent exposure.

The acute ReV, from which the acute ESL is derived, is calculated to protect human health and welfare based on the most sensitive relevant effect. As the ReV is used to evaluate air monitoring data for the protection of public health, it should be protective not only considering the relevant duration of exposure of the value (1-hour), but also considering the frequency with which exposure may occur. The same considerations apply to the acute ESL. As discussed above, short-term public exposure to benzene peaks may be frequent and intermittent depending upon the location of the receptor relative to sources. Derivation of a health-protective value for a one-time, 1-hour human exposure in a chamber would yield a higher value,

but this exposure scenario is not representative of how the public is exposed to benzene, would not take into account the hematotoxicity which may occur from intermittent exposure to lower concentrations, and thus would not be considered by TS to be protective of public health.

TCC refers to short-term values such as AEGLs. AEGLs represent threshold exposure limits for the general public and are applicable to *emergency exposure periods* ranging from 10 minutes to 8 hours. The 1-hour AEGL-1 value of 52 ppm is approximately 5 times the LOAEL demonstrated in the key study used for derivation of the acute ReV and ESL, is not designed to be health-protective considering frequent or repeated exposure, and does not consider the most sensitive effect due to repeated exposure (hematotoxicity) as it is based on mild central nervous system effects. This value is essentially designed for one-time emergency situations, and is clearly not appropriate for the protection of public health where exposure occurs on a daily basis.

In regards to the intraspecies uncertainty factor (UF), TS used a UF of 10 as there is good experimental evidence to indicate that benzene-sensitive human subpopulations may exist. Specifically, genetic polymorphisms associated with metabolic processes may confer variability in human susceptibility to benzene toxicity. The 2005 ATSDR *Toxicological Profile for Benzene* indicates...

> ***The flavoenzyme, NAD(P)H:quinone oxidoreductase (NQ01), catalyzes the reduction of 1,2- and 1,4-benzoquinone (reactive metabolites of benzene) to catechol and hydroquinone, respectively (Nebert et al. 2002), thus protecting cells from oxidative damage by preventing redox cycling. The NQ01*1 (wild type) allele codes for normal NQ01 enzyme and activity. An NQ01*2 allele encodes a nonsynonymous mutation that has negligible NQ01 activity. Approximately 5% of Caucasians and African Americans, 15% of Mexican-Americans, and 20% of Asians are homozygous for the NQ01*2 allele (Kelsey et al. 1997; Smith and Zhang 1998). Rothman et al. (1997) demonstrated that workers expressing negligible NQ01activity were at increased risk of benzene poisoning (Rothman et al. 1997). In the same group of workers, those expressing rapid CYP2E1 activity were also at increased risk of benzene poisoning. Those workers with polymorphisms for both negligible NQ01 activity and rapid CYP2E1 activity exhibited greater than 7-fold increased risk of benzene poisoning than workers not expressing these polymorphisms. These results indicate that individuals expressing rapid CYP2E1 activity may also be at increased risk for benzene toxicity.***

USEPA (2002) also discusses polymorphisms in the context of susceptible populations, and indicates that workers with rapid CYP2E1 activity (role in toxification of benzene to reactive metabolites) and homozygous for a certain NQO1 mutation (^{609}C→T; postulated role in detoxification) had a 7.6-fold increased risk of hematotoxicity compared to workers with slow CYP2E1 activity and one or more wild-type NQO1 alleles (Rothman et al. 1997). Additionally, Seaton et al. (1994)

reported up to a 13-fold difference in liver CYP2E1 among humans. Other polymorphisms (e.g., CYP2D6, GSTT1, NQOR) potentially affecting benzene metabolism could also confer differential susceptibility (higher or lower) to benzene toxicity (USEPA 2002). Other considerations relevant to potential increased susceptibility to benzene toxicity include, but are not limited to: (1) medical conditions which cause reduced bone marrow function or decreased blood factors; (2) ethanol consumption; (3) gender differences in susceptibility (e.g., the Brown et al. (1998) PBPK model suggests that women exhibit a higher blood/air partition coefficient and maximum velocity of benzene metabolism than men, and that women metabolize 23-26% more benzene than men under similar exposure scenarios); and (4) increased breathing rates in young children relative to adults and potential for increased absorption (ATSDR 2005) and greater exposure on a unit-body-weight basis (USEPA 2002).

As available information regarding genetic polymorphisms suggests potential increased hematotoxic risk in the range of 7-13 fold and the short-term ReV/ESL is based on hematotoxicity, TS believes that an intraspecies UF of 10 is reasonable. No change was made to the DSD in response to this comment.

3.0 The Chronic ESL was correctly based on the Rothman, et al (1996) study and not the Lan, et al (2004) study.

While it is agreed the Lan, et al (2004) study should not be used as the key study, it might be misleading to present a calculated alternative ESL using this study. While the Development Support Document states confirmation of findings in this study, other studies would be needed (that is, there is a failure of other studies to confirm effects below 1 ppm). There are additional reasons the Lan, et al (2004) study should not be used, including: a) uncertain exposure history of workers reported as exposed to < 1 ppm benzene and, b) uncertain relevance of alterations in clonogenic proliferation from hematopoietic progenitor cells derived from peripheral blood. The latter point is covered in an accompanying comment in Science (see Stokstad, Science (306) 3 Dec 2004, p. 1665).

TCEQ Response:

TS appreciates TCC's acknowledgment that the chronic ReV/ESL was correctly based on Rothman et al. (1996) and TCC's comments regarding the Lan et al. (2004) study. TS shares some of TCC's concerns regarding the Lan et al. study. However, TS has retained the alternate chronic ReV and ESL derived from Lan et al. (2004) for comparison purposes and to demonstrate that even if this study were to be used as the key study, following a DSD revision pursuant to the next TCC comment, it would produce a chronic ESL value greater than that from the carcinogenic assessment.

4.0 The total uncertainty factor used for the noncarcinogenic chronic ReV and ESL is excessive.

The Development Support Document uses an overall or total UF of 100 from the POD_{HEC}. This is excessive for two reasons. First, a subchronic to chronic factor of 3 is not needed because it does not appear additional time of exposure would affect the response effect level from the Rothman, et al (1996) study. Rothman, et al noted in their publication "*Neither estimated cumulative life-time benzene exposure nor number of years worked in an exposed factory was significantly associated with any hematologic outcome" (Rothman et al., 1996).* Therefore, there is no reason to suspect that the biological response from an average of 6.3 years of exposure would be quantitatively or qualitatively different than that expected to occur following 7 years of exposure. If any adjustment is made, it would seem that a data-derived adjustment of 7/6.3 (or 1.1) would be better justified than 3. In addition, the data deficiency factor of 3 is not needed because hematologic effects in humans are likely the most sensitive endpoint for benzene (see the Irons critique above). In addition, it is quite clear from subsequent publications from Zhang, that susceptible workers were present in the Rothman population. At most, an interspecies factor of 3 would be protective for the general population rather than the 10 fold factor used.

TCEQ Response:

TS agrees that the exposure duration for the Rothman et al. (1996) study (mean of 6.3 years) is likely sufficient in regards to observation of the response level for the hematotoxic effects evaluated. The DSD has therefore been revised to incorporate a subchronic-to-chronic UF of 1 for both Rothman et al. (1996) and Lan et al. (2004), which had a mean exposure duration of 6.1 years. The database UF of 3 has been retained. Human data regarding potential reproductive/developmental effects are limited and information from a two-generation reproductive/developmental study in animals does not exist. Therefore, uncertainty exists as it is unknown whether reproductive/developmental effects may occur at lower exposure levels than hematotoxicity.

Although the TCC comment refers to an "interspecies factor," it is apparent the comment is referring to the intraspecies UF. As indicated by TCC, there is evidence that the Rothman et al. (1996) study may have contained potentially sensitive individuals (e.g., Rothman et al. 1997), specifically, workers with genetic polymorphisms which conferred greater sensitivity. However, TS has elected to retain an intraspecies UF of 10 based on several considerations. Similar to the submitted comment that TCC believes an overall UF of 100 to be excessive, TS believes an overall UF of 10 in the final DSD (10 = UF of 3 for intraspecies variability x an incomplete database UF of 3) would result in a less-than-desirable level of conservatism for the chronic ReV and the protection of public health, and in fact would result in a chronic ReV which is greater than the acute ReV. TS's concern is based on several considerations, including: (1) confidence in the effect level demonstrated in Rothman et al. (1996); (2) similarity of the benchmark dose

concentration (7.2 ppm) to the study LOAEL (7.6 ppm) combined with use of a LOAEL-to-NOAEL UF of 1 for the benchmark dose concentration; (3) lymphocyte count in the low exposure group workers was reduced to approximately the 15th percentile; (4) workers are typically more healthy than members of the general population who have pre-existing conditions which may confer greater sensitivity (i.e., healthy worker effect); (5) the larger worker populations in studies which evaluated polymorphisms (e.g.,. Rothman et al. 1997, Lan et al. 2005) may not necessarily be genetically representative of the workers in Rothman et al. (1996); and (6) children receive a greater dose than adults on a body weight basis. Additional language regarding the intraspecies UF of 10 has been added to the final DSD.

5.0 The Development Support Document correctly used Acute Myelogenous Leukemia as the carcinogenic endpoint for calculating cancer risk.

The most consistent and therefore biologically plausible benzene cancer that has been observed in humans is acute myelogenous leukemia (AML). Accordingly, it is agreed that dose response evaluation of cancer risk should be based on AML.

TCEQ Response:

TS appreciates TCC's acknowledgment that AML is an appropriate cancer endpoint.

6.0 The Development Support Document overstates cancer risk by using the upper confidence level instead of the most likely estimate.

Historically, EPA has used the MLE (maximum likelihood estimate) when deriving unit risk factors (URFs) from human data, and the new cancer risk assessment guidelines do not offer any scientific rationale for changing that policy, nor is it even clear from the new cancer risk assessment guidelines that this issue was addressed during the external peer review of the new cancer risk assessment guidelines. TCC believes that use of the MLE is scientifically appropriate in most cases for URFs derived from human data, and that the upper confidence limit (UCL) should be used with human data only where substance-specific justification is presented.

Regardless, for benzene, TCEQ sites its guidelines in recommending the use of the UCL instead of the MLE because of the use of mortality rather than incidence. This rationale would predict that the AML's that are not fatal have a steeper dose response curve (and thus, a larger beta value and lower ESL) than the fatal AML's. However, for AML, the survival rate is only 20% (http://www.cancer.org/downloads/STT/CAFF2005f4PWSecured.pdf), and therefore, mortality risk would closely approximate incidence risk in this instance.

TCEQ Response:

Considering the uncertainty inherent in dose-response modeling for benzene (e.g., Crump and Allen versus Rinsky et al. exposure estimates), that AML mortality does not more closely approximate incidence, and that AMML (somewhat less conservative) as opposed to all leukemia was utilized as the cancer endpoint, TS has decided to retain use of the 95% UCL on the β for calculation of the carcinogenic ESL. TS believes this to be consistent with the ESL Guidelines. Additional language regarding use of the 95% UCL has been added to the final DSD.

7.0 TCEQ departed from the peer-reviewed ESL development process for dosimetric adjustment values.

TCEQ provided a transparent process for documenting ESL development. Yet, on a rather minor point, (240 versus 260 days per year for an occupational scenario), TCEQ changed its procedure for development of the benzene ESL. We believe the peer review guidance should take precedence for these minor differences, to lend well-deserved credibility to the peer review process.

TCEQ Response:

Consistent with the ESL Guidelines, TS has revised the DSD to incorporate use of 5/7 days to adjust from occupational to environmental exposure. Additionally, this may be more consistent with the work patterns reported for workers during periods in the 1940s, when Pliofilm production was increased due to World War II demands (e.g., Paustenbach et al. 1992).

8.0 The Development Support Document correctly did not include an early life correction factor for benzene.

The Development Support Document correctly concluded that benzene is not a chemical which is acting though a mutagenic mode of action for carcinogenesis, and therefore did not justify the need for a correction for children. This is consistent with the TCEQ Guideline as well as with the EPA's guidance regarding early-life exposures to carcinogens.

TCEQ Response:

Comment noted.

Analysis of the Underlying Biological Basis for Using Neonatal Hematology Measurements in Risk Assessments of the Developmental Toxicity of Benzene

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Introduction:

I have analyzed the suitability of using existing published studies of hematologic measurements in murine offspring following in utero exposure as a basis for risk assessment of the developmental toxicity of benzene at the request of the Aromatic Producers Association of the Council of the European Chemical Industry (CEFIC). In doing so I have focused on two reports of Keller and Snyder published in 1986 and 1988. It is my conclusion that the findings reported by Keller and Snyder do not represent a validated toxic effect and it is inappropriate to use these data for the assessment of the developmental toxicity of benzene.

Description:

In 1986 and 1988, Keller and Snyder reported the results of a series experiments of complex design in which changes in the number of hematopoietic colony forming cells and morphologically identifiable erythroid precursor cells were observed in fetal, neonatal and adult progeny of female Swiss Webster mice exposed to benzene via inhalation. In the first report, the authors examined colony forming units (CFU) for erythroid (CFU-E) and granulocytic progenitor (CFU-GM-C) cells in bone marrow and spleen of fetal, neonatal and adult progeny of female mice exposed to benzene, and in the second report, they examined the number of morphologically identifiable hematopoietic cells as well as more recognized hematologic morphometric endpoints in different tissues of fetal, neonatal and adult progeny so exposed.

Changes observed in the first report (Toxicology *42*: 171-181, 1986) included a "biphasic" effect of in utero exposure to 10 ppm benzene on CFU-E in suspensions of liver cells in randomly chosen litter groups of 2 day old neonates in which approximately half the groups exhibited an increase relative to pooled controls and the other half exhibited a decrease. No discernable dose-response was observed: 5 ppm had no effect and an increase in CFU-E was actually observed at 20 ppm. Splenic CFU-GM colonies were decreased in 10 week old male mice exposed to 10 ppm benzene for two weeks after in utero exposures to either air or 10 ppm benzene. However, the magnitude of the decrease was greater in animals previously exposed to benzene in utero. Alternatively, increases in CFU-GM colonies were measured in bone marrow with marked differences in the magnitude of these effects observed between males and females.

In the second report (Fundamental and Applied Toxicology *10*: 224-232, 1988), no changes were observed in hematologic indices of 16-day fetuses exposed to benzene in utero. Reduced numbers of circulating erythroid precursor cells and increased numbers of hepatic hematopoietic blast and granulocytic precursor cells were observed relative to controls in 2-day old mice, and 6 week old mice exposed to 20 ppm benzene in utero exhibited increased numbers of granulocytes in but not at lower exposure concentrations.

Background:

In order to interpret these findings, it is useful to review some general background information on adult and fetal hematopoiesis, as well as the concept of clonogenic, or

"colony forming" assays. The production and maintenance of blood cells (hematopoiesis) in the adult human is the result of proliferation and differentiation of precursor cells found primarily in the bone marrow. However, in rodents extramedullary hematopoiesis in spleen can persist into adult life and freqently occurs under conditions of non-specific stress. The process of hematopoiesis involves the amplification and differentiation of cell populations in which a very small number of hematopoietic stem cells (HSC) give rise to progressively more differentiated hematopoietic progenitor cells (HPC) that in turn produce morphologically recognizable precursor cells. These HPC ultimately proliferate and differentiate into the mature blood cells of the various lineages: erythrocytes, granulocytes, macrophages, lymphocytes and platelets. Extensive analyses of the kinetics of blood formation in vivo and HPC differentiation in culture has revealed that HSC and HPC represent a heterogeneous population of cells at different stages in differentiation that have different lineage potential and repopulating abilities (1). This general concept of clonal hematopoiesis is borne out in the human experience with bone marrow transplantation. The survival, proliferation and differentiation of HSC and HPC are regulated by cytokines, most of which are produced by stromal and accessory cells in the bone marrow. Together, these cells and regulatory molecules mediate hematopoiesis via a complex process that involves extensive interaction with the environment. The responsiveness of hematopoiesis to day to day environmental influences, infection, physicologic stress or injury is not equalled in any other organ system. An adult human produces well over 10^{11} blood cells/day, yet for short periods of time the kinetics of cell production can be increased over 6 fold to replace lost or damaged cells, with little or no apparent impact on the individual. Because of this reserve capacity, even an acute

massive loss of blood may have little impact on longevity if the individual survives the initial insult. Changes or fluctuations in the number of bone marrow hematopoietic progenitor cells or circulating mature blood cells can reflect a bewildering number of alternative causes including: direct loss or toxicity to circulating cells (eg. erythrocytes, granulocytes, lymphocytes, macrophages, platelets), an increase in the utilization or consumption of mature circulating elements (eg, granulocytes, platelets), increased physiologic demand, such as chronic exercise or changes in altitude, a change in the distribution or trafficking of cells within the circulation or tissues (lymphocytes or granulocytes), or an alteration in the maturation and/or proliferation of HPC in the bone marrow. Apparent alterations in the frequency of HPC may reflect such varied influences as dehydration, corticosteroid production, or, as often has been reported, variations in in vitro culture conditions.

Neither HSC or HPC can be readily identified by morphology or immunochemistry, but these cells can be enumerated by counting the number and types of colonies of morphologically recognizable cells grown in either semisolid or liquid suspension cultures supplemented with conditioned media or defined growth factors. It has been demonstrated that colony forming units (CFU) represent serial divisions and maturation of single progenitor cells that are often referred to as colony-forming cells (CFC) (2). Therefore, CFU assays permit analysis of the frequency of CFC in a population of isolated bone marrow cells under defined culture conditions. The type of CFU that are formed, as well as their number or frequency, is dependent on 1) the frequency of CFC in the starting cell population, 2) the specific mixture and concentration of cytokines or

growth factors present in the media, and 3) individual culture conditions. For example, semisolid media, e.g. methylcellulose or agar, allows for the enumeration of single progenitor cells or clones and characterization of their progeny by identification of colonies of cells undergoing maturation into morphologically recognizable blood cells. However, growth in semisolid media precludes assessment of actual stem cells that are capable of self-renewal or long term survival. In recent years, interpretation of colony growth as well as the identification of CFC, such as CFU-E, or CFU-GM, has been somewhat standardized by the availability of purified cytokines that can be used under defined culture conditions. Although the sensitivity of CFU-GM to many hematotoxic agents generally reflects experience in vivo, for some xenobiotics the sensitivity of human CFU-GM exposed in vitro can be significantly influenced by the source of the cells (i.e. mature bone marrow, peripheral blood or cord blood) or, alternatively, the mixture of cytokines used to support colony formation (3). Moreover, concentration-dependent decreases in CFU that are not as severe as to reach 50% of control values in vitro are usually not associated with measurable bone marrow suppression in vivo (4). This not withstanding, the use of conditioned media, such as that employed in the Keller studies, precludes even this minimum level of standardization. (Prior to the discovery and general availability of defined cytokines, stimulation of CFU formation required the use of "conditioned media" that was obtained from artificially stimulating cells in vitro to produce undefined "mixtures" of cytokines and natural products that were known to be capable of stimulating CFC in culture. Variations in the efficiency of different preparations of conditioned media to support CFC is dramatic and there are also marked differences in the proportion of different CFU grown out using different "batches".

These differences prevent direct comparisons of results between studies employing conditioned media.)

Fetal hematopoiesis is a complex and dynamic process that begins in the yolk sac with the appearance of primitive nucleated red blood cells and progresses in phases to the liver, spleen and bone marrow. The hepatic phase of erythropoiesis coincides with a rapid expansion in the maturation in the the primitive erythron and exhibits complex biphasic kinetics in which a initial period of rapid exponential growth in hematopoiesis transitions into a slower one (5). This is accompanied by a marked increase in the rate of hemoglobin synthesis (6). The initial more primitive phase appears to be responsive to erythropoietin while the latter phase is not. While the kinetics of erythroid development have been characterized in fetal mice, the control mechanisms that govern this process are not understood by any means, the signficiance of normally occurring changes in the relative differences of different morphologic types of primitive erythroid cells are not known and observations of fetal hematopoiesis remain largely empirical. Productive granulopoiesis does not occur in utero but begins after birth.

Finally, there are a number of differences in the regulation of hematopoiesis that are observed between mice and humans. While the biological basis for these differences is not well understood, there is evidence that they significantly impact the interpretation and extrapolation of toxicity data between these two species. For example, the frequency of early HPC found in mouse bone marrow is much greater than that observed in humans (12,13). In addition, the kinetics of HPC proliferation and colony formation in culture

are markedly different, such that murine CFC are traditionally measured or "scored" 7 days while human CFC are typically measured after 10-14 days in culture. Moreover, after birth even under conditions of hematologic stress (e.g. blood loss, toxicity etc.), hematopoiesis in humans is essentially restricted to bone marrow. In contrast, extra-medullary hematopoiesis frequently occurs in the mouse, and the murine spleen stands poised to undergo blood production with the slightest "provocation." In fact, the classic in vivo clonogenic assay to assess primitive hematopoietic cells in the mouse, the "colony forming unit-spleen (CFU-S), involves the enumeration of hematopoietic colonies forming in the spleen of irradiated mice injected with bone marrow cells taken from the test animals of interest. This means that interpreting the biological significance associated with changes in the frequency of CFC taken from mouse tissues (spleen, blood or bone marrow) necessarily requires an understanding of the redistribution and trafficking of murine HSC and HPC (i.e. a reduction in the frequency of CFC in murine bone marrow may not necessarily reflect an absolute decrease in hematopoiesis but may only represent a tissue redistribution if CFC are simultaneously elevated in the spleen. The significance of such alterations is not known.) Finally, observed species-differences in the regulation of differentiation in early HPC has been demonstrated to result in the mouse being uniquely susceptible to the development of certain hematopoietic neoplasms (14-17).

Analysis and Interpetation:

The Keller and Snyder reports represent a unique and unprecedented attempt to apply a combination of morphometric and clonogenic hematology assays developed and studied

in the adult, to the evaluation of the potential developmental toxicity of benzene in a dynamic and rapidly developing fetal system. The interpretation of the results of these studies is complicated by that fact that the application of these methodologies to developmental hematology was then and is now unprecedented, the biological significance of the endpoints unknown, and they remain largely unvalidated as predictors of biological effect or health risk. For example, changes observed in the first report included a "biphasic" effect of in utero exposure to 10 ppm benzene on CFU-E in suspensions of liver cells in which approximately half the groups exhibited an increase relative to pooled controls and the other half exhibited a decrease. An alternative explanation is that the effect observed in animals exposed to 10 ppm benzene was not a biphasic effect but rather a non-specific artifact due to wide variations in individual sets of untreated controls which appear to vary widely in both reports. In this regard, it is important to consider that these data reflect relative frequencies in suspensions of liver cells rather than absolute numbers, no dose-response was observed nor were the results accompanied by dose-dependent alterations in circulating blood cells or clinically significant hematotoxicity. Changes in the relative frequencies of CFU observed in 10 week old mice re-exposed to benzene for two weeks differed widely between spleen and bone marrow of the same animals, rendering it impossible to determine if these changes represented absolute differences in the numbers of CFU as a result of benzene exposure or alterations in the trafficking or distribution of CFU between bone marrow and spleen known to occur in adult mice due to non-specific stress.

In the second paper, the analysis of peripheral blood indices was apparently conducted at different times, because each dose level is compared to a different set of controls that differ one from the other. No dose response is observed for benzene on RBC in 16 day, 2 day or 6 week animals. No effects are observed on nucleated cells or hemoglobin types. Moreover, no biologically significant effects or dose-response is observed for the effects of benzene on peripheral blood differential counts in 6 week adults.

The Keller experiments provide no evidence of a dose-response relationship between benzene exposure and the experimental endpoints measured, and the changes observed were not accompanied by significant alterations in peripheral blood cell numbers or maturation. Moreover, no control was provided for the influence of non-specific solvent stress which is observed in animals exposed to non-hematotoxic aromatic solvents (8). For example, administration of high doses of substituted aromatic compounds, such as bromobenzene which is hepatotoxic but not hematotoxic, will result in transient alterations in bone marrow proliferative kinetics that do not result in bone marrow suppression. It is noteworthy that no alterations in the kinetics of proliferation or maturation of hemoglobin containing cells were detectable in the hepatic phase of neonates exposed in these studies. Moreover, no alterations in the maturation or development of circulating erythrocytes were observed in any exposure group and no changes in mean corpscular volume (MCV), a sensitive measure of maturation defects in erythrocytes, were observed at any dose. The effects of benzene on cell and tissue kinetics in the adult are well described (7-9). Moreover, acute or subacute exposure to even non-hematotoxic substituted benzenes have been demonstrated to result in transient

alterations in bone marrow cell cycle kinetics (8). During the hepatic phase, erythroid precursors exhibit a shortening of cell cycle time and a relative increase in the minor type of mouse hemoglobin in response to severe insult, such as anemia or infection (10,11). While the biological significance of these observations is not understood, none of these effects were observed following benzene exposure in the Keller studies.

Discussion:

The CFU-GM and BFU-E assays using human cells are often used as an indicators of HSC/HPC activity in a variety of clinical settings. Although the sensitivity of human CFU-GM to many hematotoxic agents generally reflects experience in vivo, the sensitivity of CFU-GM exposed in vitro is significantly influenced by the source of the cells (i.e. mature bone marrow, peripheral blood or cord blood) or, alternatively, the mixture of cytokines used to support colony formation (3). In this context, it should be remembered that the assay, itself, is simply a reflection of the frequency of GM-CFC in the starting cell sample capable of growing out under the precise conditions of culture and treatment employed. It provides no assessment of other complex responses that may occur following in vivo drug exposure, such as the potential of more primitive CFC to differentiate into GM-CFC, treatment-related alterations in stromal cell cytokine production which may partially ameliorate the loss of GM-CFC, or altered trafficking and redistribution of CFC between bone marrow and spleen in adult mice or bone marrow, spleen and liver in neonatal mice. Independent of any of these considerations, species differences in metabolism, plasma membrane lipid composition, stem cell ontogeny, chromosomal genetic mapping, and regulation of hematopoietic cell proliferation and

differentiation are sufficiently different to make inter-species extrapolation hazardous. As a consequence, neither animal models of hematotoxicity nor animal cells are usually relied upon as acceptable surrogates for the prediction of hematotoxicity in humans (4). A number of significant differences exist between mouse and human hematopoiesis. For example, the absolute frequency of early HPC in mouse bone marrow is known to be 25 fold greater than that observed in humans (12,13). The biological significance of this difference is not completely known, but one consequence is that murine clonal assays are more sensitive than human ones, simply because of increased statistical precision. Further, species differences in susceptibility of selective populations HPC to clonogenic suppression by drugs and chemicals are documented, with the mouse exhibiting unique susceptibility to hematotoxicity associated with certain agents (14-17).

Conclusion:

Taken together, the reports by Keller and Snyder do not meet the minimal criteria necessary for use in supporting risk assessment. This conclusion is supported by the following:

1) the biological significance of the endpoints measured has not been established.
2) none of the reported findings correlate with significant adverse health effects.
3) no dose-response relationships have been demonstrated or are apparent in the data.

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August 15, 2007

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Texas Commission on Environmental Quality
P.O. Box 13087
Austin, TX 78711-3087

Re: Texas Chemical Council Comments Regarding the Benzene Effects Screening Level Development Support Document

TCEQ Toxicology Section:

The Texas Chemical Council (TCC) submits these comments in response to the Texas Commission on Environmental Quality's (TCEQ) request for public comments on its Effects Screening Level (ESL) Development Support Document concerning benzene.

TCC members represent a major component of the manufacturing sector in Texas. The chemical industry is responsible for the employment of more than 450,000 Texans with a total annual payroll of more than $15 billion. The chemical industry has invested more than $40 billion in Texas production facilities. The Texas chemical industry generates about 25% of the state's manufacturing value and accounts for a similar percentage of manufacturing shipments.

TCC appreciates the opportunity to comment on the ESL values for benzene. TCC understands the importance of ESLs in providing the TCEQ with guidance to protect human health and welfare regarding its authority for air permitting and air monitoring. Air quality is also important to the regulated community, particularly to members of TCC.

In general, TCC believes the Draft Development Support Document for benzene is scientifically sound and demonstrates the diligence of the TCEQ to recommend supportable values. However, we do believe that TCEQ was overly conservative on a few key scientific issues which affect the ESL values. The attached comments briefly discuss these risk assessment issues that might impact the acute and chronic ESL values. While they are briefly mentioned here, many of the arguments are fully discussed in a comprehensive risk assessment document that was recently peer reviewed.[1] By offering the following comments, TCC hopes to provide perspectives to enhance the scientific basis of the ESL values for benzene.

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[1] Voluntary Children's Chemical Evaluation Program (VCCEP) Tier 1 Pilot Submission for BENZENE (CAS No. 71-43-2) Docket Number OPPTS-00274D American Chemistry Council Benzene, Toluene, and Xylenes VCCEP Consortium (http://www.tera.org/peer/VCCEP/benzene/Benzene%20VCCEP%20Submission%20Final%2030-Mar-2006.pdf)

Again, TCC appreciates the opportunity to comment on this important document and looks forward to future discussions with the TCEQ.

Sincerely,

Gregory S. Merrell

Gregory S. Merrell
Texas Chemical Council
Director of Regulatory Affairs

Texas Chemical Council (TCC)
Comments Regarding the TCEQ Development Support Document for Benzene ESL Values

1.0 The list of studies considered for the Acute ESL should explicitly state the limitations of specific studies in mice.

The Development Support Document recommends using the results of Rozen, et al (1984) as the key study. It is agreed this is an acceptable choice, however, the document includes a list of several studies in mice that were considered (Developmental Support Document table 3), and included on the list are studies that can be categorized as neonatal hematological studies. These studies have limitations and correctly were not used by TCEQ. There is a concern, however, the limitations of these studies have not been completely articulated, and by listing them unqualified on Table 3 might suggest they have comparable value for risk assessment as does the Rozen, et al study. In this regard, we are submitting a short report prepared by Dr. Richard Irons. This report was originally prepared on behalf of the Aromatic Producers Association of the Council of the European Chemical Industry (CEFIC) and was forwarded by CEFIC to the European Union (EU) with respect to the EU benzene risk assessment. The EU risk assessment correctly used more appropriate studies in their final risk assessment.

> From the EU Risk Assessment: '*Overall, investigations on the hematotoxic properties of benzene in the developing organism did not reveal signs of clinical hematotoxicity after indirect (in utero) exposure to benzene concentrations of up to 20 ppm. Evidence for some biological effect may be derived from the changes that had been observed in these studies for hematopoietic progenitor cells, however, there is large variance of the results in the CFU-assays of the studies, both within individual assays, but also when effects on different types of progenitors (CFU-E, BFU-E, CFU-GM) and between different developmental ages and from independent studies applying the same protocol are compared. Also, the reported statistical significance between groups remains doubtful, since the statistical methods applied in the earlier study of 1986 are not considerd appropriate and no justification is given. The overall results from these studies are not considered to provide indications for an increased sensitivity of the developing organism to the hematotoxic properties of benzene in comparison to the adult organism.'*

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2.0 The Acute ESL is not based on a classically "acute" effect, and the overall uncertainty factor is excessive.

Acute effects for benzene are usually regarded as those that can arise after minutes or perhaps one hour of exposure. These values are usually measured in ppm rather than ppb (see EPA

AEGL and ACGIH ERPG values). Since the pharmacokinetic factor is usually moot for these effects, uncertainty factors on the order of 3 rather than 10 are justified for intraspecies effects. Perhaps TCEQ should re-examine the rationale for an acute ESL.

3.0 The Chronic ESL was correctly based on the Rothman, et al (1996) study and not the Lan, et al (2004) study.

While it is agreed the Lan, et al (2004) study should not be used as the key study, it might be misleading to present a calculated alternative ESL using this study. While the Development Support Document states confirmation of findings in this study, other studies would be needed (that is, there is a failure of other studies to confirm effects below 1 ppm). There are additional reasons the Lan, et al (2004) study should not be used, including: a) uncertain exposure history of workers reported as exposed to < 1 ppm benzene and, b) uncertain relevance of alterations in clonogenic proliferation from hematopoietic progenitor cells derived from peripheral blood. The latter point is covered in an accompanying comment in Science (see Stokstad, Science (306) 3 Dec 2004, p. 1665).

4.0 The total uncertainty factor used for the Noncarcinogenic chronic ReV and ESL is excessive.

The Development Support Document uses an overall or total UF of 100 from the POD_{HEC}. This is excessive for two reasons. First, a subchronic to chronic factor of 3 is not needed because it does not appear additional time of exposure would affect the response effect level from the Rothman, et al (1996) study. Rothman, et al noted in their publication "*Neither estimated cumulative life-time benzene exposure nor number of years worked in an exposed factory was significantly associated with any hematologic outcome" (Rothman et al., 1996).* Therefore, there is no reason to suspect that the biological response from an average of 6.3 years of exposure would be quantitatively or qualitatively different than that expected to occur following 7 years of exposure. If any adjustment is made, it would seem that a data-derived adjustment of 7/6.3 (or 1.1) would be better justified than 3. In addition, the data deficiency factor of 3 is not needed because hematologic effects in humans are likely the most sensitive endpoint for benzene (see the Irons critique above). In addition, it is quite clear from subsequent publications from Zhang, that susceptible workers were present in the Rothman population. At most, an interspecies factor of 3 would be protective for the general population rather than the 10 fold factor used.

5.0 The Development Support Document correctly used Acute Myelogenous Leukemia as the carcinogenic endpoint for calculating cancer risk.

The most consistent and therefore biologically plausible benzene cancer that has been observed in humans is acute myelogenous leukemia (AML). Accordingly, it is agreed that dose response evaluation of cancer risk should be based on AML.

6.0 The Development Support Document overstates cancer risk by using the upper confidence level instead of the most likely estimate.

Historically, EPA has used the MLE (maximum likelihood estimate) when deriving unit risk factors (URFs) from human data, and the new cancer risk assessment guidelines do not offer any scientific rationale for changing that policy, nor is it even clear from the new cancer risk assessment guidelines that this issue was addressed during the external peer review of the new cancer risk assessment guidelines. TCC believes that use of the MLE is scientifically appropriate in most cases for URFs derived from human data, and that the upper confidence limit (UCL) should be used with human data only where substance-specific justification is presented.

Regardless, for benzene, TCEQ sites its guidelines in recommending the use of the UCL instead of the MLE because of the use of mortality rather than incidence. This rationale would predict that the AML's that are not fatal have a steeper dose response curve (and thus, a larger beta value and lower ESL) than the fatal AML's. However, for AML, the survival rate is only 20% (http://www.cancer.org/downloads/STT/CAFF2005f4PWSecured.pdf), and therefore, mortality risk would closely approximate incidence risk in this instance.

7.0 TCEQ departed from the peer-reviewed ESL development process for dosimetric adjustment values.

TCEQ provided a transparent process for documenting ESL development. Yet, on a rather minor point, (240 versus 260 days per year for an occupational scenario), TCEQ changed its procedure for development of the benzene ESL. We believe the peer review guidance should take precedence for these minor differences, to lend well-deserved credibility to the peer review process.

8.0 The Development Support Document correctly did not include an early life correction factor for benzene.

The Development Support Document correctly concluded that benzene is not a chemical which is acting though a mutagenic mode of action for carcinogenesis, and therefore did not justify the need for a correction for children. This is consistent with the TCEQ Guideline as well as with the EPA's guidance regarding early-life exposures to carcinogens.[2]

[2] EPA, Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens. EPA/630/R-03/003F, (March 2005).

Analysis of the Underlying Biological Basis for Using Neonatal Hematology Measurements in Risk Assessments of the Developmental Toxicity of Benzene

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Introduction:

I have analyzed the suitability of using existing published studies of hematologic measurements in murine offspring following in utero exposure as a basis for risk assessment of the developmental toxicity of benzene at the request of the Aromatic Producers Association of the Council of the European Chemical Industry (CEFIC). In doing so I have focused on two reports of Keller and Snyder published in 1986 and 1988. It is my conclusion that the findings reported by Keller and Snyder do not represent a validated toxic effect and it is inappropriate to use these data for the assessment of the developmental toxicity of benzene.

Description:

In 1986 and 1988, Keller and Snyder reported the results of a series experiments of complex design in which changes in the number of hematopoietic colony forming cells and morphologically identifiable erythroid precursor cells were observed in fetal, neonatal and adult progeny of female Swiss Webster mice exposed to benzene via inhalation. In the first report, the authors examined colony forming units (CFU) for erythroid (CFU-E) and granulocytic progenitor (CFU-GM-C) cells in bone marrow and spleen of fetal, neonatal and adult progeny of female mice exposed to benzene, and in the second report, they examined the number of morphologically identifiable hematopoietic cells as well as more recognized hematologic morphometric endpoints in different tissues of fetal, neonatal and adult progeny so exposed.

Changes observed in the first report (Toxicology *42*: 171-181, 1986) included a "biphasic" effect of in utero exposure to 10 ppm benzene on CFU-E in suspensions of liver cells in randomly chosen litter groups of 2 day old neonates in which approximately half the groups exhibited an increase relative to pooled controls and the other half exhibited a decrease. No discernable dose-response was observed: 5 ppm had no effect and an increase in CFU-E was actually observed at 20 ppm. Splenic CFU-GM colonies were decreased in 10 week old male mice exposed to 10 ppm benzene for two weeks after in utero exposures to either air or 10 ppm benzene. However, the magnitude of the decrease was greater in animals previously exposed to benzene in utero. Alternatively, increases in CFU-GM colonies were measured in bone marrow with marked differences in the magnitude of these effects observed between males and females.

In the second report (Fundamental and Applied Toxicology *10*: 224-232, 1988), no changes were observed in hematologic indices of 16-day fetuses exposed to benzene in utero. Reduced numbers of circulating erythroid precursor cells and increased numbers of hepatic hematopoietic blast and granulocytic precursor cells were observed relative to controls in 2-day old mice, and 6 week old mice exposed to 20 ppm benzene in utero exhibited increased numbers of granulocytes in but not at lower exposure concentrations.

Background:

In order to interpret these findings, it is useful to review some general background information on adult and fetal hematopoiesis, as well as the concept of clonogenic, or

"colony forming" assays. The production and maintenance of blood cells (hematopoiesis) in the adult human is the result of proliferation and differentiation of precursor cells found primarily in the bone marrow. However, in rodents extramedullary hematopoiesis in spleen can persist into adult life and freqently occurs under conditions of non-specific stress. The process of hematopoiesis involves the amplification and differentiation of cell populations in which a very small number of hematopoietic stem cells (HSC) give rise to progressively more differentiated hematopoietic progenitor cells (HPC) that in turn produce morphologically recognizable precursor cells. These HPC ultimately proliferate and differentiate into the mature blood cells of the various lineages: erythrocytes, granulocytes, macrophages, lymphocytes and platelets. Extensive analyses of the kinetics of blood formation in vivo and HPC differentiation in culture has revealed that HSC and HPC represent a heterogeneous population of cells at different stages in differentiation that have different lineage potential and repopulating abilities (1). This general concept of clonal hematopoiesis is borne out in the human experience with bone marrow transplantation. The survival, proliferation and differentiation of HSC and HPC are regulated by cytokines, most of which are produced by stromal and accessory cells in the bone marrow. Together, these cells and regulatory molecules mediate hematopoiesis via a complex process that involves extensive interaction with the environment. The responsiveness of hematopoiesis to day to day environmental influences, infection, physicologic stress or injury is not equalled in any other organ system. An adult human produces well over 10^{11} blood cells/day, yet for short periods of time the kinetics of cell production can be increased over 6 fold to replace lost or damaged cells, with little or no apparent impact on the individual. Because of this reserve capacity, even an acute

massive loss of blood may have little impact on longevity if the individual survives the initial insult. Changes or fluctuations in the number of bone marrow hematopoietic progenitor cells or circulating mature blood cells can reflect a bewildering number of alternative causes including: direct loss or toxicity to circulating cells (eg. erythrocytes, granulocytes, lymphocytes, macrophages, platelets), an increase in the utilization or consumption of mature circulating elements (eg, granulocytes, platelets), increased physiologic demand, such as chronic exercise or changes in altitude, a change in the distribution or trafficking of cells within the circulation or tissues (lymphocytes or granulocytes), or an alteration in the maturation and/or proliferation of HPC in the bone marrow. Apparent alterations in the frequency of HPC may reflect such varied influences as dehydration, corticosteroid production, or, as often has been reported, variations in in vitro culture conditions.

Neither HSC or HPC can be readily identified by morphology or immunochemistry, but these cells can be enumerated by counting the number and types of colonies of morphologically recognizable cells grown in either semisolid or liquid suspension cultures supplemented with conditioned media or defined growth factors. It has been demonstrated that colony forming units (CFU) represent serial divisions and maturation of single progenitor cells that are often referred to as colony-forming cells (CFC) (2). Therefore, CFU assays permit analysis of the frequency of CFC in a population of isolated bone marrow cells under defined culture conditions. The type of CFU that are formed, as well as their number or frequency, is dependent on 1) the frequency of CFC in the starting cell population, 2) the specific mixture and concentration of cytokines or

growth factors present in the media, and 3) individual culture conditions. For example, semisolid media, e.g. methylcellulose or agar, allows for the enumeration of single progenitor cells or clones and characterization of their progeny by identification of colonies of cells undergoing maturation into morphologically recognizable blood cells. However, growth in semisolid media precludes assessment of actual stem cells that are capable of self-renewal or long term survival. In recent years, interpretation of colony growth as well as the identification of CFC, such as CFU-E, or CFU-GM, has been somewhat standardized by the availability of purified cytokines that can be used under defined culture conditions. Although the sensitivity of CFU-GM to many hematotoxic agents generally reflects experience in vivo, for some xenobiotics the sensitivity of human CFU-GM exposed in vitro can be significantly influenced by the source of the cells (i.e. mature bone marrow, peripheral blood or cord blood) or, alternatively, the mixture of cytokines used to support colony formation (3). Moreover, concentration-dependent decreases in CFU that are not as severe as to reach 50% of control values in vitro are usually not associated with measurable bone marrow suppression in vivo (4). This not withstanding, the use of conditioned media, such as that employed in the Keller studies, precludes even this minimum level of standardization. (Prior to the discovery and general availability of defined cytokines, stimulation of CFU formation required the use of “conditioned media” that was obtained from artificially stimulating cells in vitro to produce undefined “mixtures” of cytokines and natural products that were known to be capable of stimulating CFC in culture. Variations in the efficiency of different preparations of conditioned media to support CFC is dramatic and there are also marked differences in the proportion of different CFU grown out using different “batches”.

These differences prevent direct comparisons of results between studies employing conditioned media.)

Fetal hematopoiesis is a complex and dynamic process that begins in the yolk sac with the appearance of primitive nucleated red blood cells and progresses in phases to the liver, spleen and bone marrow. The hepatic phase of erythropoiesis coincides with a rapid expansion in the maturation in the the primitive erythron and exhibits complex biphasic kinetics in which a initial period of rapid exponential growth in hematopoiesis transitions into a slower one (5). This is accompanied by a marked increase in the rate of hemoglobin synthesis (6). The initial more primitive phase appears to be responsive to erythropoietin while the latter phase is not. While the kinetics of erythroid development have been characterized in fetal mice, the control mechanisms that govern this process are not understood by any means, the signficiance of normally occurring changes in the relative differences of different morphologic types of primitive erythroid cells are not known and observations of fetal hematopoiesis remain largely empirical. Productive granulopoiesis does not occur in utero but begins after birth.

Finally, there are a number of differences in the regulation of hematopoiesis that are observed between mice and humans. While the biological basis for these differences is not well understood, there is evidence that they significantly impact the interpretation and extrapolation of toxicity data between these two species. For example, the frequency of early HPC found in mouse bone marrow is much greater than that observed in humans (12,13). In addition, the kinetics of HPC proliferation and colony formation in culture

are markedly different, such that murine CFC are traditionally measured or "scored" 7 days while human CFC are typically measured after 10-14 days in culture. Moreover, after birth even under conditions of hematologic stress (e.g. blood loss, toxicity etc.), hematopoiesis in humans is essentially restricted to bone marrow. In contrast, extra-medullary hematopoiesis frequently occurs in the mouse, and the murine spleen stands poised to undergo blood production with the slightest "provocation." In fact, the classic in vivo clonogenic assay to assess primitive hematopoietic cells in the mouse, the "colony forming unit-spleen (CFU-S), involves the enumeration of hematopoietic colonies forming in the spleen of irradiated mice injected with bone marrow cells taken from the test animals of interest. This means that interpreting the biological significance associated with changes in the frequency of CFC taken from mouse tissues (spleen, blood or bone marrow) necessarily requires an understanding of the redistribution and trafficking of murine HSC and HPC (i.e. a reduction in the frequency of CFC in murine bone marrow may not necessarily reflect an absolute decrease in hematopoiesis but may only represent a tissue redistribution if CFC are simultaneously elevated in the spleen. The significance of such alterations is not known.) Finally, observed species-differences in the regulation of differentiation in early HPC has been demonstrated to result in the mouse being uniquely susceptible to the development of certain hematopoietic neoplasms (14-17).

Analysis and Interpetation:

The Keller and Snyder reports represent a unique and unprecedented attempt to apply a combination of morphometric and clonogenic hematology assays developed and studied

in the adult, to the evaluation of the potential developmental toxicity of benzene in a dynamic and rapidly developing fetal system. The interpretation of the results of these studies is complicated by that fact that the application of these methodologies to developmental hematology was then and is now unprecedented, the biological significance of the endpoints unknown, and they remain largely unvalidated as predictors of biological effect or health risk. For example, changes observed in the first report included a "biphasic" effect of in utero exposure to 10 ppm benzene on CFU-E in suspensions of liver cells in which approximately half the groups exhibited an increase relative to pooled controls and the other half exhibited a decrease. An alternative explanation is that the effect observed in animals exposed to 10 ppm benzene was not a biphasic effect but rather a non-specific artifact due to wide variations in individual sets of untreated controls which appear to vary widely in both reports. In this regard, it is important to consider that these data reflect relative frequencies in suspensions of liver cells rather than absolute numbers, no dose-response was observed nor were the results accompanied by dose-dependent alterations in circulating blood cells or clinically significant hematotoxicity. Changes in the relative frequencies of CFU observed in 10 week old mice re-exposed to benzene for two weeks differed widely between spleen and bone marrow of the same animals, rendering it impossible to determine if these changes represented absolute differences in the numbers of CFU as a result of benzene exposure or alterations in the trafficking or distribution of CFU between bone marrow and spleen known to occur in adult mice due to non-specific stress.

In the second paper, the analysis of peripheral blood indices was apparently conducted at different times, because each dose level is compared to a different set of controls that differ one from the other. No dose response is observed for benzene on RBC in 16 day, 2 day or 6 week animals. No effects are observed on nucleated cells or hemoglobin types. Moreover, no biologically significant effects or dose-response is observed for the effects of benzene on peripheral blood differential counts in 6 week adults.

The Keller experiments provide no evidence of a dose-response relationship between benzene exposure and the experimental endpoints measured, and the changes observed were not accompanied by significant alterations in peripheral blood cell numbers or maturation. Moreover, no control was provided for the influence of non-specific solvent stress which is observed in animals exposed to non-hematotoxic aromatic solvents (8). For example, administration of high doses of substituted aromatic compounds, such as bromobenzene which is hepatotoxic but not hematotoxic, will result in transient alterations in bone marrow proliferative kinetics that do not result in bone marrow suppression. It is noteworthy that no alterations in the kinetics of proliferation or maturation of hemoglobin containing cells were detectable in the hepatic phase of neonates exposed in these studies. Moreover, no alterations in the maturation or development of circulating erythrocytes were observed in any exposure group and no changes in mean corpscular volume (MCV), a sensitive measure of maturation defects in erythrocytes, were observed at any dose. The effects of benzene on cell and tissue kinetics in the adult are well described (7-9). Moreover, acute or subacute exposure to even non-hematotoxic substituted benzenes have been demonstrated to result in transient

alterations in bone marrow cell cycle kinetics (8). During the hepatic phase, erythroid precursors exhibit a shortening of cell cycle time and a relative increase in the minor type of mouse hemoglobin in response to severe insult, such as anemia or infection (10,11). While the biological significance of these observations is not understood, none of these effects were observed following benzene exposure in the Keller studies.

Discussion:

The CFU-GM and BFU-E assays using human cells are often used as an indicators of HSC/HPC activity in a variety of clinical settings. Although the sensitivity of human CFU-GM to many hematotoxic agents generally reflects experience in vivo, the sensitivity of CFU-GM exposed in vitro is significantly influenced by the source of the cells (i.e. mature bone marrow, peripheral blood or cord blood) or, alternatively, the mixture of cytokines used to support colony formation (3). In this context, it should be remembered that the assay, itself, is simply a reflection of the frequency of GM-CFC in the starting cell sample capable of growing out under the precise conditions of culture and treatment employed. It provides no assessment of other complex responses that may occur following in vivo drug exposure, such as the potential of more primitive CFC to differentiate into GM-CFC, treatment-related alterations in stromal cell cytokine production which may partially ameliorate the loss of GM-CFC, or altered trafficking and redistribution of CFC between bone marrow and spleen in adult mice or bone marrow, spleen and liver in neonatal mice. Independent of any of these considerations, species differences in metabolism, plasma membrane lipid composition, stem cell ontogeny, chromosomal genetic mapping, and regulation of hematopoietic cell proliferation and

differentiation are sufficiently different to make inter-species extrapolation hazardous. As a consequence, neither animal models of hematotoxicity nor animal cells are usually relied upon as acceptable surrogates for the prediction of hematotoxicity in humans (4). A number of significant differences exist between mouse and human hematopoiesis. For example, the absolute frequency of early HPC in mouse bone marrow is known to be 25 fold greater than that observed in humans (12,13). The biological significance of this difference is not completely known, but one consequence is that murine clonal assays are more sensitive than human ones, simply because of increased statistical precision. Further, species differences in susceptibility of selective populations HPC to clonogenic suppression by drugs and chemicals are documented, with the mouse exhibiting unique susceptibility to hematotoxicity associated with certain agents (14-17).

Conclusion:

Taken together, the reports by Keller and Snyder do not meet the minimal criteria necessary for use in supporting risk assessment. This conclusion is supported by the following:

1) the biological significance of the endpoints measured has not been established.
2) none of the reported findings correlate with significant adverse health effects.
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